CHAPTER IV
RESULTS AND DISCUSSION

4.1 Isolation of Biosurfactant-Producing Microorganisms

4.1.1 Isolation of Biosurfactant Producers on Nutrient Agar (NA)

Samples were collected from various places which are petroleum contaminated soil from car service in Bangkok, shallow sea water from Pattaya, food waste from Chulalongkorn University food court, and deep sea water from Phuket. The samples were collected in zipper bags and transported to the laboratory for analyses. The method of serial dilutions of the sample and plate count in a selective medium nutrient agar (Difco, USA) (Kennedy, *et al.,* 1975) covered with crude oil (PTTEP Co., Ltd, Thailand) as a thin film was used for isolation purposes and the plates were incubated at 37°C for 24 hours. Colonies surrounded by an emulsified halo were screened as biosurfactant producer (Morikawa *et al.,* 1993) as shown in Figure 4.1

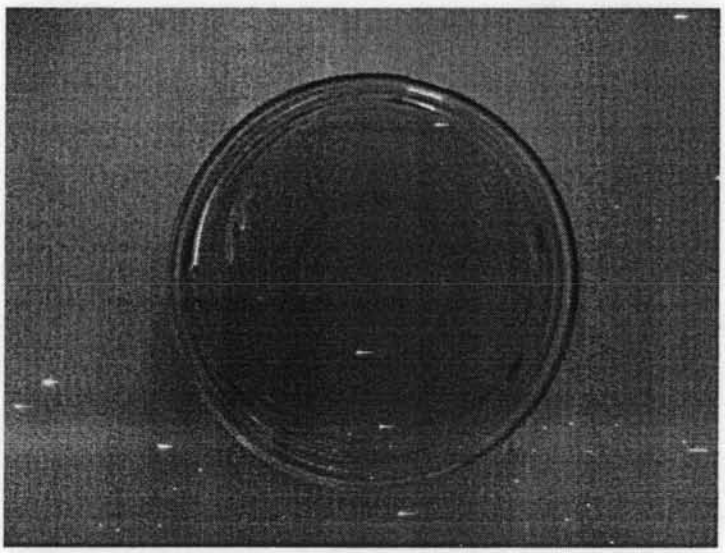

Figure 4.1 Emulsified halo around a bacterial colony that can produce biosurfactant.

Different sixty-seven microorganisms from nine samples gave sixteen bacterial colonies with clear zones as shown in Table 4.1.

Table 4.1 Summary of isolated culture that can produce biosurfactants

Source of sample	Number of samples	Number of total isolated cultures	Number of isolated cultures that created a clear zone*
Car service (Soil)	2	40	13
Pattaya (Sea water)	1	11	1
Food court (Food waste)	5	2	0
Phuket (Deep sea water)	1	14	2
Total	9	67	16

* Clear zone around colony on agar nutrient containing crude oil.

4.2 Determination of Biosurfactant-Producing Bacteria Activity

4.2.1 Determination of Oil Displacement Test (Morikawa *et al.*, 1993)

After bacterial colonies in NA slants were transferred to nutrient broth (NB) containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for 24 hours, the oil displacement test was performed for selecting the potential biosurfactant-producing bacteria as shown in Figures 4.2 and 4.3.

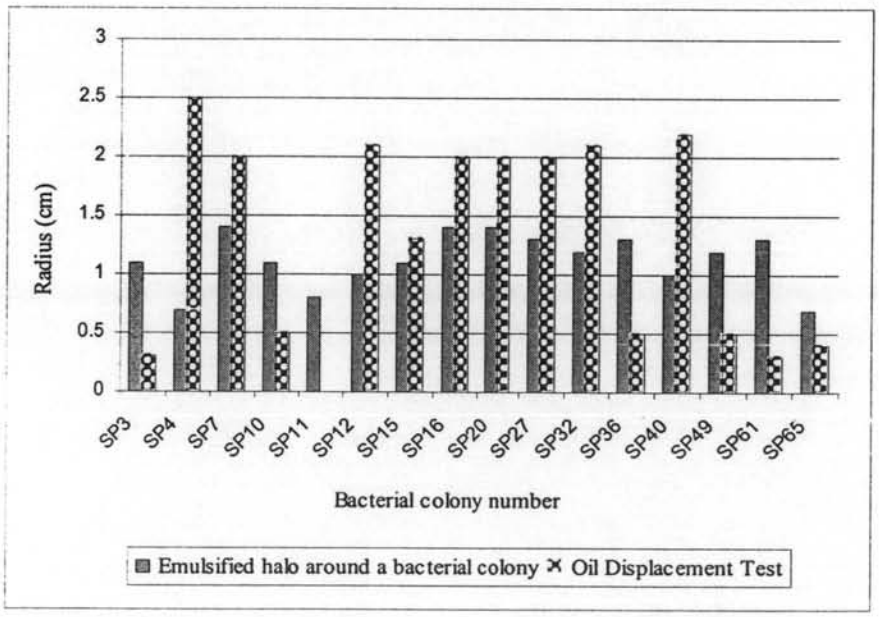

Figure 4.2 Oil displacement test & the appearance of clear zone of 16 bacterial colonies.

