CHAPTER IV
RESULTS AND DISCUSSION

4.1 Characterization of Mineral medium and Palm Oil

In this present study, the MM with palm oil was used as the culture medium for the biosurfactant production by *Pseudomonas aeruginosa* SP4. The MM contained 2.0 g/l of $NaNO_3$, 1.0 g/l of K_2HPO_4, and 0.5 g/l of KH_2PO_4, and palm oil was used as a sole carbon source. The C/N and C/P ratios of the culture medium were kept constant at 16/1 and 14/1, respectively, because they were reported to be the optimum ratios for the rhamnolipid production by *Pseudomonas aeruginosa* (Guerra-Santos *et al.*, 1984). Table 4.1 displays the characteristics of the MM and palm oil used in this present study. It was found that COD and TOC of the MM were zero while those of palm oil were relatively high. However, pH of MM was higher than that of palm oil. The SS of the MM and palm oil were zero.

Table 4.1 Characteristics of mineral medium (MM) and palm oil

Parameters	Mineral medium (MM)	palm oil
COD (mg/l)	0	1,250,800
TOC (mg/l)	0	42,130
Total nitrogen (mg/l)	290	1,500
Total phosphorus (mg/l)	291	90
Suspended solids (mg/l)	0	0
Surfactant concentration (xCMC)	0	0
pH	8.6	4.6
Surface tension (mN/m)	70.89	-

4.2 Effect of Cycle Time on Biosurfactant Production

In this part, the reactor performance was evaluated by using 6 parameters: % COD removal, % oil removal, surface tension reduction, mixed liquor suspended solid (MLSS), suspended solid (SS), and pH. The effect of cycle time on the reactor performance was studied at three different cycle times (1, 2, and 3 d/cycle times) with an oil loading rate (OLR) of 2 kg/m^3d.

4.2.1 Effect of Cycle Time on COD removal

The Chemical Oxygen Demand (COD) measurement is the method to determine the concentration of organic compounds in a sample in terms of mg/l of oxygen required to oxidize the organic compounds in the sample solution. In this experiment, CODs of both influent and effluent were measured in order to determine the degradation capability of the microbes for utilizing organic substances as their nutrients. Therefore, the constant COD can be used to indicate the steady-state condition. The influent COD mainly came from palm oil because the COD of the MM was zero, while the effluent COD was related to the remaining palm oil, the excreted biosurfactant, and some metabolites. As shown in Figure 4.1, the effluent COD fluctuated in the beginning of all three cycle times. It was also found that each cycle time required different period of time to reach steady-state condition (Cassidy *et al.*, 2002). For cycle time of 1 and 2 d/cycle, the SBR system reached the steady-state condition after 4 operation days, while the steady-state condition of the cycle time of 3 d/cycle was observed after 9 operation days. Figure 4.1 shows that the effluents COD of 1/d cycle time significantly increased with increasing time for the first 4 days but it remained constant at around 2,200 mg/l, corresponding to the COD removal of about 72% as shown in Figure 4.2. In the same trend, the effluent COD of the cycle time of 2 d/cycle also significantly increased before remained constant after 4 operation days, indicating the steady-state condition. Compared to the cycle time of 1 d/cycle, the 2 d/cycle gave a lower average effluent COD of 1,600 mg/l (days 10-14) resulting in a higher COD removal of nearly 90%, as illustrated in Figure 4.3 and 4.4. Interestingly, the cycle time of 3 d/cycle gave the highest effluent COD of 14,1120 mg/l leading to the lowest COD removal of about 41 %. The results suggest

that the microorganisms could not effectively utilize palm oil at the cycle time of 3/d cycle. Regarding to the COD removal results, the system operated at 2 d/cycle provided the maximum performance.

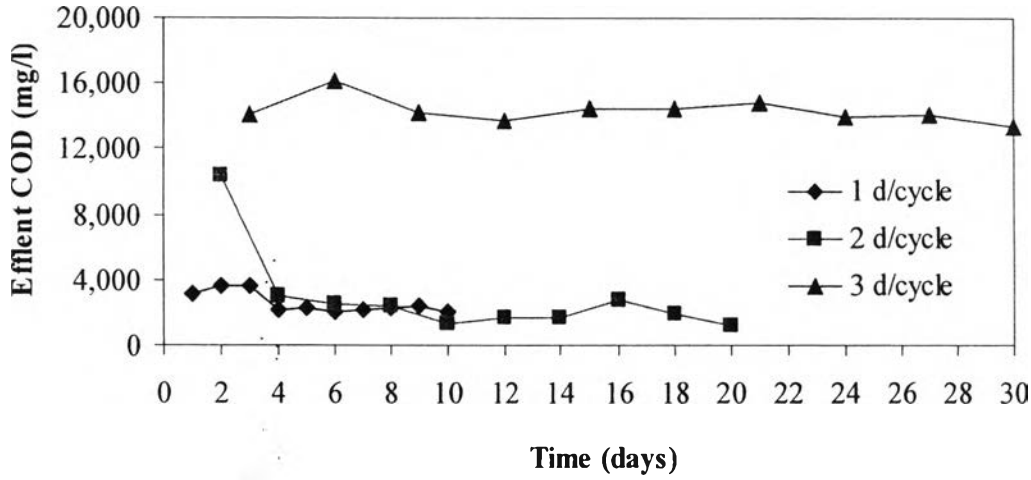

Figure 4.1 Effluent COD as a function of operation time and cycle time at an oil loading rate of 2 kg/m^{3}d.

