CHAPTER IV
RESULTS AND DISCUSSION

4.1 Isolated Biosurfactant-Producing Microorganisms

4.1.1 Numbers of Isolated Microorganisms

Samples from petroleum contaminated soil, deep sea water, and oil sludge were screened on NA plates. Then, each of the isolated bacterial colonies was tested on NA plates covered with 30 µl of crude oil. After that, the NA plates were incubated at a temperature of 37±2°C for 24 h. The clear zones around the bacterial colonies are measured (Morikawa *et al.,* 1993), as shown in Figure 4.1. Table 4.1 shows numbers of microbes found in each source that show the potentials in biosurfactant production. There were overall 53 stains found in the 4 sources but only 10 stains showed the ability in biosurfactant production as indicated by the clear zone. As expected, a large variety of biosurfactant-producing bacteria was found in petroleum-contaminated sources (car service and oil sludge samples). Only the ten isolated stains capable in produce biosurfactants were selected for further investigation.

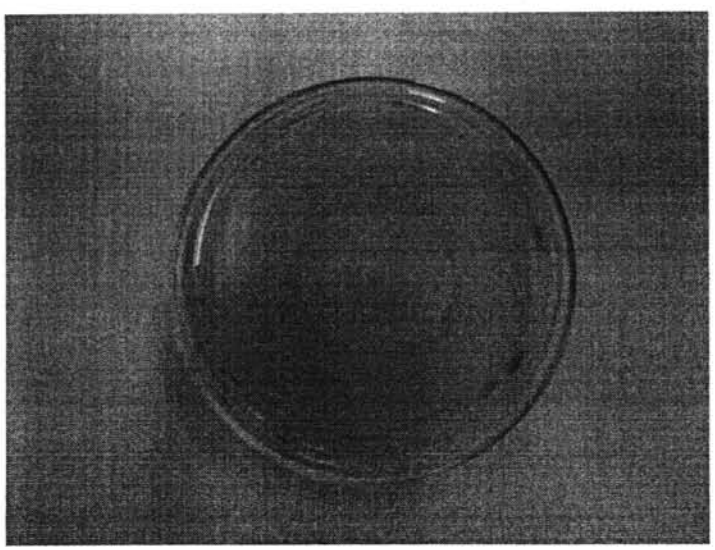

Figure 4.1 Clear zone around a bacterial colony on the oil layer..

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	20	3
Rayong (Sea water)	1	11	1
Chonburi (Sea water)	3	2	0
Oil Sludge (PTT, Bangchak)	2	20	6
Total	8	53	10

* Clear zone around colony on agar nutrient containing crude oil. (We have to specify the diameter of clear zone how large that use consider to have a biosurfactant-producing bacteria)

4.1.2 Surface Activities of Isolated Microbes

In this part, only 10 active stains in producing biosurfactants which were isolated from the car service, sea water and oil sludge were tested their surface activities using two methods: the oil displacement test and the surface tension measurement. The NB containing 2% palm oil were used to cultivate each tested stain at 37°C for 48 using the shaker at 200 rpm. The centrifuged culture broths cultivated from all six stains isolated from the oil sludge sample show the superior surface activity as shown in Figure 4.2.