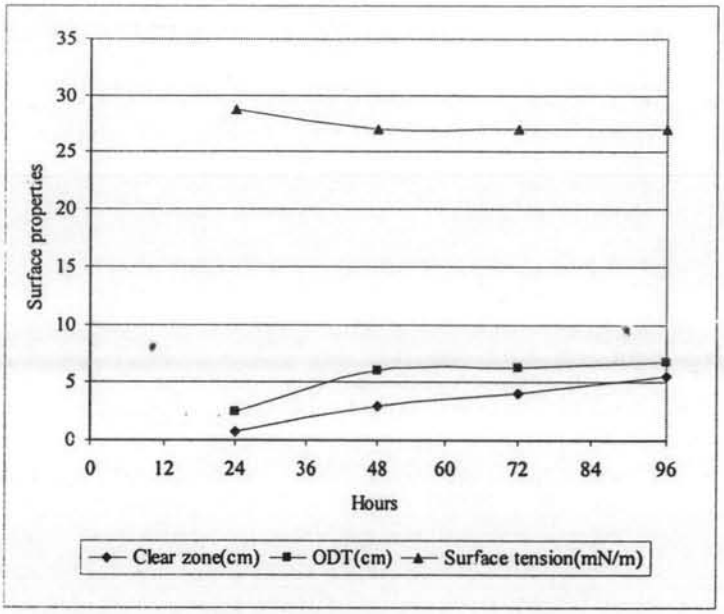

Figure 4.3 Surface properties of bacteria SP4.

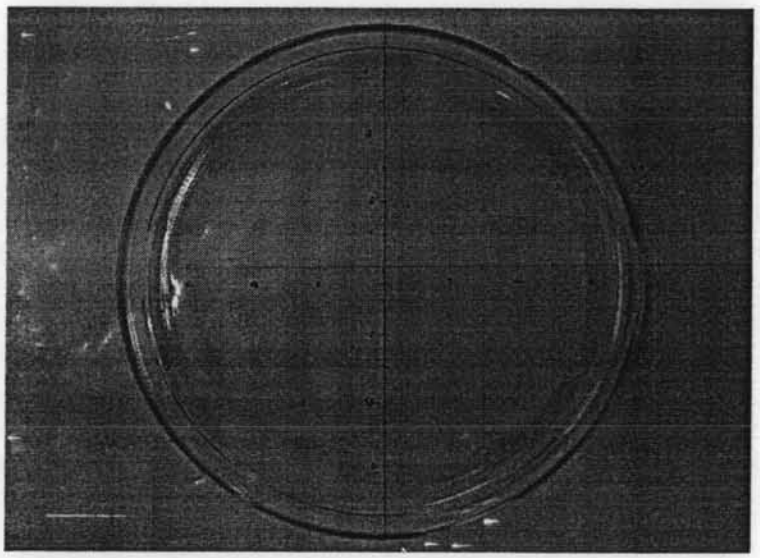

Figure 4.4 Activity of biosurfactant-producing bacteria for oil displacement test.

4.2.2 The Percentage Reduction of Surface Tension of Biosurfactant-Producing Bacteria

The isolated cultures from the first part (4.1.1) were cultivated in NB containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for

24 hours. Then, the culture supernatant (clear solution) was measured for surface tension by Wilhelmy plate DCAT II tensiometer, as shown in Table 4.2.

Table 4.2 Surface tension and the percentage reduction of surface tension of the culture supernatant from 16 isolates in NB containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for 24 hours

Isolate	Surface tension (mN/m)	% Reduction of Surface tension
SP 3	45	4.255
SP 4	**28.7**	**38.936**
SP 7	38	19.149
SP10	**32**	**31.915**
SP11	44.5	5.319
SP12	**32.5**	**30.851**
SP15	33	29.787
SP16	39	17.021
SP20	38	19.149
SP27	36.5	22.340
SP32	36	23.404
SP36	34.5	26.596
SP40	**30**	**36.170**
SP49	39	17.021
SP61	42	10.638
SP65	41.5	11.702

From Table 4.2, four isolates (SP4, SP10, SP12, and SP40) were given high performance ratings for producing biosurfactants by considering the percentage reduction of surface tension higher than 30% (Desai and Banat, 1997) which is determined from:

$$\% \text{ reduction of surface tension} = [(\gamma_{bc} - \gamma_{ac}) / \gamma_{bc}] \times 100$$

Where; γ_{bc} = surface tension before cultivation

γ_{ac} = surface tension after cultivation

* γ_{NB} = 47 mN/m

Table 4.3 Surface tension, the percentage reduction of surface tension, and oil displacement test of the culture supernatant from the 4 isolated cultures (SP4, SP10, SP12, and SP40) in NB containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for 24 hours

Isolate	Surface tension (mN/m)	% Reduction of surface tension	Oil Displacement Test (cm^2)
SP 4	**28.7**	**38.936**	**19.643**
SP10	32	31.915	0.786
SP12	32.5	30.851	13.860
SP40	30	36.170	15.211

From Table 4.3, the isolate number SP4 was given the highest value for the oil displacement test (19.643 cm^2) and the percentage reduction of surface tension (38.936%). Also, it was given the lowest value for surface tension (28.7mN/m). Therefore, the isolate SP4 was classified and studied for biosurfactant production.

