Chapter 3

Results and Discussion

1. Optimization for growth and biosurfactant production

1.1 Time course on growth and biosurfactant production by *Bacillus* MUV4 in shake flask cultivation

Time course on growth and biosurfactant production by *Bacillus* MUV4 in Mckeen medium (pH 7.0) contained 1.0% glucose as carbon source at 30 ^{o}C was shown in Figure 5. The organism grew rapidly within the first 6-18 h cultivation with OD_{660}=4.97. This was correlated to the slightly decline of pH which due to the sugar was metabolized by cells and formed acidic metabolites. After 18 h, pH slightly increased and constant after 36 h which may cause by the depletion of carbon source. The maximum growth was observed at 36 h giving OD_{660} of 6.82. After that the growth was slightly decreased due to the death of cells during stationary phase of growth. The organism started to produce the biosurfactant after the pH gradually increased until 48 h of cultivation the maximum biosurfactant was obtained with ODA and EC values were 9.76 cm^2 and 0.89%, respectively. The production of biosurfactant by *Bacillus* MUV4, which was growth associated with maximum activity during stationary phase of growth.

1.2 Effect of nutrients on biosurfactant production

1.2.1 Carbon sources

The effect of various carbon sources on growth and biosurfactant production from *Bacillus* MUV4 was shown in Figure 6. The organism could grow in the medium containing all of the carbon sources tested (glucose, sucrose, mollases and glutamate) with significant difference ($p<0.05$). The maximum growth (OD_{660}=7.52) occurred when sucrose was used as carbon source but the

maximum biosurfactant activity (ODA and EC values were 9.76 cm^2 and 0.89%, respectively) could be obtained when glucose was used as carbon source. This finding was in agreement with the experimental result of Cooper *et al*. (1981) and Suthivanichakul (1999), which showed that glucose was the best carbon source for producing biosurfactant by *Bacillus subtilis* and *Bacillus licheniformis*, respectively. *Bacillus* MUV4 grew slowly and produced minimal amount of biosurfactant in the medium with hydrophobic carbon sources (palm oil, weathered oil and n-hexadecane). There was a report showed that hydrocarbon compound inhibited the biosurfactant production of *Bacillus subtilis* (Cooper *et al*., 1981). Makkar and Cameotra (1997b) demonstrated that the organism was able to grow on n-hexadecane and pristane but did not produced biosurfactant on n-hexadecane and pristane. These results shown that weathered oil at 0.1% gave the highest ODA and %EC values comparing with using palm oil and n-hexadecane. (Figure 7). The optimal concentration of weathered oil gave higher ODA and %EC than the others oil was 0.3% (Figure 8). However, when the organism was cultivated in the medium contained 0.3% weathered it lower grew and produced biosurfactant than those used glucose as carbon source. These result concluded that glucose was the best carbon source for growth biosurfactant production by *Bacillus* MUV4. The similar result of biosurfactant production by *B. subtilis* C9 required 1.0% glucose to produce maximum biosurfactant. This biosurfactant C9-BS lowered the surface tension of water to 28.5 dyne/cm and gave maximum emulsification activity comparing with using hexadecane either as the sole carbon source or in combination with glucose inhibited the biosurfactant production (Kim *et al*., 1997). It can be concluded from those results that the carbon source affected both cell growth and biosurfactant production and glucose was selected as suitable carbon source for *Bacillus* MUV4.

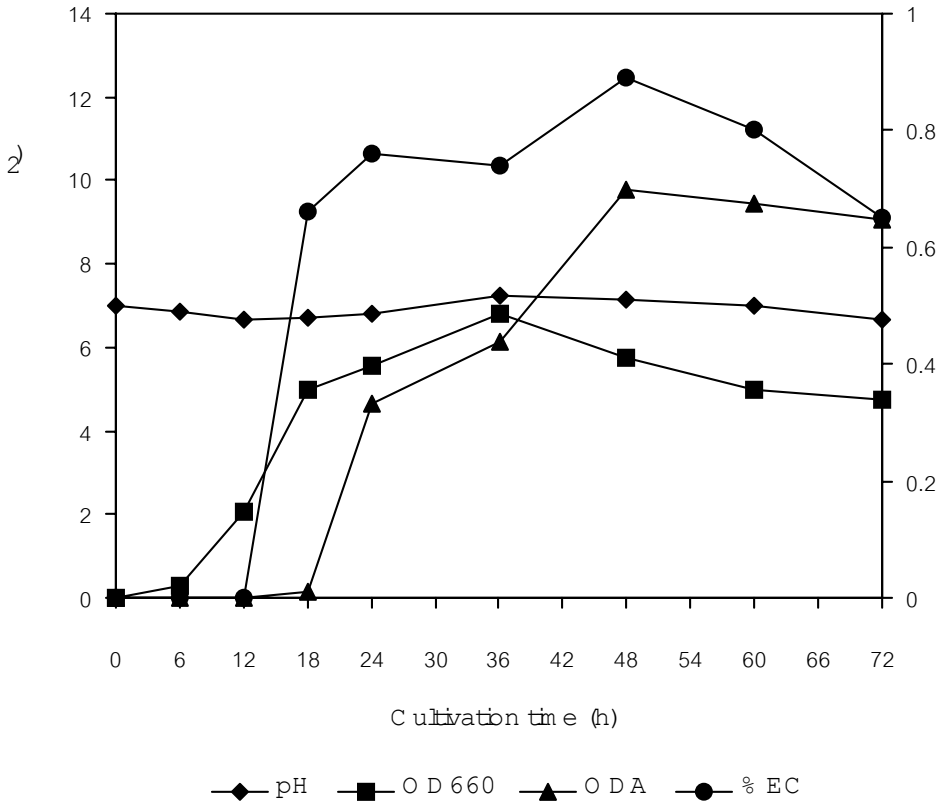


Figure 5 Growth and biosurfactant production by *Bacillus* MUV4 in Mckeen medium under shake-flask cultivation (200 rpm, 30 ^{o}C)

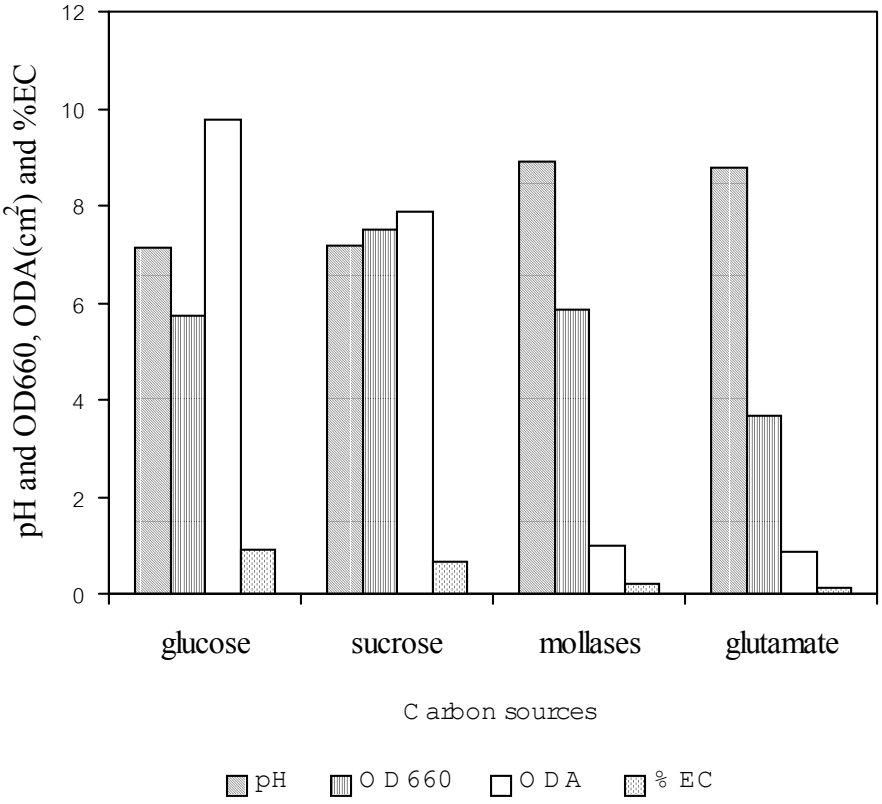


Figure 6 Effect of carbon sources (2.0%) on growth and biosurfactant production by *Bacillus* MUV4 in Mckeen medium under shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h