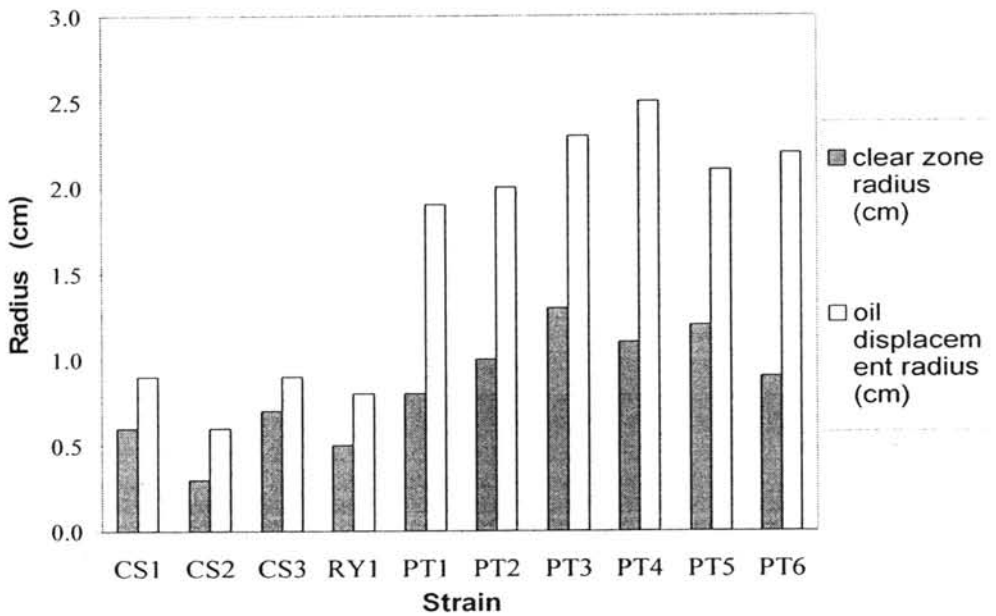

Figure 4.2 Surface activities of 10 isolated stains using oil displacement test and clear zone at 24 hours.

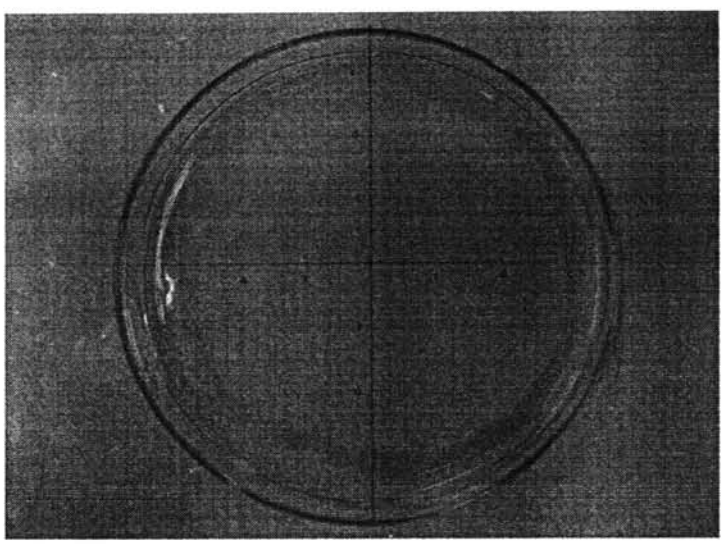

Figure 4.3 Activity of biosurfactant-producing bacteria for oil displacement test.

Apart from the measurement of surface activity by using the oil displacement test and the clear zone, the culture broth samples before and after cultivation with 10 isolated stains were measured for surface tension using the Dunouy ring tensiometer. Table 4.2 shows the surface tensions and the surface tensions reductions of all the culture supernatant of the 12 isolated stains. The surface tension of the nutrient broth (γ_{NB}) was found to be 47 mN/m. The reduction of surface tension is calculated by the following equation:

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

Where; γ_{bc} = surface tension before cultivation = γ_{NB} = 47 mN/m

γ_{ac} = surface tension after cultivation

As shown in Table 4.2, PT4 shows the highest surface activity as indicated by the highest surface tension reduction. Table 4.2 shows a comparison of the stains which are classified to provide active surface activity greater than 30% surface tension reduction. Bacteria PT4 that exhibited the highest surface activities was then selected for further investigation.