4.3 Microorganism Identification

Table 4.4 Determination taxonomy of microorganism SP4 from Bergey's Manual (Holt *et al.*, 1994)

Characteristics	Bacteria colony SP4 Reaction	*Pseudomonas aeruginosa* Bergey's Manual	*Pseudomonas putida biovar A* Bergey's Manual
Gram reaction	Gm-	Gm-	Gm-
Reduction of nitrate	+	+	N/A
Indole production of trytophane	-	N/A	N/A
Fermentative of acid from glucose	-	N/A	N/A
Arginine dihydrolase	+	+	+
Urease production	-	N/A	N/A
Hydrolysis of esculin	-	N/A	N/A
Hydrolysis of gelatin	+	+	-
b-galactosidase production (p-nitro phenyl-b-galactopyranoside)	-	N/A	N/A

Assimilation of :			
Glucose	+	+	+
Arabinose	-	-	-
Mannose	-	-	N/A
Mannital	+	+	N/A
N-acetyl-glusamine	+	N/A	N/A
Maltose	-	-	-
Gluconate	+	+	+
Caprate	+	+	+
Adipate	+	+	-
Malate	+	+	+
Citrate	+	+	+
Phenyl-acetate	-	-	N/A
Cytochrome oxidase	+	+	+

From Bergey's manual of determinative bacteriology (Holt *et al.*, 1994) as used in table 4.4, the bacteria colony SP4 was classified as *Pseudomonas aeruginosa*. Therefore, the bacterial colony SP4 will be called *Pseudomonas aeruginosa* SP4.

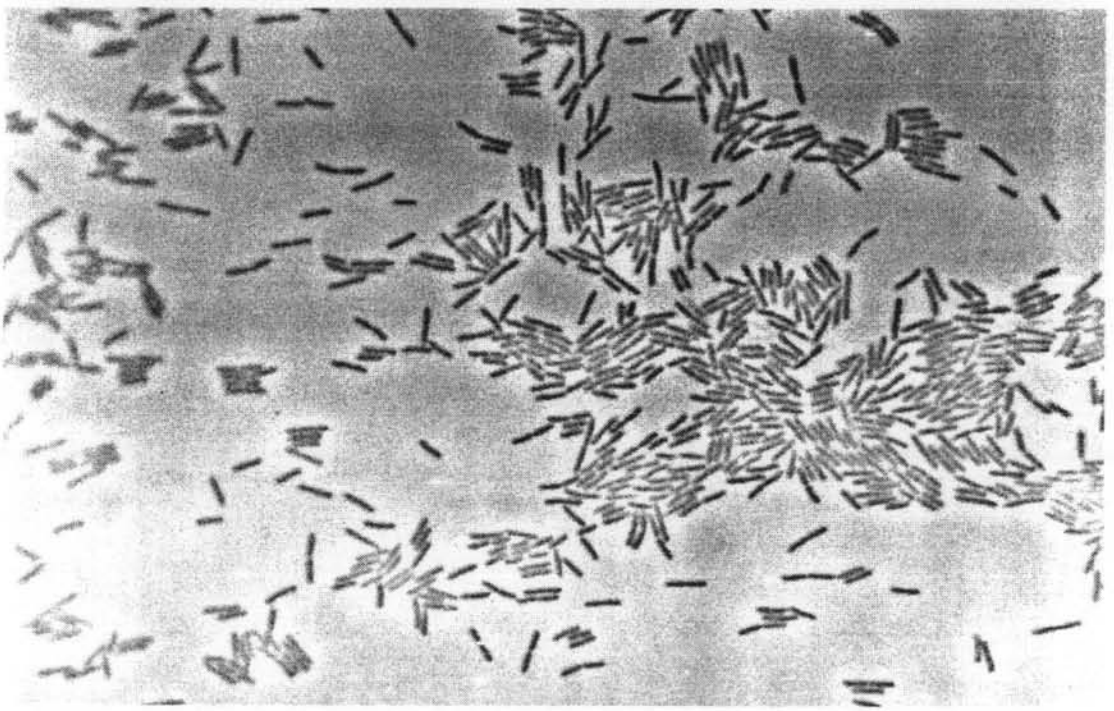

Figure 4.5 *Pseudomonas aeruginosa* SP4 under microscope.

4.4 Optimization of Culture Medium for Biosurfactant Production

After the cultivation of *Pseudomonas aeruginosa* SP4 in 3 mediums that-nutrient broth (NB), basal medium (BM), and defined medium (DM)-each of the mediums was varied of the percentage of palm oil from 2 to 10. The culture mediums were incubated at 37°C in a shaking incubator at 200 rpm for 72 hours. Tables 4.5–4.9 show the comparisons of the three mediums at the various palm oil percentages.