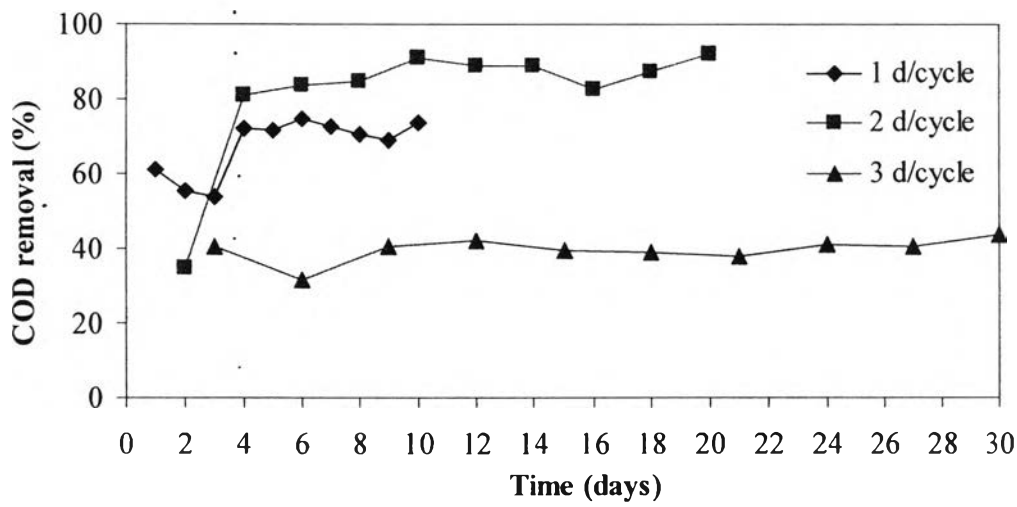

Figure 4.2 COD removal as a function of operation time and cycle time at an oil loading rate of 2 kg/m^{3}d.

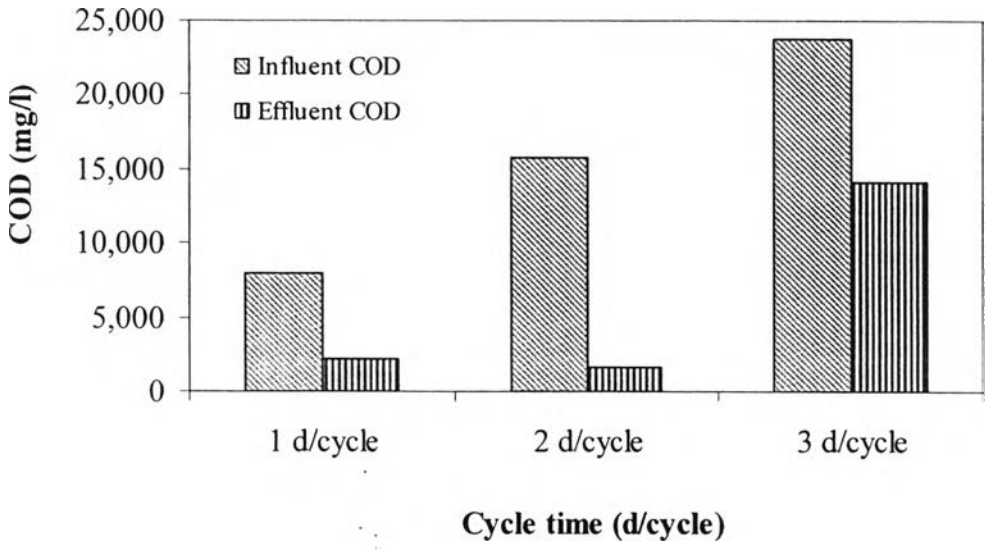

Figure 4.3 The average COD of influent and effluent during steady-state operation at an oil loading rate of 2 kg/m^{3}d and different cycle times.

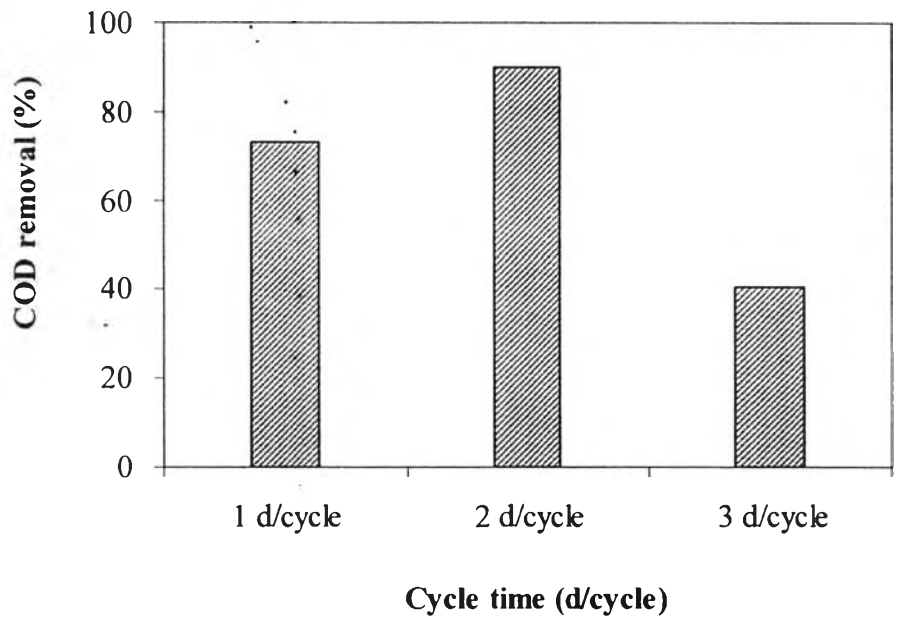

Figure 4.4 COD removal during steady-state operation at an oil loading rate of 2 kg/m^{3}d and different cycle times.