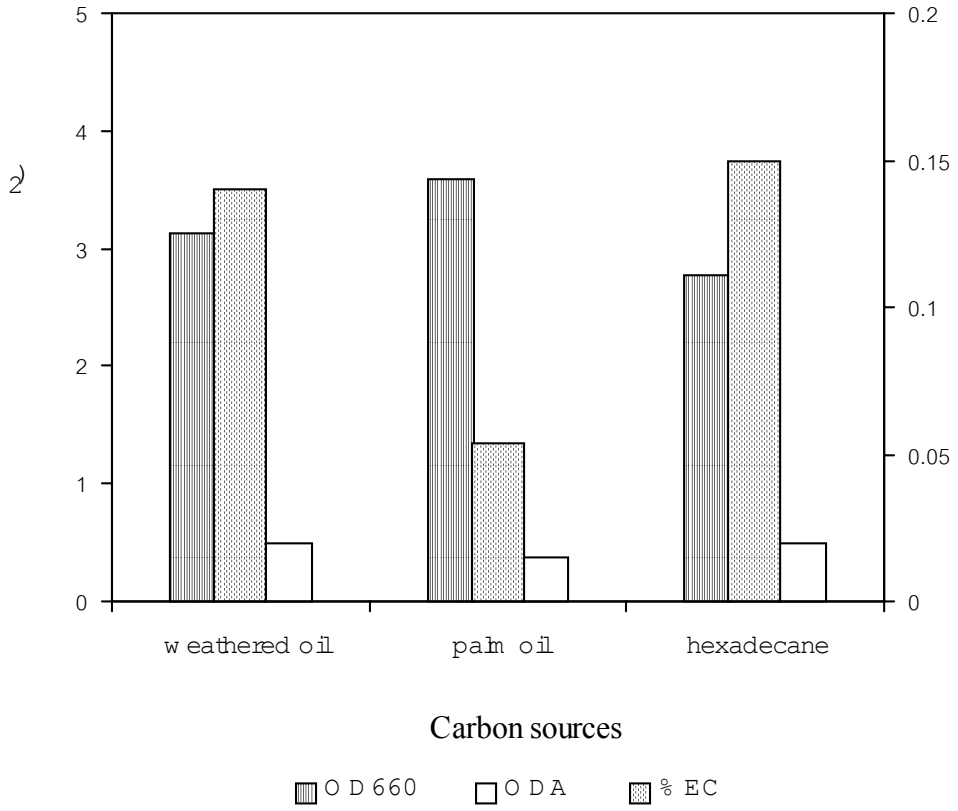


Figure 7 Effect of hydrophobic carbon source (0.1%) on growth and biosurfactant production by *Bacillus* MUV4 in Mckeen medium under shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h

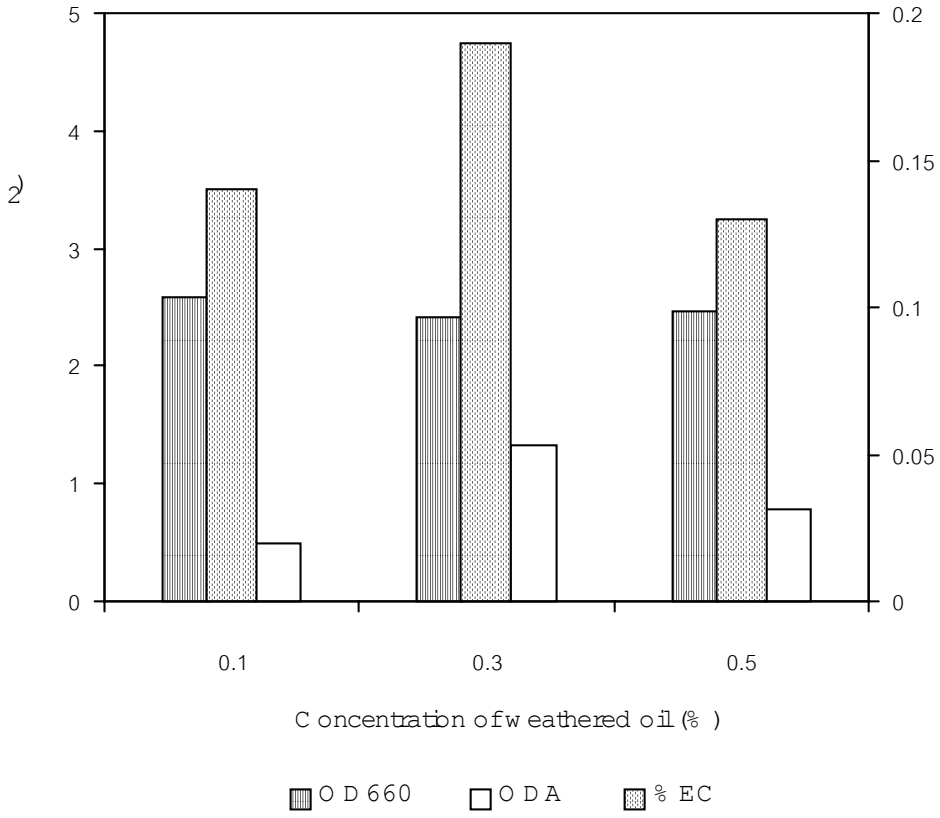


Figure 8 Effect of concentration of weathered oil on growth and biosurfactant production by *Bacillus* MUV4 in Mckeen medium under shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h

1.2.2 Concentrations of carbon source

The effect of glucose concentration on growth and biosurfactant production by *Bacillus* MUV4 was examined (Figure 9). The biosurfactant production from *Bacillus* MUV4 increased significantly as the glucose concentration increased up to 2.5%. The ODA values were 8.16, 10.36 and 10.74 cm^2 and EC values were 0.61, 0.67 and 0.93% at 1.0, 2.0 and 2.5% glucose concentrations, respectively. When the concentration of glucose increased over to 2.5%, biosurfactant activity slightly decreased due to the pH of culture broth decreased. Growth of *Bacillus* MUV4 was higher than 5.0 (OD_{660}) at 48 h of cultivation in the medium with the glucose concentration 2.0-4.0%. Therefore, 2.5% of glucose was suitable for cell growth and biosurfactant production. This could be compared with the results of Yakimov *et al.* (1995), who reported that glucose levels over 20 g/l did not lead to improve the production of biosurfactant by *Bacillus licheniformis* BAS50.

1.2.3 Nitrogen sources

The effect of nitrogen sources on growth and biosurfactant production by *Bacillus* MUV4 in the Mckeen medium contained 2.5% glucose as carbon source was shown in Figure 10. Growth and biosurfactant production of under inorganic nitrogen sources such as KNO_3, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)HPO_4$, $(NH_4)H_2PO_4$, NH_4HCO_3 and $NaNO_3$ were compared to those under organic nitrogen sources, DL-glutamic acid, L-glutamic acid and monosodium glutamate. The result found that $(NH_4)HPO_4$ gave the highest cell growth (OD_{660}=7.32) (Figure 10 (A)) among organic nitrogen sources while L-glutamic acid as nitrogen source gave higher cell growth (OD_{660}=5.28) than monosodium glutamate (OD_{660}=5.09). The organism lowly grew in the medium containing $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3 as a nitrogen source. The cell growth values (OD_{660}) of 0.54, 0.76, 1.45 and 2.53 were observed, respectively. This is contrary to the result from *B. licheniformis* F2.2 as 2.0 g/l NH_4NO_3 was the best nitrogen source for biosurfactant production. (Sutthivanitchakul *et al*., 1999).