Table 4.2 Surface tension and the percentage reduction of surface tension of the culture supernatant obtained from 16 strains in NB containing 2% palm oil at a temperature of 37±2°C in a shaker at 200 rpm for 48 h

Source	strain	surface tension (mN/m)	%reduction of surface tension
Car service	CS1	38.2	18.72
	CS2	45.0	4.26
	CS3	32.0	31.91
Sea water	RY1	43.3	7.87
Oil sludge	PT1	33.0	29.79
	PT2	32.5	30.85
	PT3	30.0	36.17
	PT4	26.5	43.62
	PT5	29.4	37.45
	PT6	34.7	26.17

Table 4.3 Surface tension, the percentage reduction of surface tension, and oil displacement test of the culture supernatant from the 5 isolated stains (CS3, PT2, PT3, PT4, and PT6) in NB containing 2% palm oil at a temperature of 37±2°C in a shaker at 200 rpm for 48 h

strain	surface tension (mN/m)	% Reduction of surface tension	Oil displacement test (cm^2)	Oil displacement test (cm)
CS3	32.0	31.91	2.55	0.9
PT2	32.5	30.85	12.57	2.0
PT3	30.0	36.17	16.63	2.3
PT4	**26.5**	**43.62**	**75.46**	**2.5**
PT6	29.4	37.45	13.86	2.1

4.1.3 Optimization of Culture Medium for Biosurfactant Production

To optimize biosurfactant production, bacteria PT4 was cultivated in nutrient broth (NB) containing different oil contents (2 to 10 % of palm oil) at 37°C in the shaker bath for 48 h. Figure 4.4 shows the results of surface tension, oil displacement and dried all weights obtained at different oil concentrations. As can be seen in Figure 4.4, both surface activity and dried weight cell do not vary with oil concentration. Hence, the minimum oil concentration of 2 % was selected for further experiments.

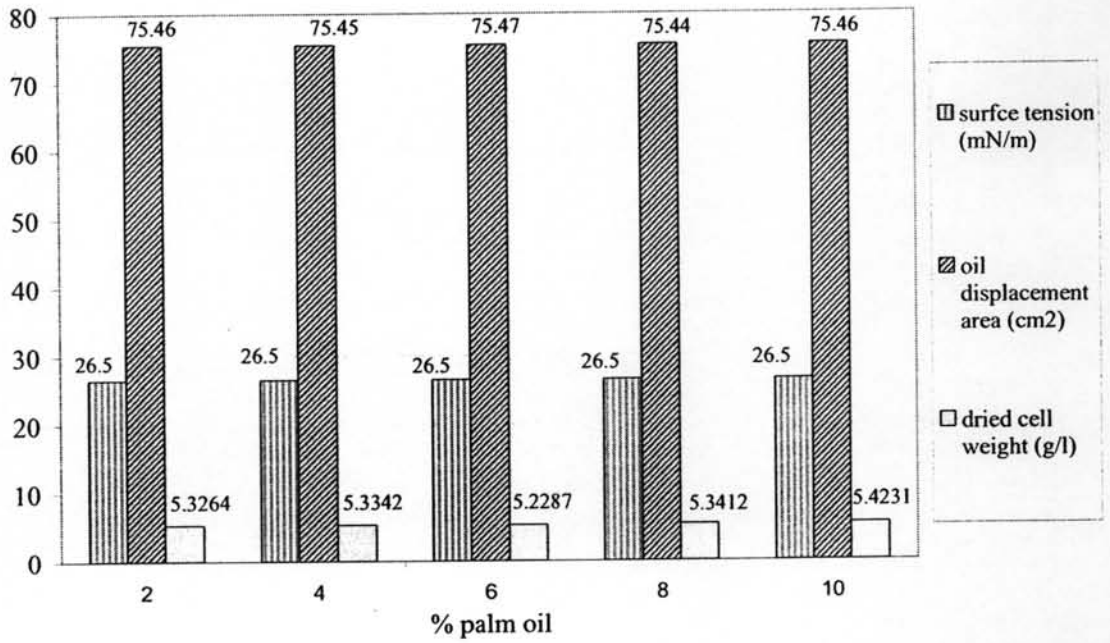

Figure 4.4 The comparisons of the Nutrient Broth at the various palm oil percentages using PT4 at different palm oil concentrations and 37°C.