Table 4.5 Comparison of 3 culture mediums for the percentage reduction of surface tension and oil displacement test after cultivation in NB, BM, and DM containing 2% palm oil and incubation at 37°C in a shaking incubator at 200 rpm for 72 hours

Media & surface properties	% Reduction of surface tension	Oil displacement test (cm^2)
Nutrient broth	**43.2**	**132.8**
Basal medium	40.9	19.6
Defined medium	21.3	3.1

Table 4.6 Comparison of 3 culture mediums for the percentage reduction of surface tension and oil displacement test after cultivation in NB, BM, and DM containing 4% palm oil and incubation at 37°C in a shaking incubator at 200 rpm for 72 hours

Media & surface properties	% Reduction of surface tension	Oil displacement test (cm^2)
Nutrient broth	**40.8**	**95.1**
Basal medium	40.9	69.4
Defined medium	20.8	4.5

Table 4.7 Comparison of 3 culture mediums for the percentage reduction of surface tension and oil displacement test after cultivation in NB, BM, and DM containing 6% palm oil and incubation at 37°C in a shaking incubator at 200 rpm for 72 hours

Media & surface properties	% Reduction of surface tension	Oil displacement test (cm^2)
Nutrient broth	**43.8**	**75.5**
Basal medium	39.4	40.7
Defined medium	20.5	1.5

Table 4.8 Comparison of 3 culture mediums for the percentage reduction of surface tension and oil displacement test after cultivation in NB, BM, and DM containing 8% palm oil and incubation at 37°C in a shaking incubator at 200 rpm for 72 hours

Media & surface properties	% Reduction of surface tension	Oil displacement test (cm^2)
Nutrient broth	**41.6**	**95.1**
Basal medium	37.8	19.6
Defined medium	24.1	3.1

Table 4.9 Comparison of 3 culture mediums for the percentage reduction of surface tension and oil displacement test after cultivation in NB, BM, and DM containing 10% palm oil and incubation at 37°C in a shaking incubator at 200 rpm for 72 hours

Media & surface properties	% Reduction of surface tension	Oil displacement test (cm^2)
Nutrient broth	**40.2**	**113.1**
Basal medium	37.9	12.6
Defined medium	27.6	4.5

However, the best culture medium of each of the various percentages of palm oil was selected by considering the percentage reduction of surface tension and the oil displacement test. Therefore, the best culture medium of each of various percentage of palm oil was performed for selecting the best culture medium for *Pseudomonas aeruginosa* SP4. The comparisons are shown in Tables 4.10 and 4.11

Table 4.10 Comparison of the percentage reduction of surface tension of nutrient broth containing 2%, 4%, 6%, 8%, and 10% palm oil

% Palm Oil	Media	% Reduction of surface tension
2	**Nutrient broth**	**43.2**
4	Nutrient broth	40.8
6	Nutrient broth	43.8
8	Nutrient broth	41.6
10	Nutrient broth	40.2

Table 4.11 Comparison of the oil displacement test of nutrient broth containing 2%, 4%, 6%, 8%, and 10% palm oil

% Palm Oil	Media	Oil displacement test (cm^2)
2	**Nutrient broth**	**132.8**
4	Nutrient broth	95.1
6	Nutrient broth	75.5
8	Nutrient broth	95.1
10	Nutrient broth	113.1

From Tables 4.10 and 4.11, nutrient broth containing 2% palm oil was shown to be the best culture medium for culturing *Pseudomonas aeruginosa* SP4 which gave a percentage reduction of surface tension was 43.2% and an oil displacement test (ODT) was 132.8 cm^2. Considering the effect of carbon sources on the production of *Pseudomonas aeruginosa* SP4, we found that 2%v/v palm oil as carbon source gave the best surface activity. The present result is similar to the result of Chongchin *et al.* (1999) who reported that the best optimum of palm oil as a carbon source was 2%v/v. Therefore, nutrient broth containing 2% palm oil was selected for culturing *Pseudomonas aeruginosa* SP4 for the next section.

4.5 Microbial Growth Determination

The *Pseudomonas aeruginosa* SP4 was cultivated in nutrient broth and nutrient broth containing 0.02% glucose and was incubated at 37°C in a shaking

incubator at 200 rpm. The absorbance of both culture mediums was determined every 3 hours for 48 hours, as shown in Figure 4.6.