4.2.2 Effect of Cycle Time on Oil removal

Oil removal was used to directly indicate the palm oil consumption by the microbe for the biosurfactant production. Figure 4.5 shows the effluent average oil concentration and the percentage of oil removal at different cycle times. It was found that the oil removal of 89.48%, 96.66%, and 71.70% was observed at 1, 2, and 3 d/cycle, respectively. The results suggest that the microorganism could effectively consume palm oil at the cycle time of 3 d/cycle. The decrease in the oil removal at 3 d/cycle indicated that overload condition. The results clearly suggest that the microbial ability to degrade carbon source depends on the operation time.

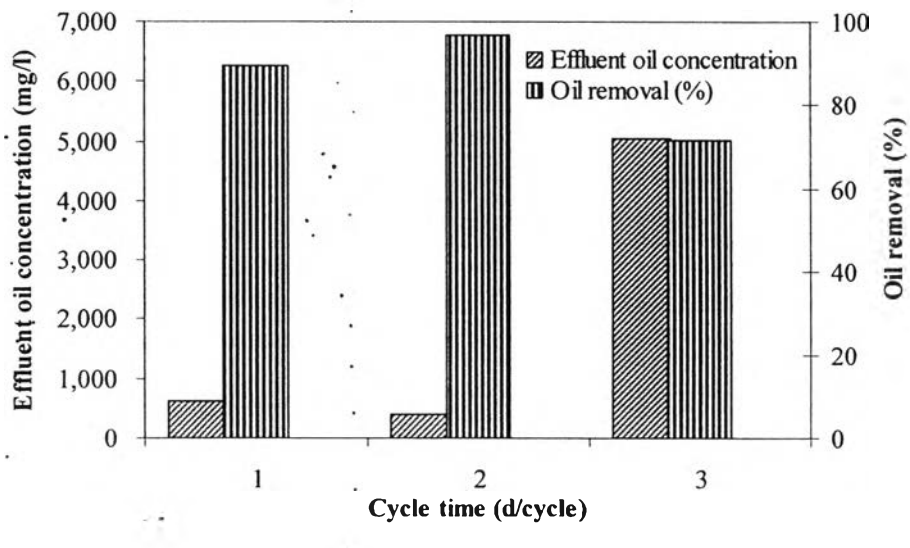

Figure 4.5 Oil removal and effluent oil concentration as a function of cycle time during steady-state operation at an oil loading rate of 2 kg/m^{3}d.

4.2.3 Effect of Cycle Time on Surface Tension (ST) and Surface Tension Reduction

Surface activity of a surfactant is determined from its ability to lower surface tension of medium which is the surface free energy per unit area required to bring a surfactant molecule from the bulk phase to the surface (Rosen, 1978). When the surfactant concentration is higher than the CMC, it is supposed that additional surfactant molecules aggregate into micelles in the bulk phase and do not contribute to significant future change at the interface. Therefore, the surface tension of the medium remains constant at the surfactant concentrations higher than its CMC. In

this present study, the surface tension of the culture supernatant of *Pseudomonas aeruginosa* SP4 was measured to estimate the extent of biosurfactant production. The higher the surface tension reduction, the higher the biosurfactant yield. Figure 4.6 illustrates the surface tension and the surface tension reduction of the culture supernatant as a function of operation time and cycle time, while Figure 4.7 displays the average percentage of surface tension reduction during the steady-state conditions at different cycle times. It was found that the surface tension of the culture medium was lowered from 70.89, 70.96, and 70.97 mN/m to 30.37, 28.82, and 30.93 mN/m, corresponding to the surface tension reduction of 57.15%, 59.39%, and 56.41%, at 1, 2, and 3 d/cycle, respectively. The presence of biosurfactant produced is responsible for the reduction of surface tension of the culture medium. It was reported that, the biosurfactant produced by *Pseudomonas aeruginosa* 47T2 using olive oil and sunflower oil as carbon sources could lower the surface tension of culture media to 34 and 37 mN/m, respectively (Haba *et al.*,2000). The rhamnolipid biosurfactant-containing supernatant obtained from the batch culture of *Pseudomonas aeruginosa* J4 was formed to reduce the surface tension of pure water from 72 to 31 mN/m (Wei *et al.*,2005).

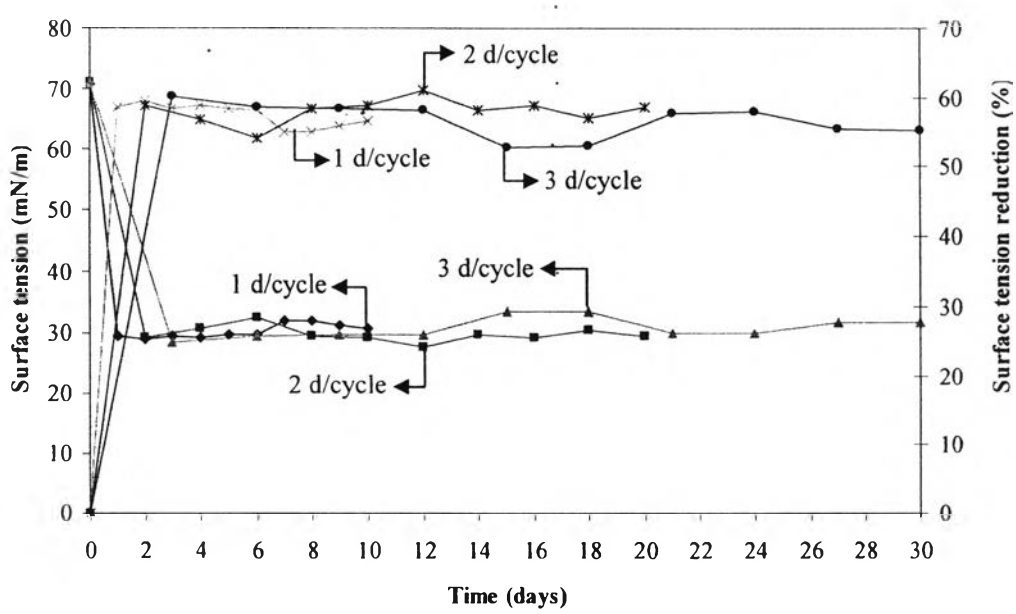

Figure 4.6 Profiles of surface tension and surface tension reduction at different cycle times with an oil loading rate of 2 kg/m^{3}d.