4.1.4 Growth Curve of Stain PT4

A growth curve of PT4 was carried out at 2 % oil and 37°C. The absorbance was used to indicate microbial concentration in the culture medium. Figure 4.5 shows the profile of microbial concentration with a long lay period of 24 h. According to the growth curve as shown in Figure 4.5, the optimum cultivation time of 30 h corresponding to the maximum bacterial concentration was selected for further investigation.

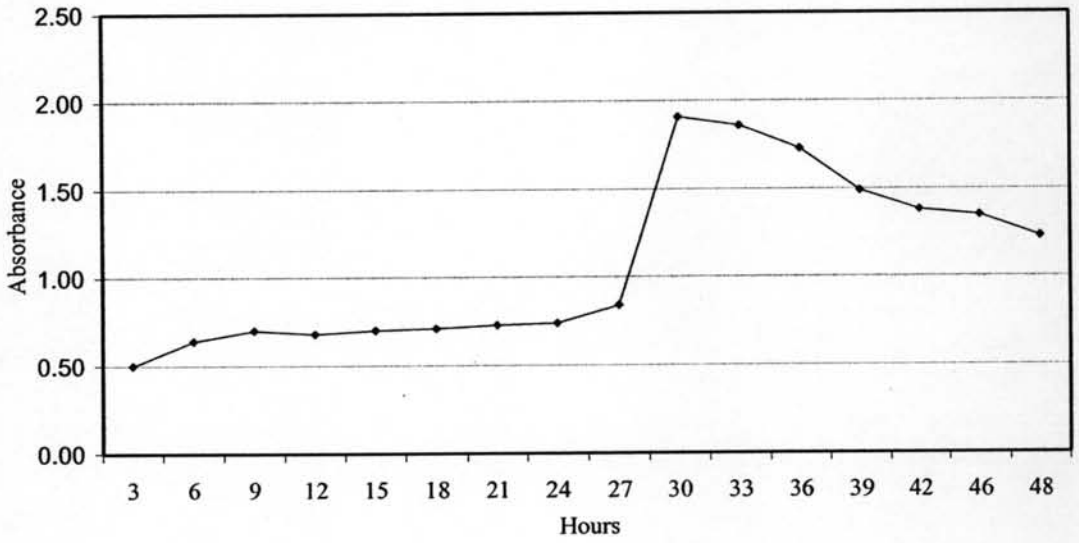

Figure 4.5 Growth curve of PT4 at 37°C using absorbance measurement at 600 nm.

The PT2 stain was cultivated in a suitable medium (NB+2% palm oil) for 96 h. Then, surface tension, dry cell weight, and oil displacement (Morikawa *et al*, 1993) were determined every 3 h for 96 h. The results are shown in Figure 4.6. The lag phase appeared in the first range of 0-6 h and the log growth phase was found in the range of 6 to 45 h after that it reached the stationary phase around 50 h. Moreover, the biosurfactants appeared after first 6 h and reached a maximum around 60 h. Based on the growth curve as well as the oil displacement profile, 51 h was selected as an optimum cultivation time for further study.