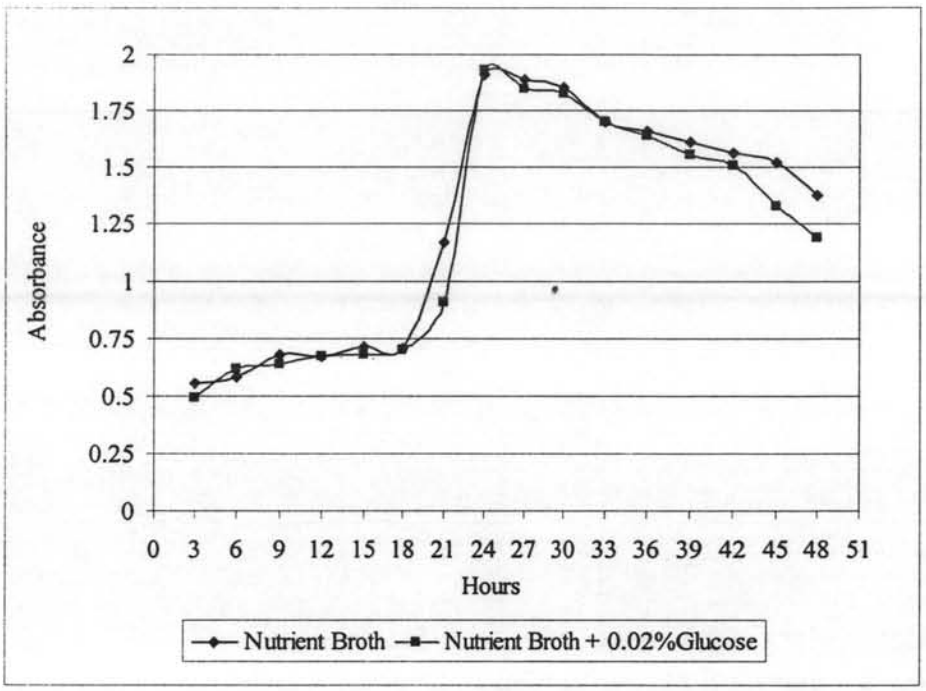

Figure 4.6 Microbial growth determination of absorbance of culture medium at 600 nm after culturing *Pseudomonas aeruginosa* SP4 in nutrient broth and nutrient broth containing 0.02% glucose and incubation at 37°C in a shaking incubator at 200 rpm.

From Figure 4.6, it can be seen that at the mid-log phase (approximately 22 hours) the nutrient broth gave more density of *Pseudomonas aeruginosa* SP4 than the nutrient broth containing 0.02% glucose. Also, nutrient broth's time to dead phase was slower than the nutrient broth containing 0.02% glucose. Therefore, nutrient broth without 0.02% glucose was selected as the medium for the inoculums.

Moreover, the suitable time for culturing *Pseudomonas aeruginosa* SP4 in nutrient broth without 0.02% glucose was the mid-log phase.

4.6 Determination of Optimum Inoculums

The *Pseudomonas aeruginosa* SP4 was cultivated in nutrient broth without 0.02% glucose for 22 hours. Then, *Pseudomonas aeruginosa* SP4 was transferred from the culture medium to nutrient broth containing 2% palm oil using various

amounts of inoculums (2%, 4%, 6%, and 8%). After incubation at 37°C in a shaking incubator at 200 rpm for 24 hours and 48 hours, surface tension, dry cell weight, and oil displacement (Morikawa *et al.,* 1993) tests were performed. Table 4.12 shows the results.

Table 4.12 Comparison of the percentage inoculums after culturing in nutrient broth containing 2% palm oil and incubating at 37°C in a shaking incubator at 200 rpm

Hours	Percent inoculums (v/v)	Surface tension (mN/m)	Oil Displacement Test (cm^2)	Dried cell weight (g/l)
24	**2**	**29.2**	**28.3**	**1.8740**
	4	29.4	28.3	2.3760
	6	29.5	28.3	3.7240
	8	31.8	19.6	4.9120
48	**2**	**27.1**	**119.1**	**5.7460**
	4	27.1	102.1	2.0080
	6	28.1	84.9	7.6640
	8	27.9	113.1	12.6580

From the results in Table 4.12, the 2% inoculums gave the lowest surface tension value (29.2 mN/m and 27.1 mN/m) and the highest oil displacement test value (28.3 cm^2 and 119.1 cm^2) for 24 hours and 48 hours, respectively. Therefore, the 2% inoculums are used for the biosurfactants production.

4.7 Growth Curve of Biosurfactant-Producing Bacteria

The *Pseudomonas aeruginosa* SP4 was cultivated in a suitable medium (nutrient broth including 2% palm oil) for 120 hours. Then, pH, surface tension, dry weight cell, and oil displacement (Morikawa *et al.,* 1993) were determined every 3 hours for the first 24 hours and every 6 hours until 120 hours. The results are shown in Figure 4.7 where it can be seen that the lag phase was 0-6 hours and the log phase was between 6 and 36 hours after that it was in the stationary phase and the dead phase from 54 to 120 hours. Moreover, biosurfactant started to produce from the first 6 hours.