Bacillus MUV4 could not produce biosurfactant when KNO_3, NH_4NO_3, $(NH_4)_2SO_4$ and $NaNO_3$ were used as inorganic nitrogen source. While (NH_4inorganic nitrogen sources. However, in basal Mckeen medium contained L-glutamic acid as nitrogen source the maximum biosurfactant (ODA 11.33 cm^2 and 2.60% EC) was obtained. The report of Johnson, *et al*. (1992) demonstrated that when $(NH_4)_2SO_4$, KNO_3 and urea were used as nitrogen source, KNO_3 was the best nitrogen source for biosurfactant production by *Rhodotorula glutinis* IIP-30. Emulsification activity was maximized with nitrate followed by ammonium salt and urea. Another the report showed that a maximum amount of biosurfactant was produced when urea and nitrate were supplied as nitrogen source (Makkar and Cameotra, 1997a). However, this result the *Bacillus* MUV4 had lowly oil displacement activity (ODA) and emulsification capacity (EC) when using various organic nitrogen sources.

Islam (2000) studied the effect of nitrogen sources on biosurfactant production by *Pasteurella* PA6 and *Acinetobacter* S7. It was found that NH_4NO_3 was the best nitrogen source for growth by *Pasteurella* PA6 followed by NH_4HCO_3 but the maximum biosurfactant production was obtained when $(NH_4)_2SO_4$ was used as nitrogen source (ODA and EC values of 6.16 cm^2 and 1.40%, respectively). For *Acinetobacter* S7, NH_4NO_3 was the best nitrogen source for biosurfactant production. This is similar to biosurfactant production of *Bacillus licheniformis* F2.2 (Sutthivanitchakul *et al*., 1999).

When L-glutamic acid and monosodium glutamate (0.5%) were compared as nitrogen sources. The culture grown in the medium contained L-glutamic acid gave higher ODA and EC (11.35 cm^2 and 2.60%, respectively) than those from monosodium glutamate. No significantly differences in ODA and EC were detected among L-glutamic acid and monosodium glutamate treatment, therefore monosodium glutamate was selected as nitrogen source due to its lower cost.

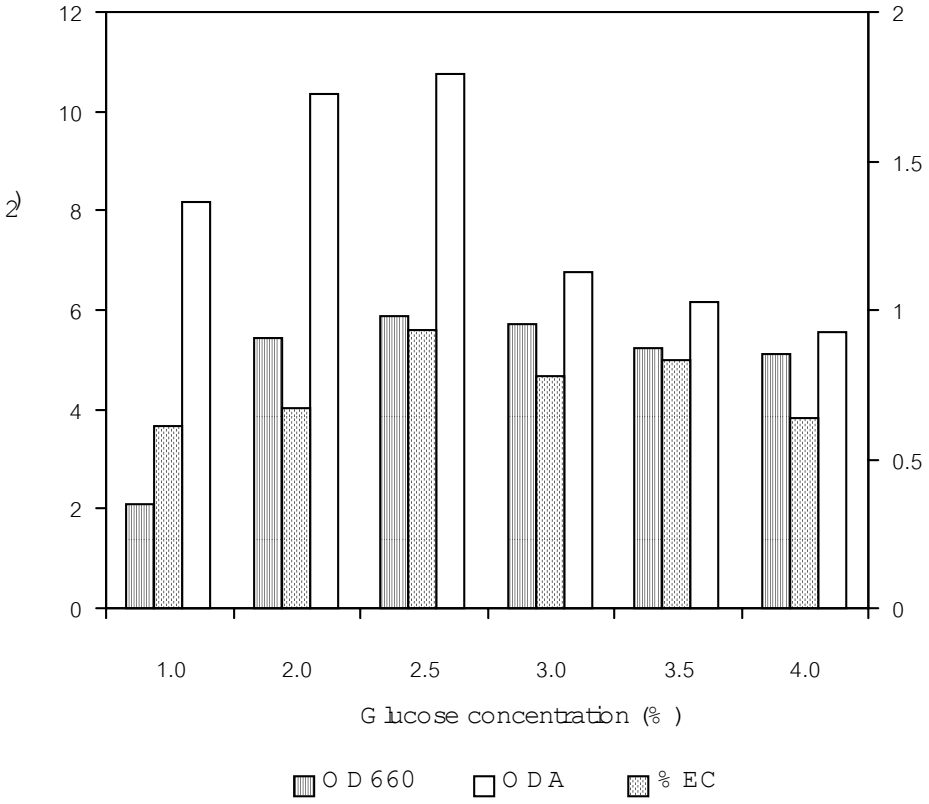
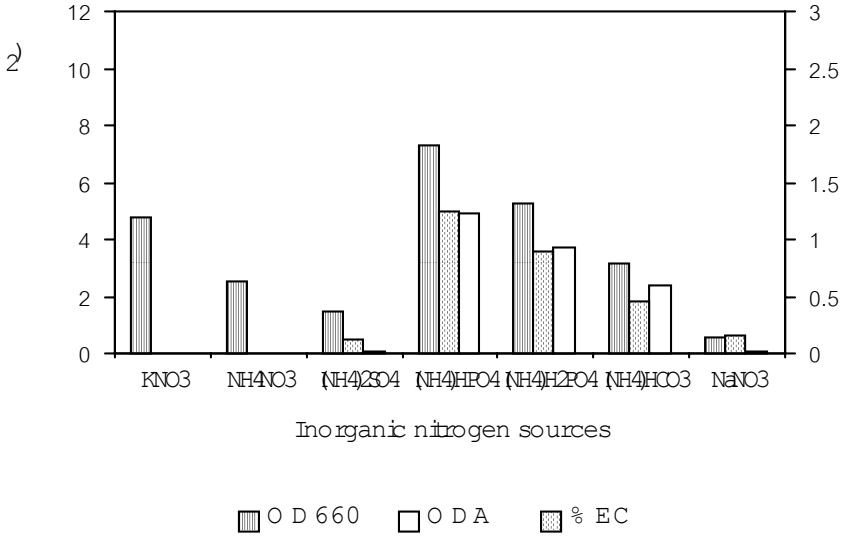


Figure 9 Effect of glucose concentrations on growth and biosurfactant production by *Bacillus* MUV4 in shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h



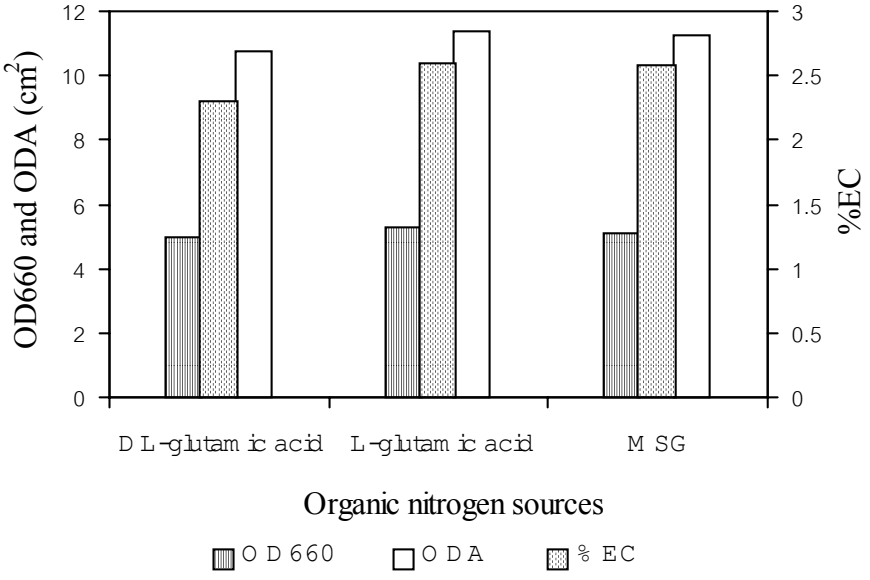


Figure 10 Effect of nitrogen sources (0.5%) on growth and biosurfactant production by *Bacillus* MUV4 in shake-flask cultivation (200 rpm, 30 ^{o}C) for 48

1.2.4 Effect of monosodium glutamate concentrations

The effect of monosodium glutamate concentration on growth and biosurfactant production by *Bacillus* MUV4 was shown in Figure 11 (A,B). The growth and biosurfactant production of the bacterium increased significantly ($p < 0.05$) as concentration of monosodium glutamate increased up to 1.0%. The maximum cell growth with the OD_{660} values of 1.66, 4.11, 6.93 and 8.23 were achieved in the presence of monosodium glutamate at 0.1, 0.3, 0.5 and 1.0%, respectively. At 1.0% monosodium glutamate the culture gave the highest ODA, EA and EC (44.28 cm^2, 80% and 2.94%, respectively). In the following experiment, monosodium glutamate at 1.0% was used as nitrogen source.