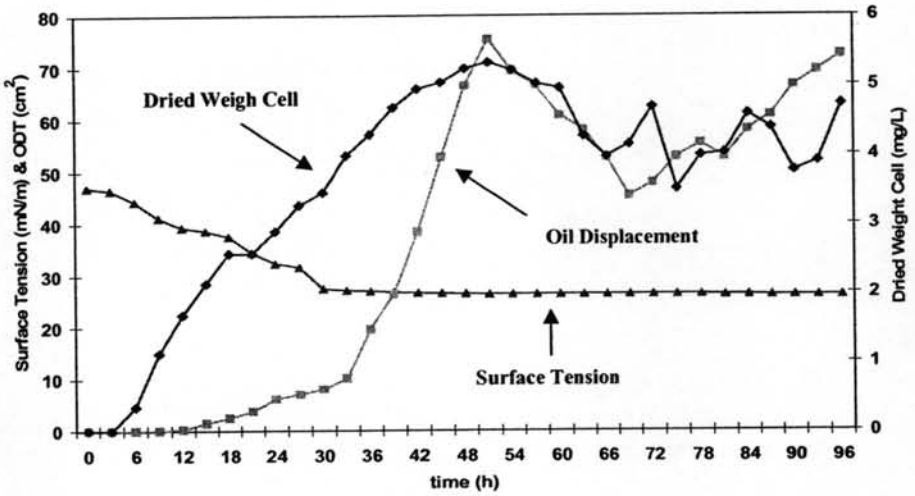

Figure 4.6 Growth curve of PT4 in NB with palm oil at 37°C.

Figure 4.7, shows the growth curve of PT4 in NB with 2 % extracted oil from oil sludge as the carbon source. The lowest surface tension and the highest oil displacement of biosurfactants were found at 40 h. The results, suggest that PT4 is compatible with the sludge oil more than palm oil because PT4 is the microorganism isolated from the oil sludge sample.

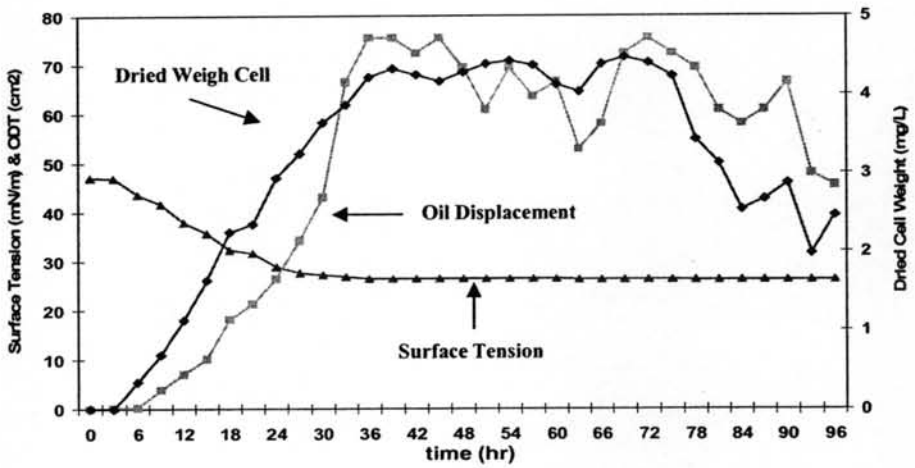

Figure 4.7 Growth curve of PT4 in NB with 2 % extracted oil at 37°C.

To determine the CMC, the broth obtained from 51 h cultivation time of PT4 using 2 % palm oil as the carbon sources was extracted to obtain a crude biosurfactant sample. The water content in the crude biosurfactant sample was analyzed by using thermal gravimetric analysis. After that, the crude biosurfactant sample was diluted to obtain the different concentrations to measure surface tension. Figure 4.8 show the plot of surface tension and biosurfactant concentration.