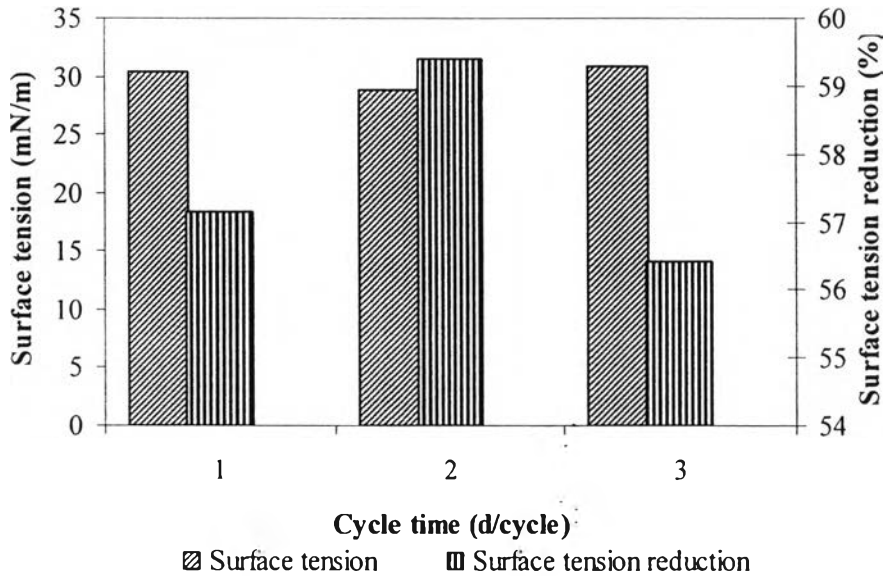

Figure 4.7 Surface tension and surface tension reduction during steady-state operation at different cycle times with an oil loading rate of 2 kg/m^{3}d.

4.2.4 Microbial Concentration

4.2.4.1 Mixed Liquor Suspended Solid (MLSS)

MLSS is used to represent the concentration of microorganisms in a reactor. In this study the sample was taken during the aeration step in order to determine the microbial concentration in terms of suspended solids (both organic and inorganic substances) in the reactor. Figure 4.8 shows that the MLSS at the steady-state conditions was 2,597, 3,467 and 11,580 mg/l at 1, 2, and 3 d/cycle, respectively. An increase in the cycle time increased the microbial concentration in the reactor. The microbial concentration was highest at 3d/cycle because this cycle time could provide the longest time for microbial growth as compared to 1 and 2d/cycle.

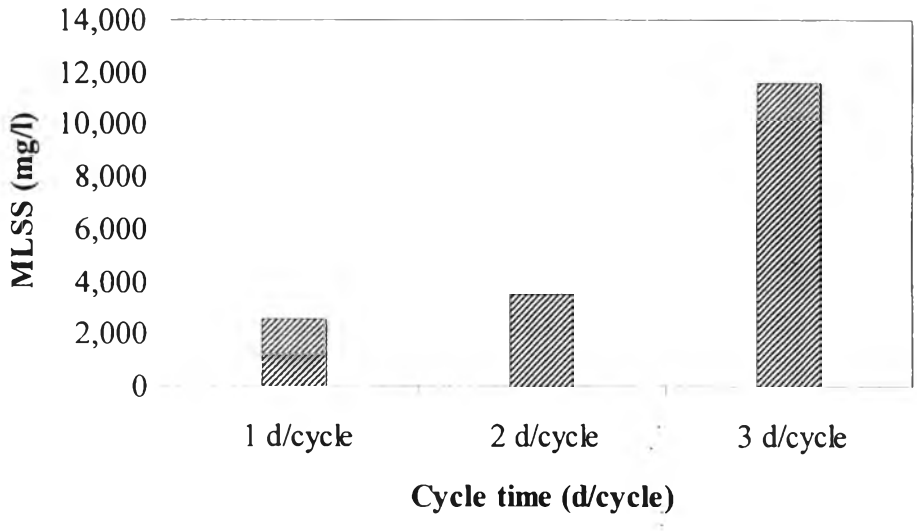

Figure 4.8 MLSS during steady state operation with an oil loading rate of 2 kg/m^{3}d at different cycle time.

4.2.4.2 Suspended Solid (SS)

SS is referred to solid materials, including both organic and inorganic substances in a sample. This suspended solids can be separated by filtering through a glass fiber filter. The filter paper is subsequently dried and weighed to determine the amount of total suspended solids. In this experiment, SS was measured during the settle step and used to represent the cells wash out. Figure 4.9 displays the effluent SS at different cycle times. The effluent SS was 630, 403, and 5,279 mg/l at 1, 2, and 3 d/cycle time, respectively. It was observed that the highest MLSS and SS were obtained at 3 d/cycle, suggesting that a longer cycle time promotes the microbial growth in the reactor. Thus, the cells wash out includes both living and non-living microbial cells. In a Comparison between 1 and 2 d/cycle, the effluent SS of 1 d/cycle was slightly higher than that of 2 d/cycle, suggesting that 2d/cycle was able to maintain the bacterial cell in the reactor.