1.2.5 Effect of yeast extract and bacto peptone concentrations

The effect of yeast extract on growth and biosurfactant production from *Bacillus* MUV4 was shown in Figure 12. Cell growth of the organism increased when the concentrations of yeast extract increased. The OD_{660} values of 8.18, 9.18, 9.49 and 9.84 were achieved in the presence of yeast extract at 0, 0.1, 0.3 and 0.5%, respectively (Figure 12 (A)). The maximum cell growth with the OD_{660}=9.84 was achieved in the presence of yeast extract at 0.5% but the maximum biosurfactant production was obtained with ODA 78.5 cm^2, 81.82 %EA and 4.72 %EC when cultured in the medium contained 0.3% yeast extract (Figure 12 (B)). This results indicated that yeast extract was necessary for growth and production of biosurfactant. Addition 0.3% of yeast extract was suitable for biosurfactant production by *Bacillus* MUV4. Cooper and Paddock (1984) demonstrated that yeast extract was important for growth and glycolipid biosurfactant production in *T. bombicola*. In the medium contained 5.0% glucose and 0.5% yeast extract gave high biomass and glycolipid (12.4 and 18.0 g/l, respectively). Ghurye and Vipulanadan (1994) reported that an addition of yeast extract to the medium salt during fermentation resulted in the highest biomass production and biosurfactant for microorganism obtained from activated sludge.

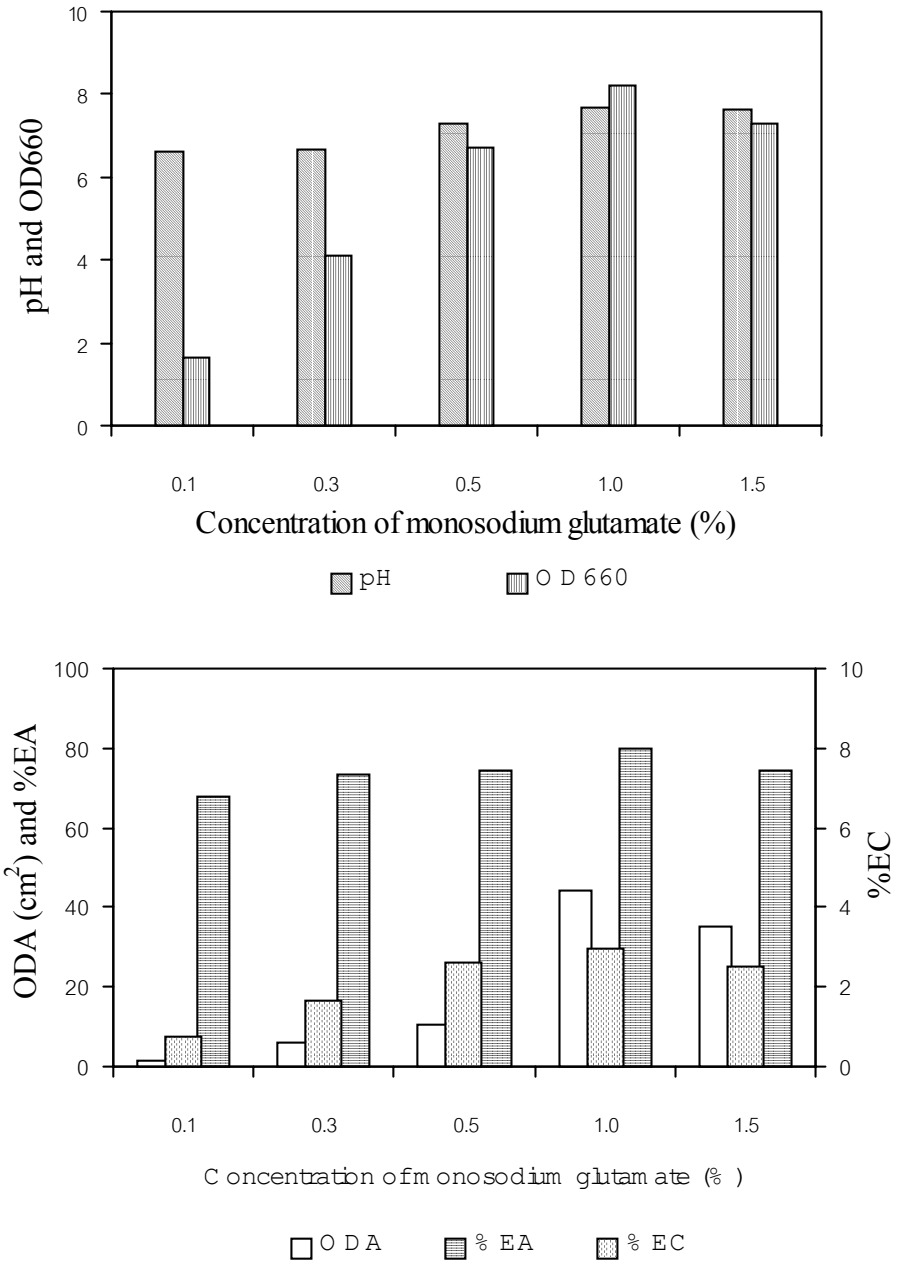
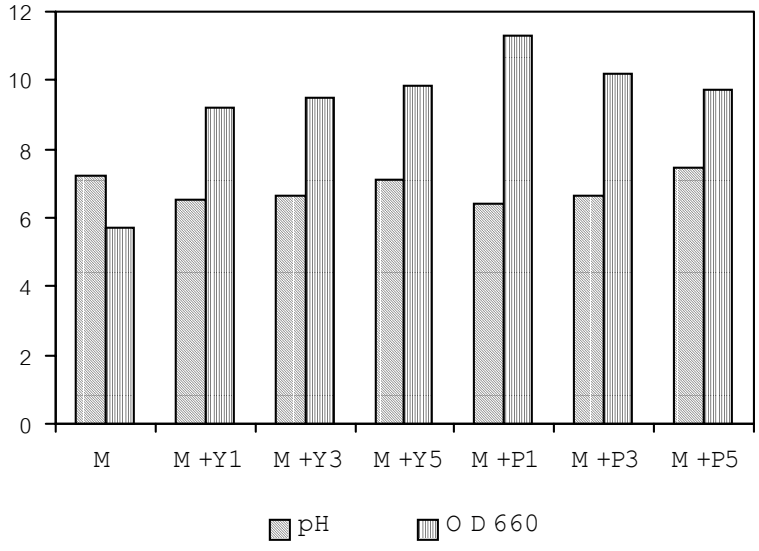


Figure 11 Effect of monosodium glutamate concentrations on growth (A) and biosurfactant production (B) by *Bacillus* MUV4 in shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h