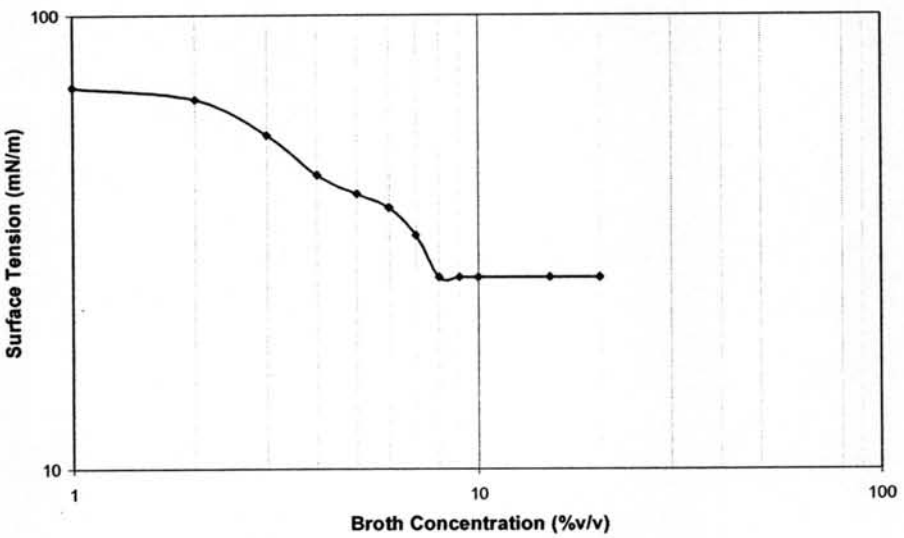

Figure 4.8 Surface tension at different concentrations of biosurfactant produced from PT4 using 2 % palm oil in NB at 37°C.

4.2 Microorganism Identification

The PT4 stain was taken for identification and the results are shown in Table 4.4. It was identified as *Bacillus Subtilis* (Arima *et al.,* 1968).

Table 4.4 Characteristics of the bacterial strain PT2

Characteristics	Reaction
Fermentative production of acid from :	
- esculine	+
- salicine	+
- cellobiose	+
- maltose	+
- lactose	-
- melibiose	+
- inuline	+
- melezitose	-
- D-raffinose	-
- starch	+
- glycogene	+
- xylitol	–
- ß-gentiobiose	+
- D-turanose	-
- D-lyxose	-
- D-tagatose	-
- D-fucose	-
- L-fucose	-
- D-arabitol	-
- L-arabitol	-

Table 4.4 (con't) Characteristics of the bacterial strain PTT2

Characteristics	Reaction
- gluconate	-
- 2-keto-gluconate	-
- 5-keto-gluconate	-
ß-galactosidase production (ortho-nitro-phenyl-ß-D-galactopyranoside)	+
Arginine dihydrolase production	-
Lysine decarboxylase production	-
Ornithine decarboxylase production	-
Citrate utilization	-
H_2S production	-
Urease production	-
Tryptophane deaminase production	-
Indole production of tryptophane	-
Acetoin production	+
Hydrolysis of gelatin	+
Reduction of nitrate	+

Remark : + = Positive reaction

- = Negative reaction

4.3 Results of Oil Recovery Activities

For the experiments of oil recovery activity, a cylinder packed with Ottawa sand was flooded with a motor oil complex. Then, a biosurfactant solution was flushed through the column. Figure 4.9 shows the oil recovery profiles using two broth solution

produced by PT4 under palm oil and motor oil as the carbon sources. As can be seen from Figure 4.19, the broth produced from PT4 using motor oil as the carbon source exhibits a greater oil recovery activity than the broth produced from PT4 using palm oil. As expected, the biosurfactants produced from motor oil can solubilize motor oil better than that produced from palm oil.