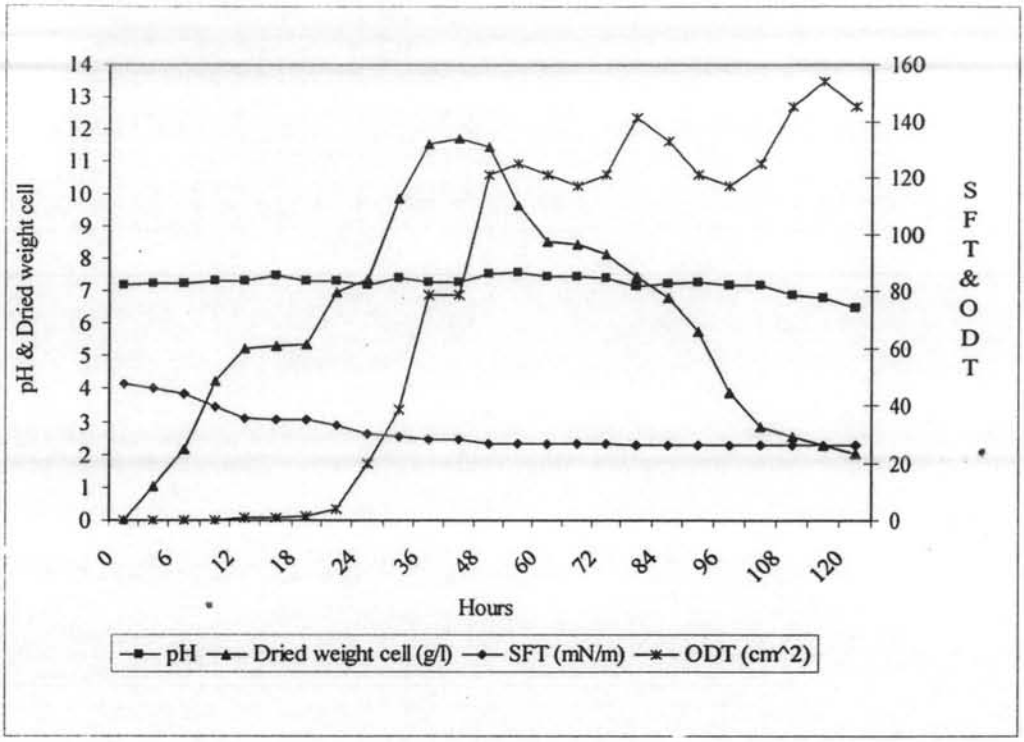

Figure 4.7 Growth curve of biosurfactant-producing bacteria.

From Figure 4.7, it can be seen that biosurfactant began to be produced and released into the culture medium in the early logarithmic phase, and the surface tension of the culture broth began to decrease in the middle of the logarithmic phase.

By examining the surface tension of the culture broth, we found that at 48 hours of culturing *Pseudomonas aeruginosa* SP4, the surface tension was 26.9 mN/m. This finding is nearly similar to that of Dubey *et al.* (2001), who reported that the *Pseudomonas aeruginosa* BS2 possessed the potential surface-active properties, as it effectively reduced the surface tension of water from 72 to 27 mN/m. Also, Thaniyavarn *et al.* (2004) reported that the surface tension of the culture broth for biosurfactant from *Pseudomonas sp.* A41 was 30 mN/m.

The lowest surface tension and the highest oil displacement of biosurfactant was at 114 hours, but this time was not selected because it takes a long time and is not economically in the real operational situation. And it was observed that *Pseudomonas aeruginosa* SP4 was grown in the stationary phase and was grown associated. This observation was in good agreement with the result from Koch *et al.* (1988). Therefore, the stationary phase (at 48 hours) was selected for the biosurfactant production. Also, the Critical Micelle Concentration (CMC) at 48 hours was derived according to

Sheppard and Mulligan (1987) that was 3.6% v/v and the surface tension at the CMC was 28.3 mN/m, as shown in figure 4.8. Also, Critical Micelle Concentration (CMC) in term of mg/L was determined by using Thermal Gravimetric Analysis to find net weight of biosurfactant in supernatant solution. Thus, the CMC of free-cell broth was 61 mg/L.

This result is likely to that of Chongchin *et al.* (1999) who reported that the CMC of *Pseudomonas sp.* A41 is 3.6 %v/v but their research work *Pseudomonas sp.* A41 was cultured at a different medium.

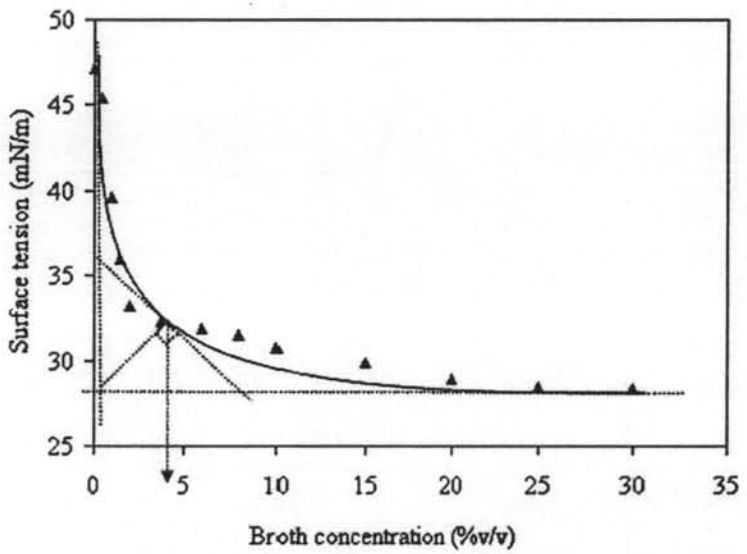

Figure 4.8 The Critical Micelle Concentration (CMC) of the culture broth which produces biosurfactants from *Pseudomonas aeruginosa* SP4.