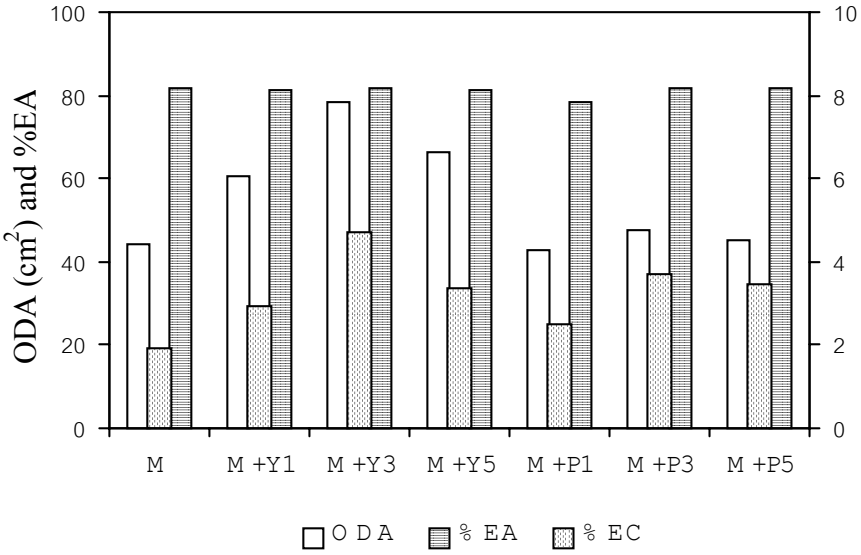


Figure 12 Effect of yeast extract and bacto peptone on growth (A) and biosurfactant production (B) by *Bacillis* MUV4 in shake-flask cultivation (200 rpm, 30 ^{o}C) for 48 h (M+Y1=Medium+ 0.1% yst extract, M+Y3=Medium +0.3%yst extract,M+Y5=Medium+ 0.5 % yst extract, M+P1=Medium+0.1% bacto peptone, M+P3=Medium+0.3% bacto peptone and M+P5=Medium +0.5% bacto peptone) M=Medium contained 2.5% glucose as carbon source and 1.0% monosodium glutamate as nitrogen source

Studies on growth and biosurfactant production from *Bacillus* MUV4 with various bacto peptone concentrations in the optimized medium (pH 7.0) containing 2.5% glucose and 1.0% monosodium glutamate without yeast extract cultivated at 30 ^{o}C were carried out in shake flask cultivation. Cell growth was increased as concentration of bacto peptone increased with the OD_{660} over 11.0. The maximum biosurfactant (ODA 47.73 cm^2, 81.82% EA and 3.68% EC) was obtained from the medium with 0.3 % bacto peptone (Figure 12 (B)). Islam (2001) showed that yeast extract and bacto peptone effect growth and biosurfactant production by *Acinetobacter* S7 and *Pasteurella* PA6. However, Fox and Bala (2000) found that using potato medium containing yeast extract and bacto peptone had no effect on surfactin production by *Bacillus subtilis* and supplemented the medium with difference concentrations (0.01-0.1%) of yeast extract found that the addition of yeast extract had no effect on biomass production of *Bacillus subtilis* (Abu-Ruwaida *et al*., 1991b).

1.3 Time course of growth and biosufactant production under optimal medium in shake-flask cultivation

Cultivation of *Bacillus* MUV4 in the optimal medium, which contained 2.5% glucose as carbon source, 1.0% monosodium glutamate and 0.3% yeast extract as nitrogen source (initial pH 7.0) at 30 ^{o}C on shaker was shown in Figure 13. *Bacillus* MUV4 grew rapidly during 12 h of cultivation and slightly increased to the maximum growth (OD_{660}=12.96) at 36 h and the dry cell weight was 3.0 g/l. The biosurfactant was produced after 6 h of cultivation and the maximum biosurfactant was obtained after 60 h cultivation with the maximum ODA, EA and EC values were 78.50 cm^2, 81.82 and 5.18, respectively. These results indicated that biosurfactant production by *Bacillus* MUV4 was growth associated. This result was similar to growth and biosurfactant production of *Rhodotorula glutinis* IIP-30 (Johnson *et al*., 1992), *Bacillus subtilis* C9 (Kim *et al*., 1997) and *Pasteurella* PA6 and *Acinetobater* S7 (Islam, 2001).

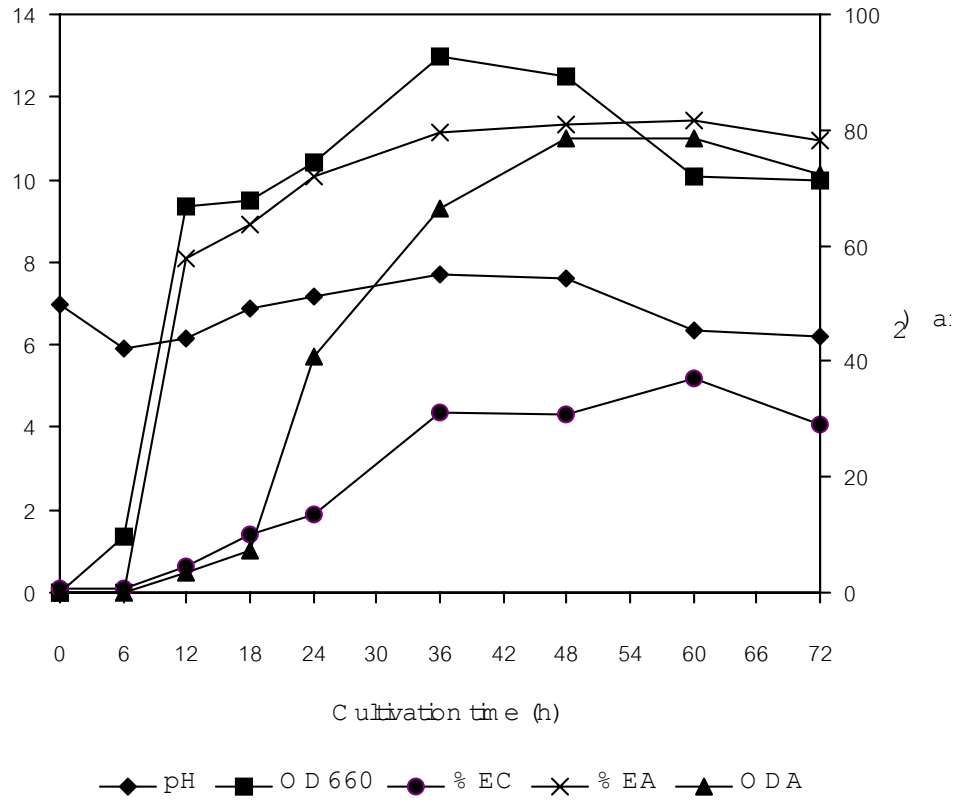


Figure 13 Growth and biosurfactant production by *Bacillus* MUV4 when cultivated in the optimal medium (2.5%glucose as carbon source, 1.0% monosodium glutamate and 0.3% yeast extract as nitrogen source) in shake-flask cultivation (200 rpm, 30 ^{o}C)

2. Effect of environmental condition on biosurfactant production in fermentor

2.1 Effect of pH (uncontrolled pH and controlled pH 7.0)

Bacillus MUV4 was cultivated in the 2.0-l fermentor containing 1.5-l optimal medium, which contained 2.5% glucose as carbon source, 1.0% monosodium glutamate and 0.3% yeast extract as nitrogen source with the aeration rate of 0.5 vvm, agitation speed of 200 rpm and incubation temperature of 30 ^{o}C, with and without controlled pH (pH 7.0). The result showed that *Bacillus* MUV4 grew well at both conditions (Figure 14 (A)) but the biosurfactant production was higher under uncontrolled pH condition than controlled pH condition (Figure 14 (B)). Under uncontrolled pH condition, the pH of culture broth declined slightly at early of stage of growth after 6 h the pH gradually increased when the organism started to produce the biosurfactant. The maximum growth was observed at 48 h of cultivation with 7.41 OD_{660}. The maximum biosurfactant production was obtained at 60 h of cultivation with ODA, EA and EC of 60.79 cm^2, 74.54% and 4.21%, respectively. After 60 h of cultivation, the biosurfactant production was decreased.