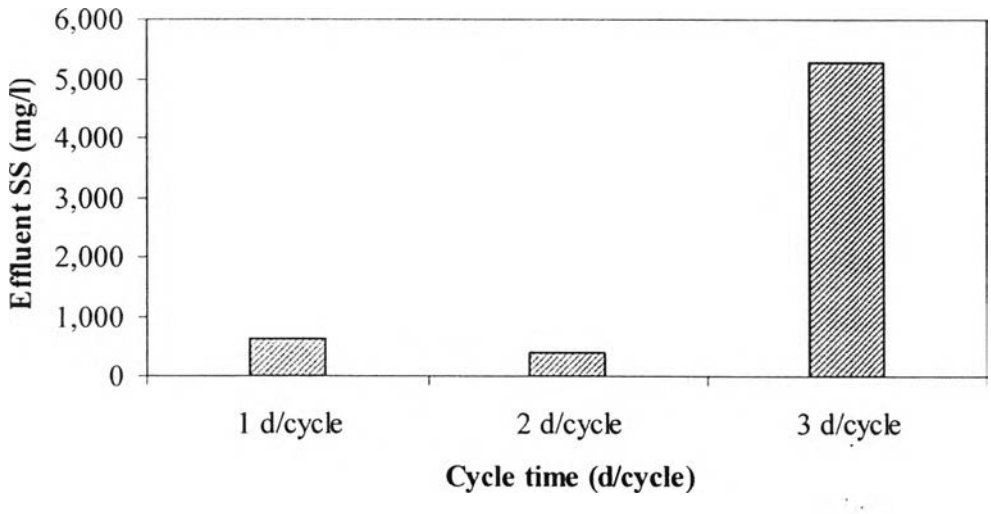

Figure 4.9 Effluent SS during steady-state operation at 1 d/cycle (days 5-7), 2 d/cycle (days 10-14), and 3 d/cycle (days 9-15) with an oil loading rate of 2 kg/m^3d.

4.2.5 The effluent pH

The pH of culture medium is one of the environmental factors that play an important role on the biosurfactant production because the pH can directly affect cellular growth or activity. A little change in pH of the culture medium can significantly alter the biosurfactant productivity. In this present study, after removing microbial cells by centrifugation, the supernatant pH was measured as a function of the operation time. As shown in Figure 4.10, the effluent pHs at all three cycle times were in the range of 5 to 6. Table 4.2 displays that average effluent pHs of 1 and 2 d/cycle were approximately 5.6, while that of 2 d/cycle was about 6.0, which is the most suitable pH for the production of biosurfactant by *Pseudomonas aeruginosa* SP4 using SBRs (Guerra-Santos *et al.*, 1984). It was reported that the rhamnolipid production by *Pseudomonas* spp. was maximized in the pH range of 6 to 6.5, and the optimum pH for the maximum biosurfactant production was found to be 6.25. It seemed that the optimum pH for biosurfactant production obtained from this present study was lower than that of the previous work (Guerra-Santos *et al.*, 1984). This might result from the different bacterial strains.

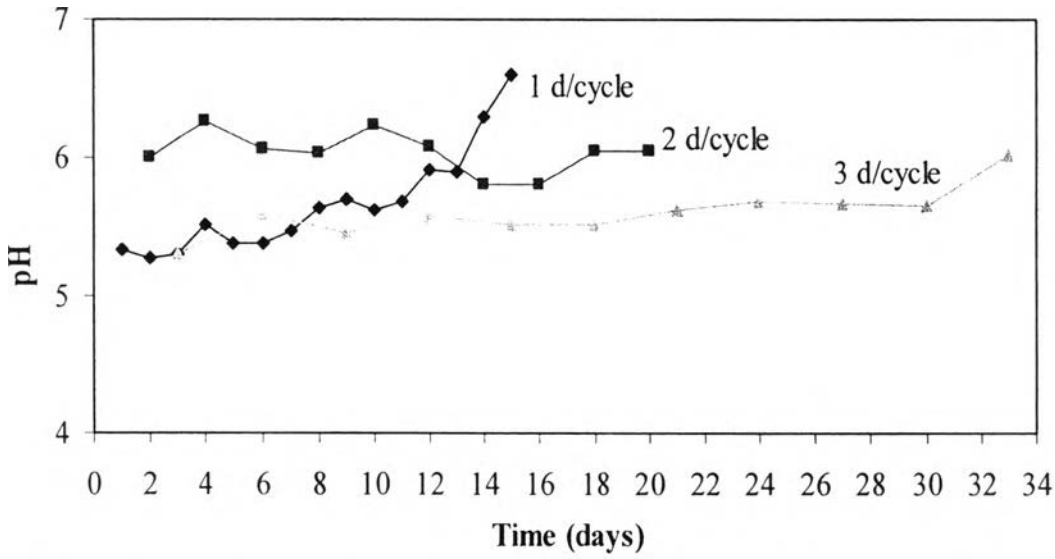

Figure 4.10 Effluent pH profiles with an oil loading rate of 2 kg/m^{3}d at different cycle times.

Table 4.2 The average effluent pH with an oil loading rate of 2 kg/m^{3}d at different cycle times.