4.8 Extraction of Biosurfactants and Analysis of Biosurfactants by Thin Layer Chromatography (TLC)

To directly detect the crude concentrated biosurfactants (glycolipids) an independent test that has been previous used were carried out. This included detection by Thin Layer Chromatography (TLC) (Koch *et al.,* 1988). The crude concentrated biosurfactant was extracted by 2:1 chloroform to ethanol (Zhang and Miller, 1992). Then, the crude concentrated biosurfactant was spotted on a TLC plate (Silica gel 60, 20×20 cm). The TLC plate was immersed in a tank consisting of 65% chloroform, 25% methanol, and 4% water, as shown in Figure 4.9.

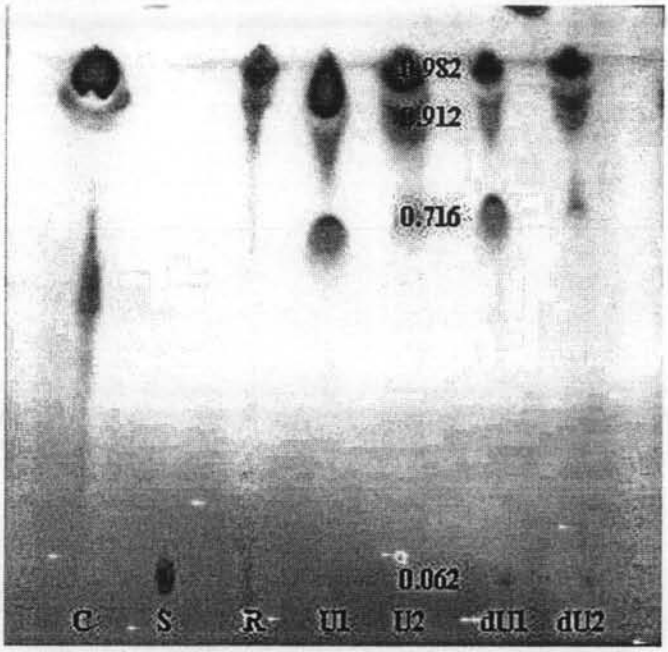

Figure 4.9 The separation of biosurfactants on a TLC plate.

From Figure 4.9, in the thin layer analysis the crude concentrated biosurfactants was applied to the silica gel thin layer plate and four typical crude concentrated biosurfactant spots were revealed after iodine staining. Each of the codes were classified as C= *Candida Antartica*, S= *Surfactin*, R= *Rhamnolipid*, U= Unknown sample (biosurfactants from *Pseudomonas* SP4), and dU= Dilution of unknown sample (biosurfactants from *Pseudomonas* SP4). Also, the rate of flow of each of the points was calculated as shown in Table 4.13.

Rf = Distance of moving substance on TLC plate / Distance of moving solvent

Table 4.13 Rate of flow (Rf value) of biosurfactants

Distance of moving solvent (cm)	17.000	
Distance of moving substance (cm)	Point 1	16.700
	Point 2	15.500
	Point 3	12.000
	Point 4	1.050
Rate of Flow	Point 1	0.982
	Point 2	0.912
	Point 3	0.706
	Point 4	0.062

The biosurfactant from *Pseudomonas aeruginosa* SP4 (dU) was classified into four points. Moreover, at point number 1 the biosurfactant from *Pseudomonas aeruginosa* SP4 (dU) had a Rf value the same as surfactin, and other points hadn't the same Rf value when compared with biosurfactant from *Candida Antartica* and rhamnolipid surfactants. Therefore, the biosurfactants from *Pseudomonas aeruginosa* SP4 (dU) consisted of four compounds of surfactants and one of them expected to be a surfactin.

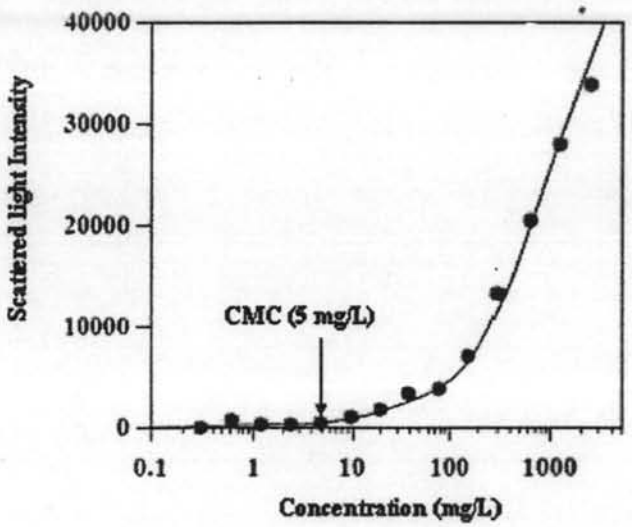

Figure 4.10 The critical micelle concentration of biosurfactants from *Pseudomonas aeruginosa* SP4.