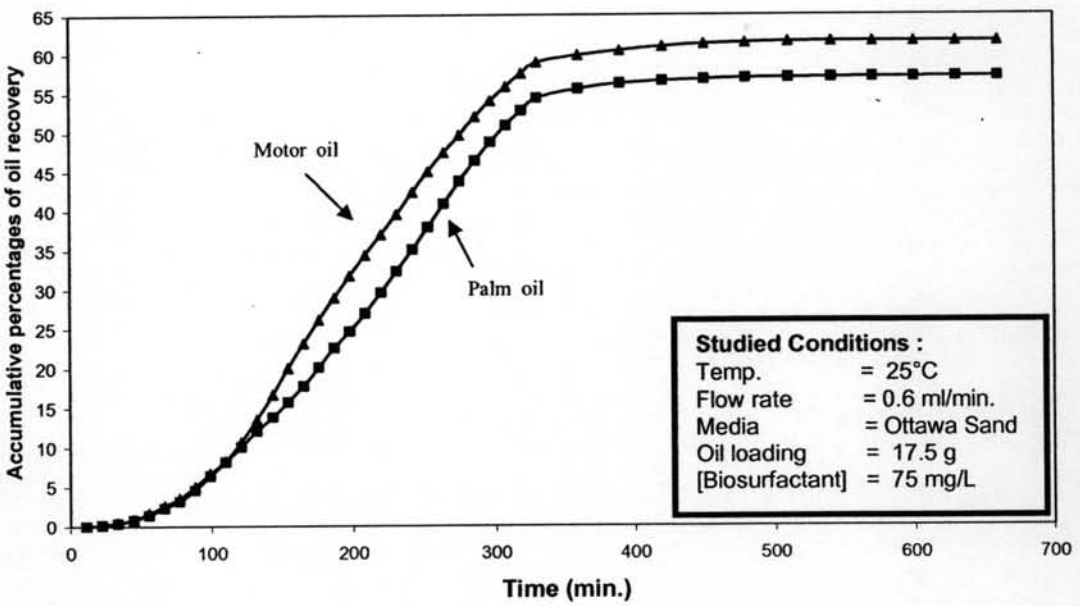

Figure 4.9 The oil recovery curve using two nutrient broth solution produced by PT4 with palm oil and motor oil at 37°C and cultivation times of 51 and 40 h, respectively.

In addition, the biosurfactant-containing broth produced from *Pseudomonas* SP4 (Paisanjit, 2005) was also investigated its oil recovery activity. Figure 4.10 shows the effect of carbon source used to cultivate *Pseudomonas* SP4 on the oil recovery activity which save a similar trend as the biosurfactants produced by *Bacillus* (PT4).

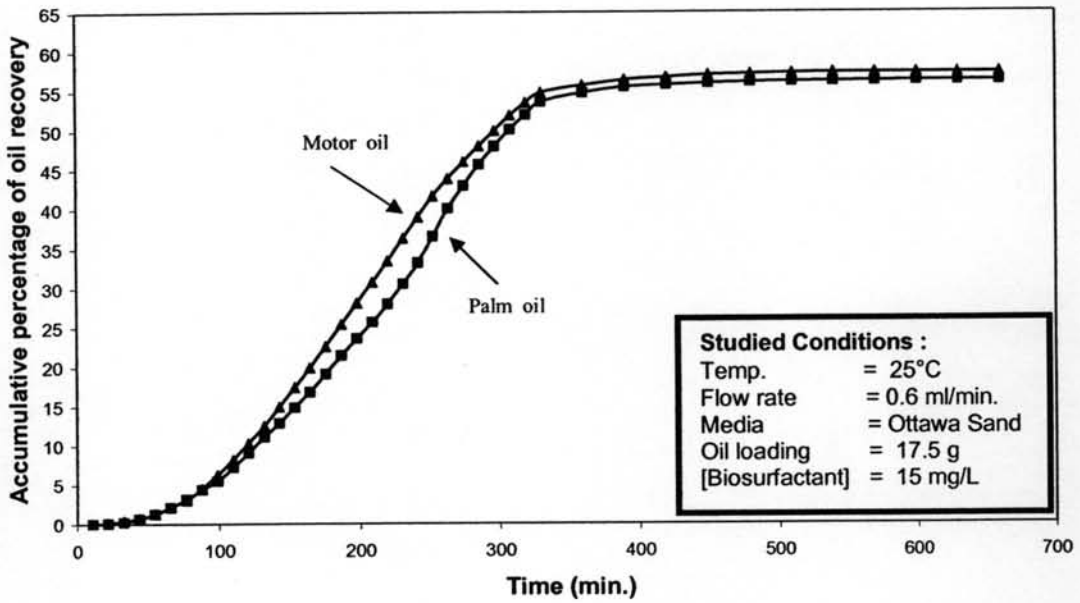

Figure 4.10 The oil recovery curves using two nutrient broth solutions produced *Pseudomonas* SP4 with palm oil and motor oil at 37°C.