Under controlled pH (pH 7.0) condition, The organism was slightly grew after 6 h of cultivation until 48 h the maximum growth was 6.69 OD_{660}. The biosurfactant production was also slightly increased at 12 h of cultivation and the maximum biosurfactant (ODA, EA and EC values of 32.1 cm^2, 72.72% and 2.8%, respectively) was obtained after 60 h of cultivation. The growth and biosurfactant production under uncontrolled pH at 7.0 was higher than those under controlled pH at 7.0 indicated that the pH of culture broth affected on both growth and biosurfactant production of *Bacillus* MUV4.

2.2 Effect of aeration rate

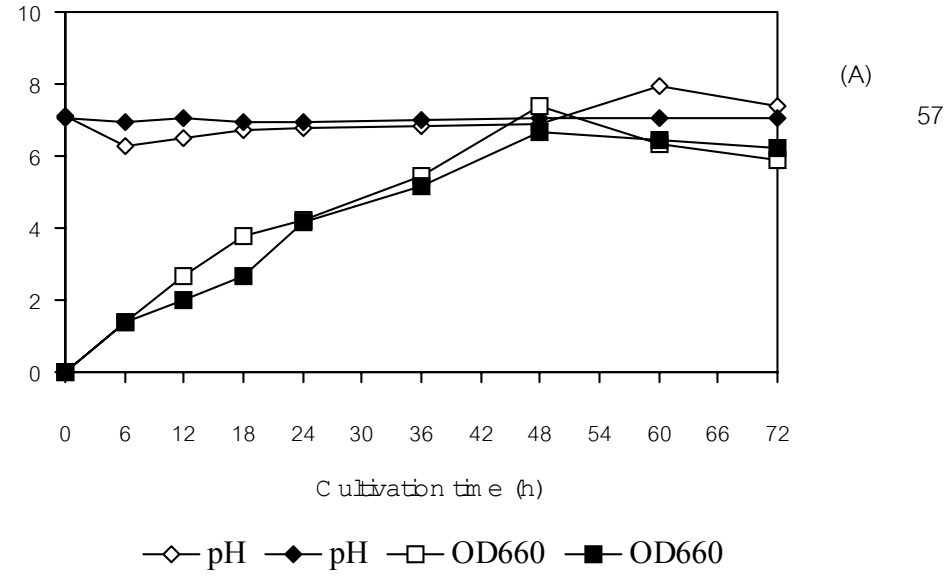
The effect of aeration rates on growth and biosurfactant production by *Bacillus* MUV4 was shown in Figure 15. Higher aeration rates gave higher cell

growth (Figure 15 (A)). The growth observed under no aeration rate may be due to the effect of agitation speed (200 rpm). The biosurfactant production of *Bacillus* MUV4 increased significantly as the aeration rates increased. The maximum biosurfactant production was occurred at the aeration rate 1.0 vvm (ODA, EA and EC of 72.34 cm^2, 81.48% and 4.94%, respectively)(Figure 15 (B)). An increasing of the aeration rate caused an increasing in biosurfactant production of *Pseudomonas aeruginosa* LBI (Benincasa *et al*., 2001)

2.3 Time course on growth and biosurfactant production of *Bacillus* MUV4 under optimal condition in fermentor

Bacillus MUV4 was cultivated in the optimal medium (initial pH 7.0) at 30 ^oC in the fermentor with agitation speed 200 rpm under uncontrolled pH for 72 h. The result was shown in Figure 16. The maximum growth was observed at 48 h of cultivation with the OD_{660} of 7.13. ODA and %EC values slowly increased at 6-24 h cultivation. The maximum biosurfactant was obtained at 60 h cultivation which was stationary phase of growth.

When growth and biosurfactant production of *Bacillus* MUV4 under optimal condition in fermentor and in shake-flask culture were compared . The result indicated that both growth and biosurfactant production of *Bacillus* MUV4 in fermentor were lower than those of shake-flask culture. Antifoam was added simultaneously then may affect the results of biosurfactant production.



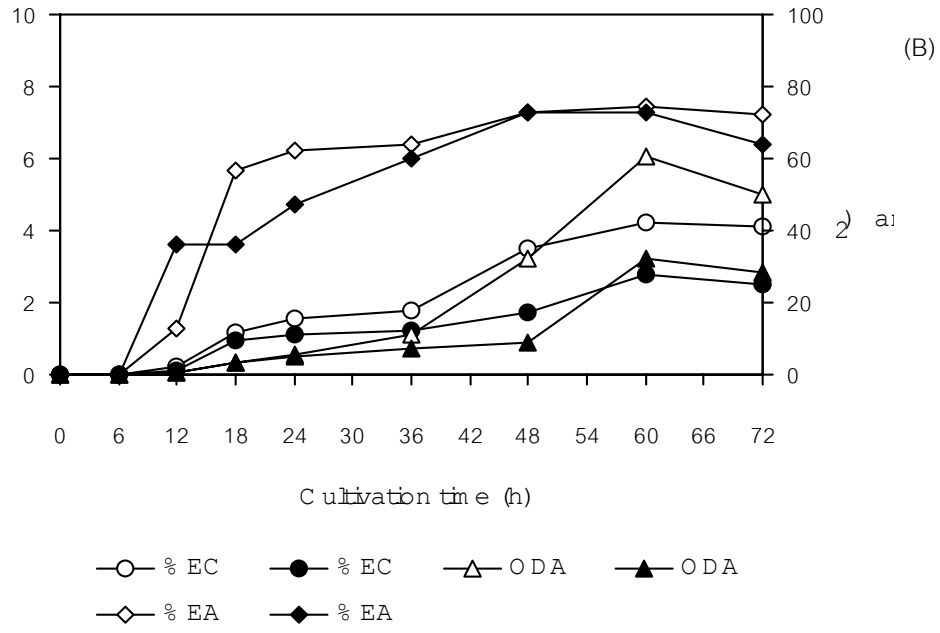


Figure 14 Growth (A) and biosurfactant production (B) by *Bacillus* MUV4 during cultivation under uncontrolled pH (open symbol) and controlled pH (closed symbol) in the 2 l fermentor with 1.5 l of working volumn (200rpm and 0.5vvm)