Cycle time (d/cycle)	Avg. effluent pH
1	5.66
2	6.04
3	5.6

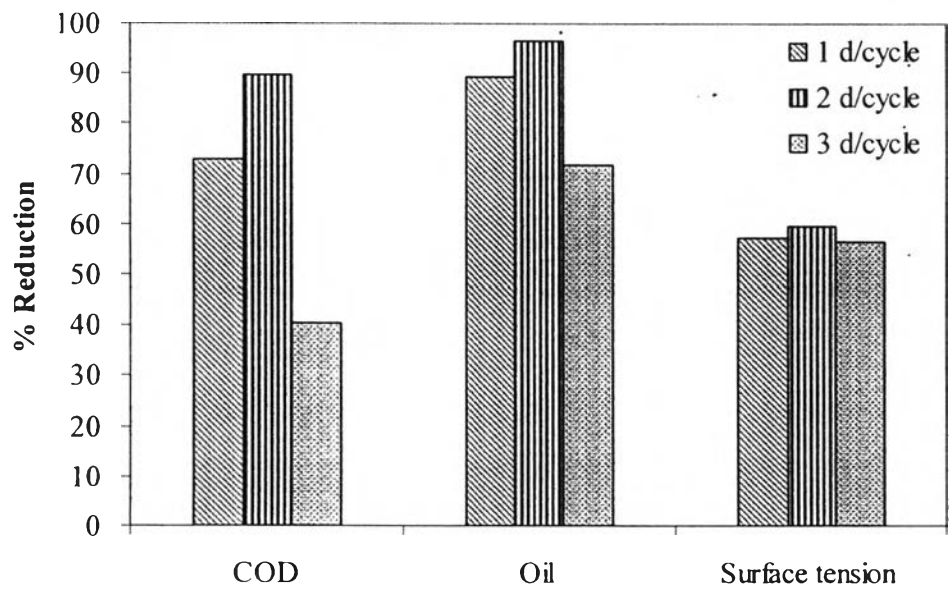

Figure 4.11 The average percentages of COD, oil, and surface tension reduction during steady-state operation with an oil loading rate of 2 kg/m^{3}d at different cycle times.

Figure 4.11 compares the average percentages of COD, oil, and surface tension reduction during steady-state operations at different cycle times. The results clearly demonstrate that the cycle time significantly affects the biosurfactant production. At the cycle time of 2 d/cycle, the highest COD removal of 89.8% was achieved, suggesting the highest activity of the microbe in utilizing palm oil as an organic substance. The highest percentages of oil, COD and surface tension reduction also indicated that the optimum cycle time at the optimum oil loading rate of 2 kg/m^3d for the biosurfactant production by *Pseudomonas aeruginosa* SP4 using SBRs was d/cycle. There optimum conditions were used for further investigation.

4.3 Measurement of Biosurfactant Concentration

After the optimization of cycle time for the biosurfactant production by *Pseudomonas aeruginosa* SP4, the surface tension of the culture medium inside the bioreactor was determined as a function of aeration time in order to investigate the profile of the biosurfactant production. Figure 4.12 displays the surface tension profile at 2d/cycle and with an oil loading rate of 2 kg/m^3d. The surface tension of the culture media slightly decreased during the first 4 h of aeration time but it was sharply decreased at an aeration time of 6 h before remained constant around 28-31 mN/m. The results demonstrated that the biosurfactant was produced substantially after 6 h of the aeration step and the biosurfactant production remained high throughout the aeration period, implying that the rate of biosurfactant production exceeded the rate of biosurfactant degradation after 6 h of aeration time (Cassidy and Huduk, 2001).

To determine the biosurfactant concentration, the critical micelle dilution (CMD) method was performed. The samples taken during the aeration period at different aeration times were serially diluted, and the surface tension was subsequently measured. Figure 4.13 shows the surface tension of serial dilutions at different aeration times of 10, 20, 30, and 40 h. The surface tension decreases with increasing surfactant concentration and reaches the minimum value at the CMC. Beyond the CMC, the surface tension remains unchanged with increasing surfactant concentration.
It was found that the increasing dilution of the sample taken from the aeration time of 40 h from 95:5 to 90:10 caused an increase in the surface tension from 29-31 mN/m

to 57.90 mN/m. From, the CMD results, the system operated at 2 d/cycle and an oil loading rate of 2 kg/m^{3}d provided the highest biosurfactant concentration of 1.05 times of the CMC at the aeration time of 40h.

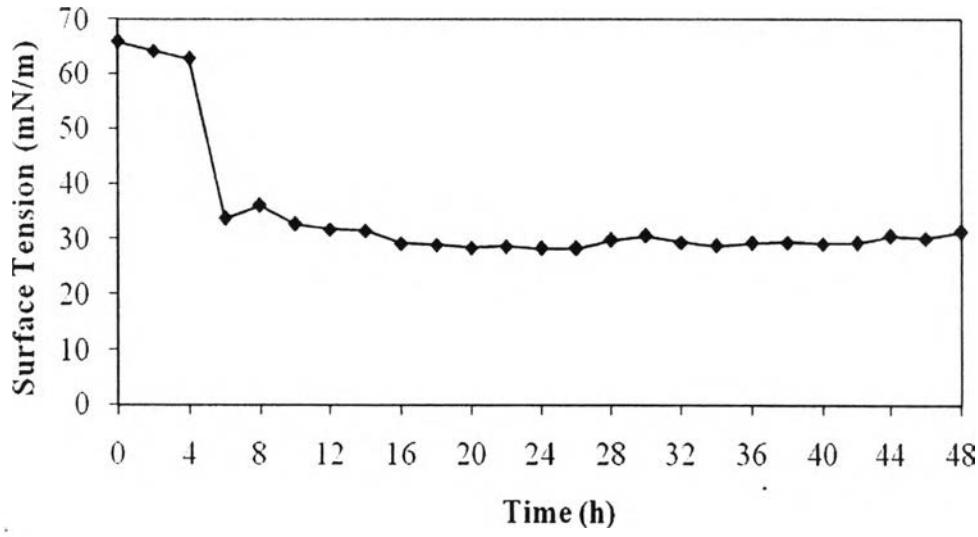

Figure 4.12 The surface tension profile at 2 d/cycle and an oil loading rate of 2 kg/m^{3}d.