Moreover, three commercially synthetic surfactants (Alfoterra, tween 80 and SDBS) were also tested for oil recovery activity in order to compare with the biosurfactants produced from two stains of *Pseudomonas* and *Bacillus*. Figure 4.11 illustrates the oil recovery activities of the three synthetic surfactants, Alfoterra, Tween 80 and SDBS.

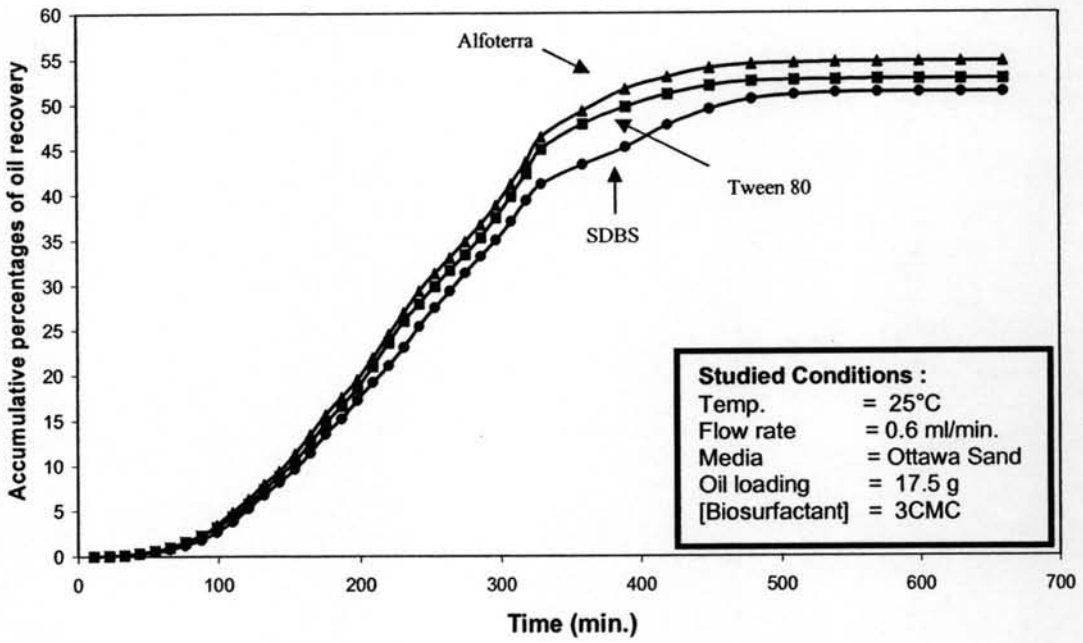

Figure 4.11 Oil recovery profiles of different synthetic surfactants.

Table 4.5 shows the oil recovery activities of the two biosurfactants produced from *Pseudomonas* and *Basillus* stains compared to the three synthetic surfactants. Both biosurfactants exhibit higher activities in oil recovery than the synthetic surfactants. This is because most biosurfactants have two tails which can enhance the oil solubilization as compared to the tested synthetic surfactants which have only one tail.

Table 4.5 Comparisons of oil recovery activity of two biosurfactants and three commercially synthesis surfactants (studied conditions: temp 25°C, 3 CMC, oil loading 17.5 g).

Surfactant used	Activity of motor oil recovery, %
Biosurfactant produced from *Bacillus Subtilis* PT2	**63.56**
Biosurfactant produced from *Pseudomonas Aeroginosa* SP4	58.56
Tween 80	53.65
SDBS	52.17
Alfoterra	55.16