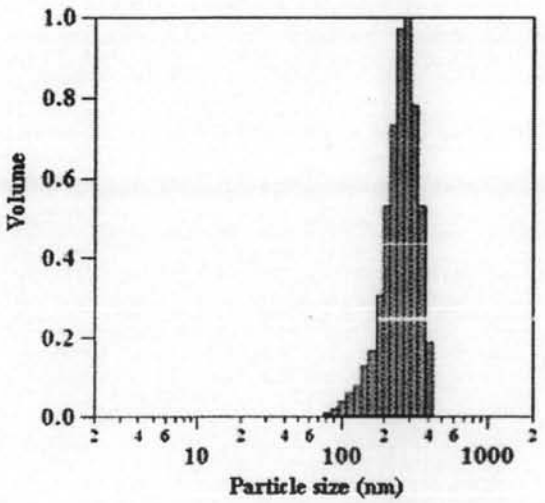

Figure 4.11 The micelle size of biosurfactants from *Pseudomonas aeruginosa* SP4 at 39.1 mg/L.

From figure 4.10 and 4.11, it can be seen that the critical micelle concentration was 5 mg/L and the particle size at 39.1 mg/L was 300 nm of crude biosurfactant from *Pseudomonas aeruginosa* SP4 were derived by dynamic light scattered measurement.

4.9 Preliminary Test of Biosurfactants in Free-Cell Broth for Oil Recovery from Ottawa Sand

All biosurfactants produced from potential cultures were tested for how well they are recover oil from Ottawa sand. A cylinder was packed with Ottawa sand and flooded with a motor oil complex (the motor oil was held by the pores of the sand). Then, the biosurfactants solution was flushed through the column for recovering the motor oil from the Ottawa sand. After that, the recovered oil was analyzed by the total organic carbon analyzer (TOC), as shown in Figure 4.12.

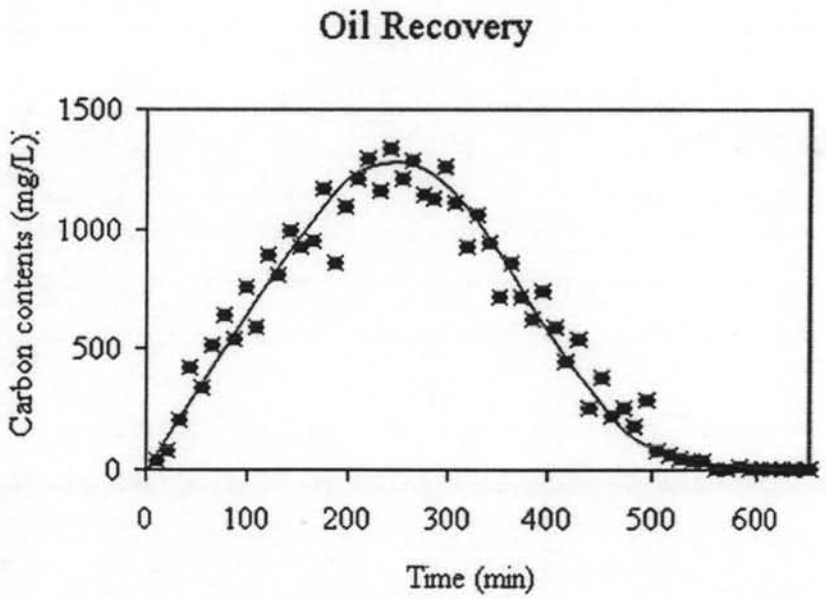

Figure 4.12 The recovered oil curve from total organic carbon analyzer (TOC).

From Figure 4.12, the diesel motor oil complex that was recovered reached to maximum point around 220 minutes. Then, the rate of recovered oil decreased until 600 minutes and the percentage of oil recovery was 76.37. Also, this percentage of oil

recovery is better than the percentage of oil recovery by use 0.2%Alfoterra145-4PO + sodium dioctyl sulfosuccinate +1% NaCl (Thakerngwat *et al.*, 2005).

Table 4.14 The comparison of biosurfactant and chemical surfactant for oil recovery from Ottawa sand

Surfactant used	The percentage of oil recovery from Ottawa Sand
Biosurfactant from *Pseudomonas aeruginosa* SP4	**76.37%**
0.2%Alfoterra145-4PO + sodium dioctyl sulfosuccinate +1% NaCl (Thakerngwat *et al.*, 2005)	67.85%
0.2%Alfoterra145-4PO + sodium dioctyl sulfosuccinate +5% NaCl (Thakerngwat *et al.*, 2005)	77.87%