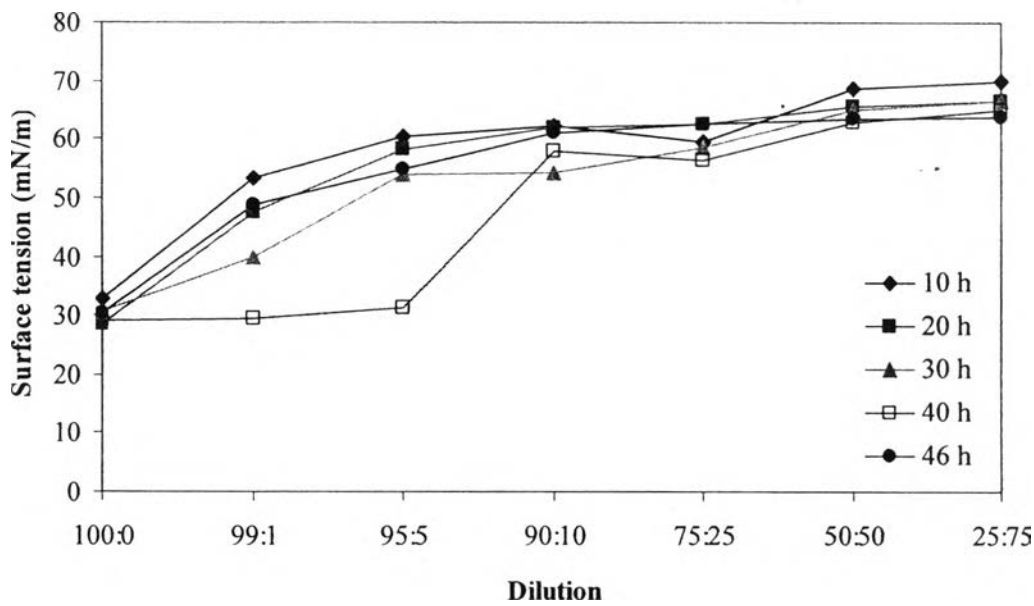

Figure 4.13 The surface tension of serial dilutions at different aeration times at 2 d/cycle and an oil loading rate of 2 kg/m^{3}d.

4.4 The Influence of C/N Ratio on Microbial Growth and Biosurfactant Production

According to the work of Santos *et al.* (2002), the C/N ratio was found to significantly affect both microbial and biosurfactant production. Thus, the influence of the C/N ratio on the microbial growth and biosurfactant production in the SBR system was investigated in the present study. The C/N ratio used in this present study was varied from 16/0.57 to 16/3 while the C/P ratio was still maintained at 14/1 using the optimum operating conditions at an OLR of 2 kg/m^3d and 2 d/cycle. As illustrated in Figure 4.14, the C/N ratio significantly affects the microbial cell concentration, resulting in the change in both MLSS and SS as the C/N ratio is varied. The lowest microbial cell concentration was observed at the C/N ratio of 16/0.57, which could be obtained in the absence of nitrogen source in the mineral medium. The MLSS at the C/N ratio of 16/3 was higher than that at the C/N ratio of 16/0.57, indicating that nitrogen concentration could play an important role in the microbial growth and manipulation (Haba *et al.*, 2000). Moreover, the remaining palm oil in the culture medium at the C/N ratio of 16/3 was much lower than that at the C/N ratio of 16/0.57. As displayed in Figure 4.15, the remaining palm oil at the C/N ratio of 16/3 formed thinner layer at the top of the liquid culture as compared to that at the C/N ratio of 16/0.57. It seems that the higher the microbial cell concentration, the higher the palm oil consumption was. However, the highest C/N ratio of 16/3 can not serve as an appropriate ratio for the microbial growth because of the toxicity of the ammonium nitrogen in the system. Therefore, the optimum C/N ratio for the biosurfactant production by *Pseudomonas aeruginosa* SP4 was 16/1, corresponding to the optimum ratio for the biosurfactant production by *Pseudomonas aeruginosa* DSM2659 (Guerra-Santos *et al.*, 1984).

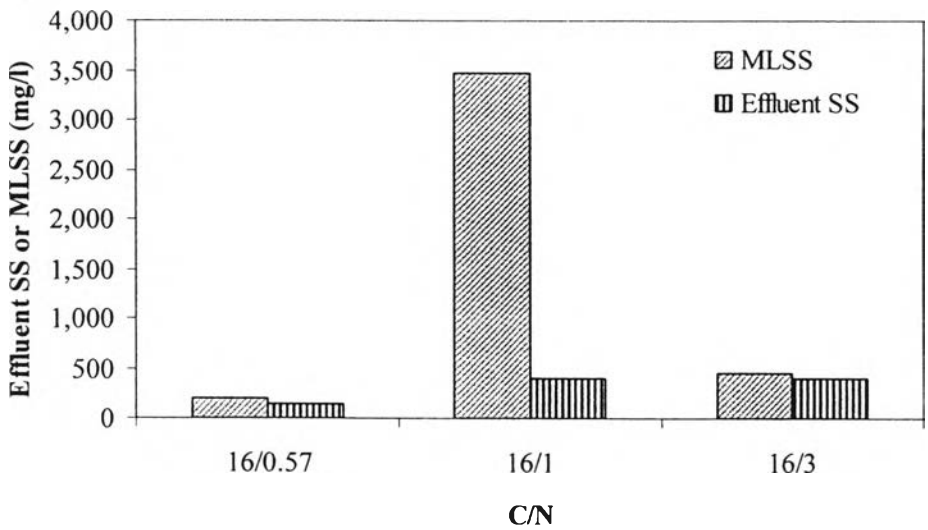

Figure 4.14 Effluent SS and MLSS at different C/N ratios of with an oil loading rate of 2 kg/m^{3}d and 2 d/cycle.