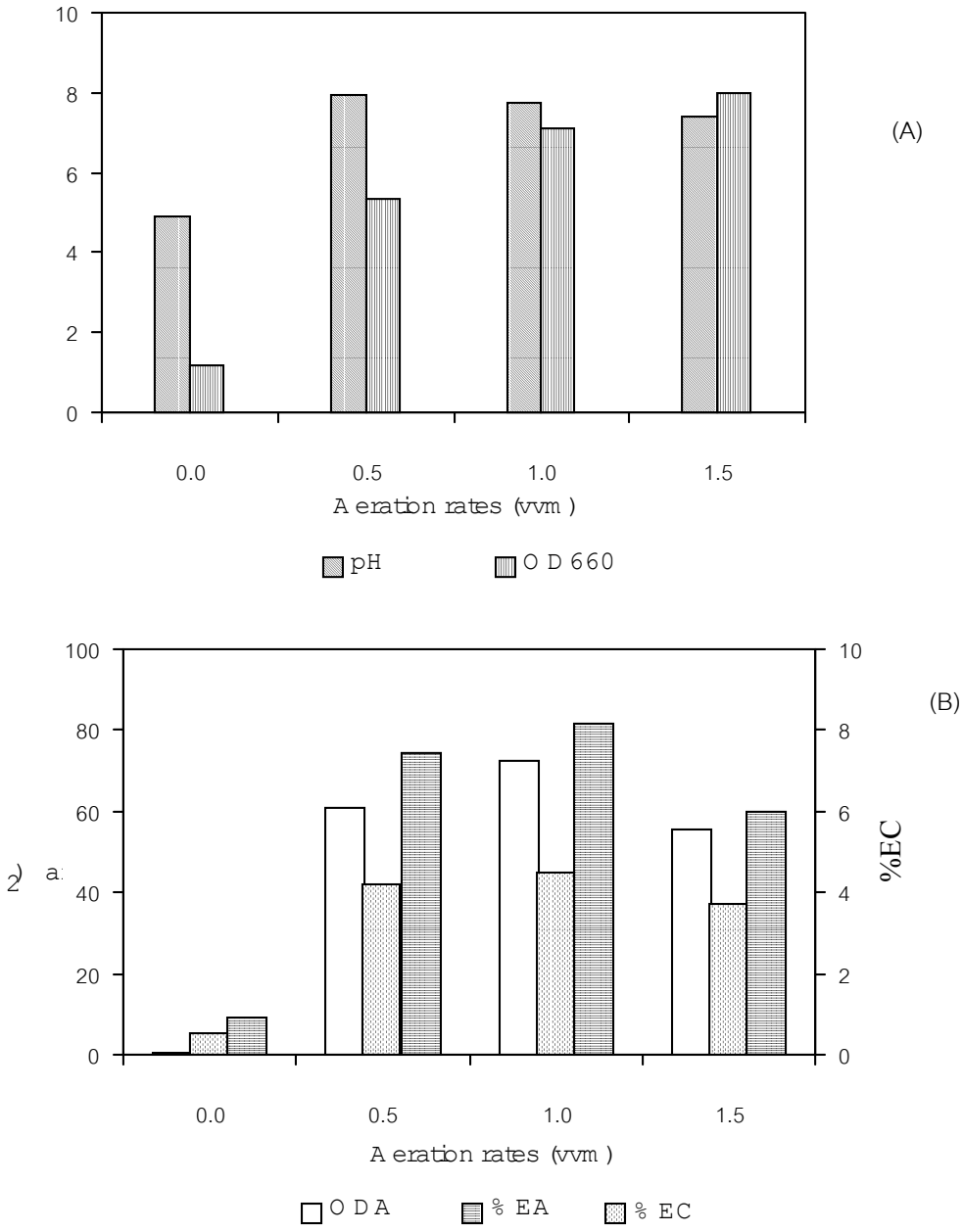


Figure 15 Effect of aeration rates on growth (A) and biosurfactant production (B) by *Bacillus* MUV4 during cultivation in (agitation speed 200 rpm, 30 ^{o}C) for 60 h

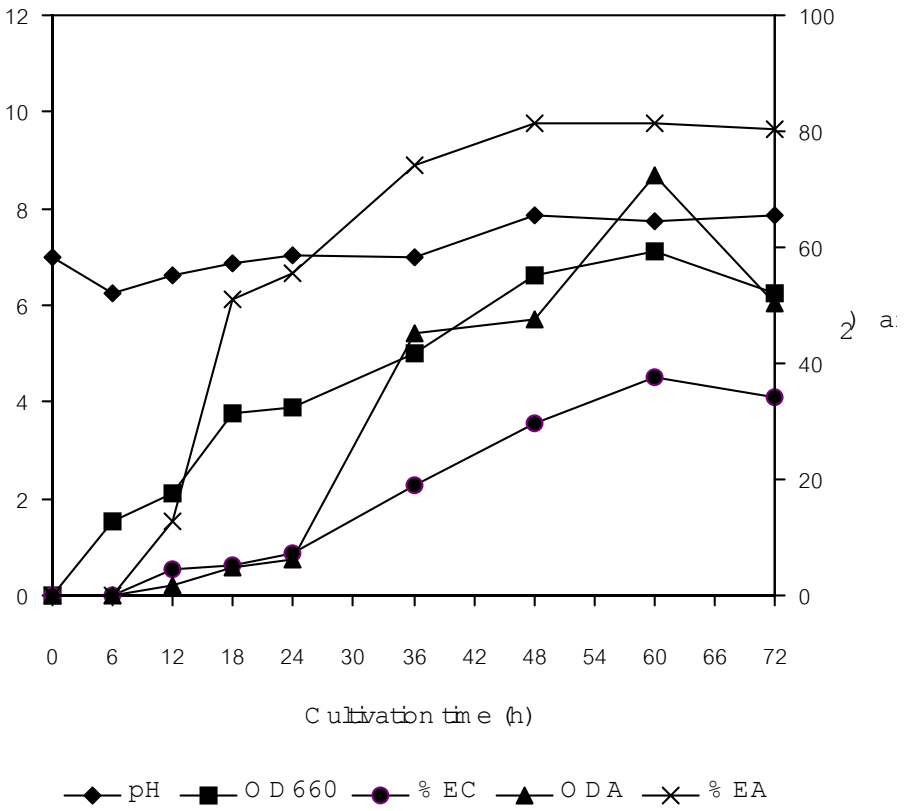


Figure 16 Growth and biosurfactant production by *Bacillus* MUV4 during cultivation in fermentor at 30 ^{o}C with agitation speed 200 rpm and aeration rate 1.0 vvm for 60 h

3. Properties of biosurfactant

3.1 Solubility of the acid precipitated biosurfactant

The solubility of the acid precipitated biosurfactant was performed by adding 10 mg biosurfactant in 10 ml of various solvents and mixed well. The biosurfactant-solvent mixtures were allowed to stand for 24 h and the solubility was observed. The biosurfactant-solvent mixtures were centrifuged and then the solutions were evaporated to remove the solvent. The crude samples were dissolved in 0.02 M Tris-HCl buffer pH 7.3 and tested the oil displacement activity. It was found that the crude samples in water, alkaline water, methanol, ethanol, acetone, ethyl acetate, acetonitrile and chloroform were positive detection by oil displacement activity (ODA). This indicated that the acid precipitated biosurfactant was good soluble in high polar solvent such as water and alkaline water but was slightly soluble in methanol, ethanol, acetone, ethyl acetate, acetonitrile and chloroform because it was observed a little of residual precipitate in the solution. The acid precipitated biosurfactant was insoluble in hexane which showed negative value of oil displacement activity (Table 3) and it had much residual precipitate in solution. This finding was similar to that of Kim *et al.* (1997), who reported that BS-C9 biosurfactant was soluble in alkaline water and chloroform but not in acetonitrile, ethyl acetate and n-hexane. Surfactant BL86 was found to be soluble in alkaline water, tetrahydrofuran (THF), methanol, chloroform, dichloromethane, xylane and toluene but was not soluble in n-hexadecane (Horowitz *et al*., 1990).

Table 3 Solubility test of the acid precipitated biosurfactant in various solvents

Solvents	Solubility
Water	+ +
Alkaline water	+ +
Methanol	+
Ethanol	+
Acetone	+
Ethyl acetate	+
Acetonitrile	+
Chloroform	+
Hexane	-

+ + good soluble + low soluble - insoluble

3.2 pH stability of biosurfactant

The effect of pH on stability of biosurfactant was shown in Figure 17. After the culture broth and acid precipitated biosurfactant (partially purified biosurfactant) solution was adjusted to various pH ranging from 2.0 to 14.0 compare to control (pH 7.74), and kept for 12 h at 4 ^{o}C. It was found that the activity of culture broth and acid precipitated biosurfactant of *Bacillus* MUV4 was relatively stable to pH change. pH had much effect on ODA and EC. Relative ODA and EC of culture broth was stable at the pH range 6.0-10.0. It retained more than 70% when the pH decreased to pH 6.0 and increased to pH 10.0 compared to control (pH 7.74) while relative EA was stable at the pH range 4.0-14.0. It retained more than 80% when the pH decreased to pH 4.0 and increased to pH 14.0. Relative ODA, EA and EC of acid precipitate biosurfactant were stable at the pH range 6.0-12.0. The maximum ODA, EA and EC values of biosurfactant both from culture broth and acid precipitation had retained more than 80% at pH 8.0, it may due to the pH was little changed compared to control (pH=7.74 and 7.44, respectively). At the lower end of pH scale (<4.0), the

activity of biosurfacatant was reduced due to precipitation. Similar results had been reported for surfactin (Cooper *et al*., 1981), lichenycin B (McInerney *et al*., 1985), biosurfactant BF 2.2 (Sutthivanitchakul *et al*., 1999) were stable at a wide range of pH 6.0 to 12.0 and biosurfactant from *Bacillus* sp. strain KP-2 was stable at a pH range 7.0 to 10.0. The biosurfactant was not stable at the pH below 6.0 due to biosurfactant in the culture broth was precipitated (Roongsawang *et al*., 1999). In this experiment, the biosurfactant from culture broth of *Bacillus* MUV4 had more than 70% of both ODA and EC activity remained at pH 6.0-10.0. Islam (2001) reported that the biosurfactant from culture broth of *Pasteurella* PA6 and *Acinetobacter* S7 had more than 70% of %EC and ODA remained at pH 4.0-8.0 and 2.0-6.0, respectively.