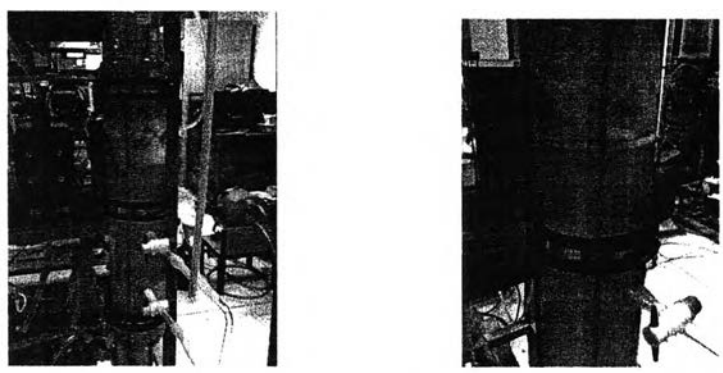

Figure 4.15 Different thickness of the remaining palm oil layer at the top of liquid culture in the SBR reactor at different C/N ratios of 16/0.57 (a) and 16/3 (B) with an oil loading rate of 2 kg/m^{3}d and 2 d/cycle time.

Surface tension measurement could be used as an indirect method to estimate the biosurfactant concentration (Guerra-Santos *et al.*, 1984). Figure 4.16 illustrates the surface tension and surface tension reduction at different C/N ratios. It was observed that the lowest surface tension and the highest surface tension reduction were found at the C/N ratio of 16/1, indicating the highest biosurfactant concentration in the SBR reactor as compare to the C/N ratios of 16/0.57 and 16/3, confirming that

the C/N ratio of 16/1 was an optimum ratio for the biosurfactant production by the strain SP4.

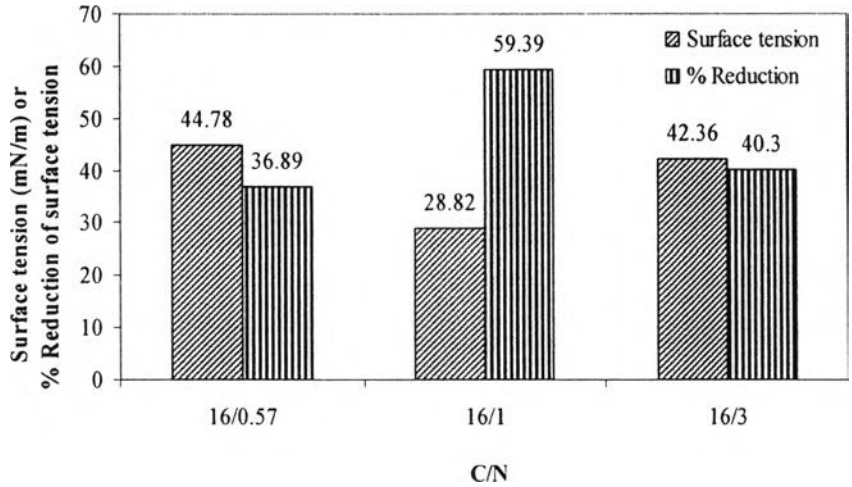

Figure 4.16 Surface tension and surface tension reduction at different C/N ratios using an oil loading rate of 2 kg/m^{3}d with 2 d/cycle.

Another important factor governing the microbial growth is pH of the culture medium. As shown in Figure 4.17, the difference in C/N ratio also causes the change in the pH of the culture medium. The average effluent pH at the C/N ratio of 16/0.57, 16/1, and 16/3 was found to be 5.41, 6.04, and 5.82, respectively. Among these three pH value, only the pH at the C/N ratio of 16/1 was in the suitable pH range for the biosurfactant production (Guerra-Santos *et al.*, 1984).

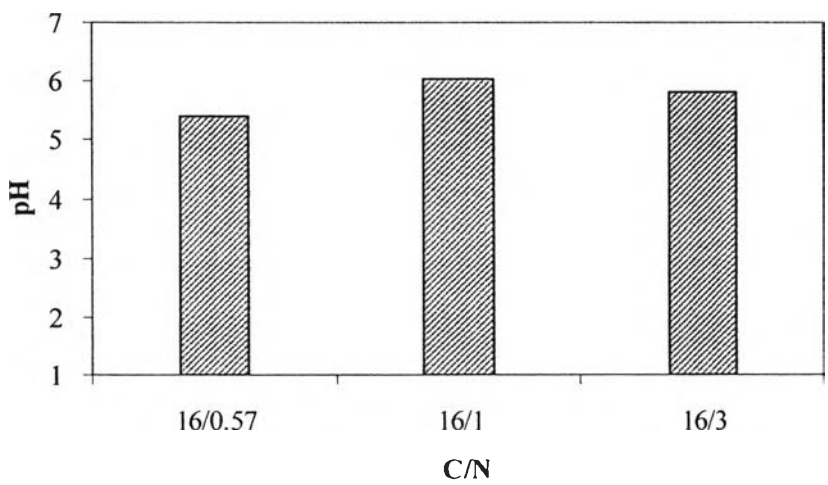

Figure 4.17 The average effluent pH at different C/N ratios using an oil loading rate of 2 kg/m^{3}d with 2 d/cycle.