3.3 NaCl concentration stability of biosurfactant

The effect of NaCl concentrations on the stability of biosurfactant from *Bacillus* MUV4 was shown in Figure 18 (A),(B). The biosurfactant was dissolved in 0-35% NaCl at 25 ^{o}C for 20 min and activity of biosurfactant was measured. The result showed that the activity of biosurfactant was reduced when the NaCl concentrations increased. The maximum activity of biosurfactant (ODA, EA and EC of 95.95 cm^2, 74.24% and 95.94%, respectively) was retained when the NaCl concentration was presented at 5%. The NaCl concentration had much effect on ODA and EA. At 20% NaCl the relative ODA of culture broth was less than 10% and EA was not detectable while % relative EC still higher than 60% (Figure 18(A)), compared to the stability of acid precipitated biosurfactant, the relative ODA less than 10% at 15% NaCl, relative EA was not detectable while relative EC was retained more than 25%. This result suggested that the biosurfactant in the culture broth was stable to NaCl concentration than those of the acid precipitated biosurfactant. Islam (2001) reported that the biosurfactant from culture broth of *Pasteurella* PA6 and *Acinetobacter* S7 had more than 70% of %EC and ODA remained at 5-15% and 5-20% NaCl. Roongsawang *et al.* (1999) reported that the ability of biosurfactant BF2.2 from

Bacillus licheniformis F2.2 to reduce surface tension dropped when NaCl concentration exceeded 10%.

3.4 Temperature stability of the biosurfactant

The temperature stability of biosurfactant was examined by incubating the culture broth or the acid precipitated biosurfactant solution at various temperatures for 48 h. The samples were taken every 6 h for measuring the ODA, %EA and %EC values. The result showed that %relative ODA, EA and EC values were decreased when the incubation time and temperature increased. Temperature had much effect on ODA and EC than EA value. The EA value of the biosurfactant was slightly affected by temperature even at 100 ^{o}C for 12 h, it still retained EA activity more than 80% (Figure 19, 20). This is contrast to the report of Horowitz *et al*. (1990) showed that the surfactant from *Bacillus licheniformis* 86 was stable for 20 min incubation at temperature of 25 to 120 ^{o}C. The biosurfactant KP-2 from *Bacillus* sp. KP-2 showed a high surface activity (2.4-2.6 v/v CMC) at the temperature ranging from 25 to 121 ^{o}C for 20 min (Rungsawang *et al*., 1999). Similar to the biosurfactant from *Serratia marcencens* and *Arthrobacter protophormiae* stable over a wide range of temperature 30 to 100 ^{o}C and 10 to 120 ^{o}C, respectively (Pruthi and Cameotra, 1997a; Pruthi and Cameotra, 1997b). Compared to biosurfactant from *Nocardia* sp., remained stable even after exposure to the high temperature of 100 ^{o}C for 3 h (Kim *et al*., 2000).

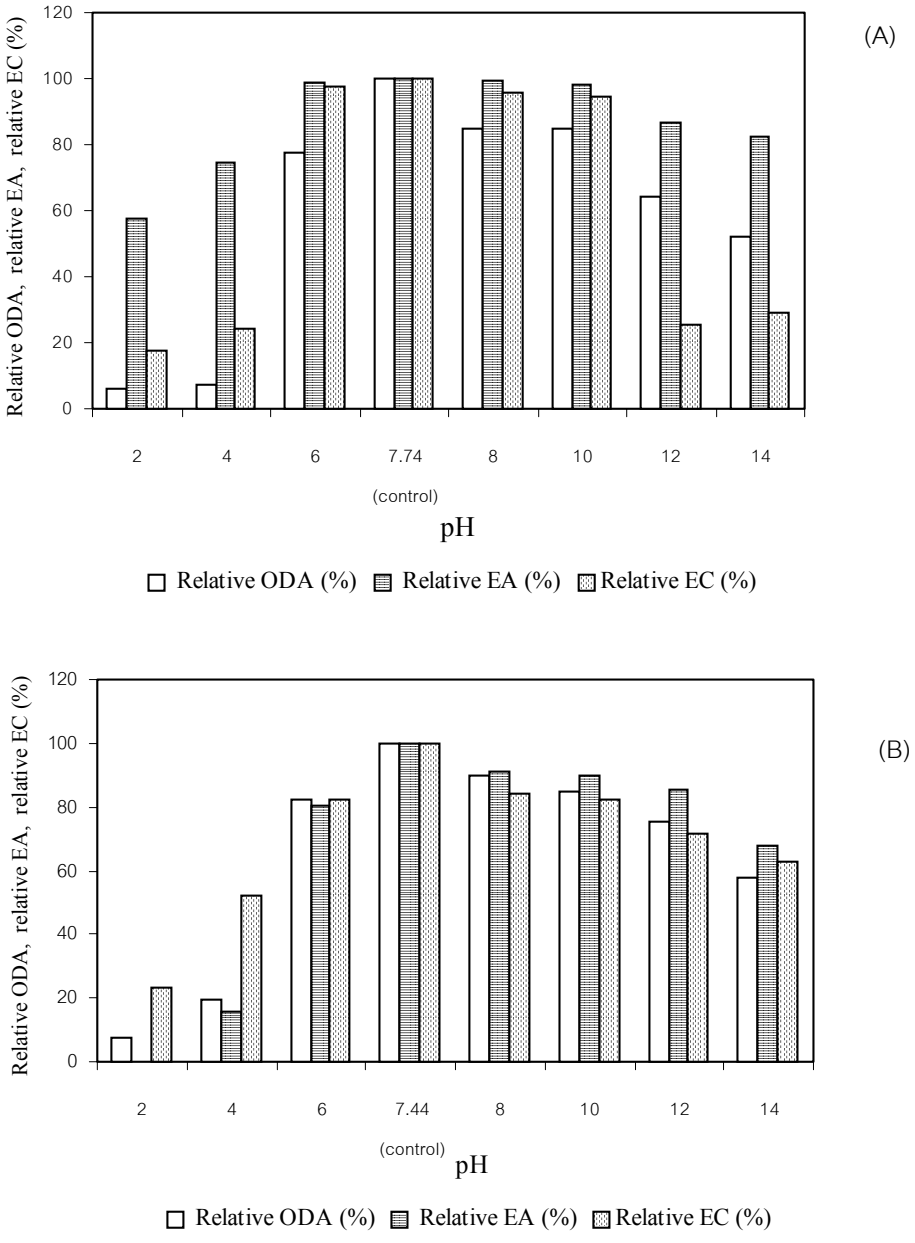


Figure 17 Effect of pH on stability of culture broth (A) and acid precipitated biosurfactant (B) from *Bacillus* MUV4 (biosurfactant was adjusted with 1 M HCl or 1 M NaOH to pH 2-14 and allowed to stand for 24 h at 4 ^{o}C before measuring ODA, EA and EC values)

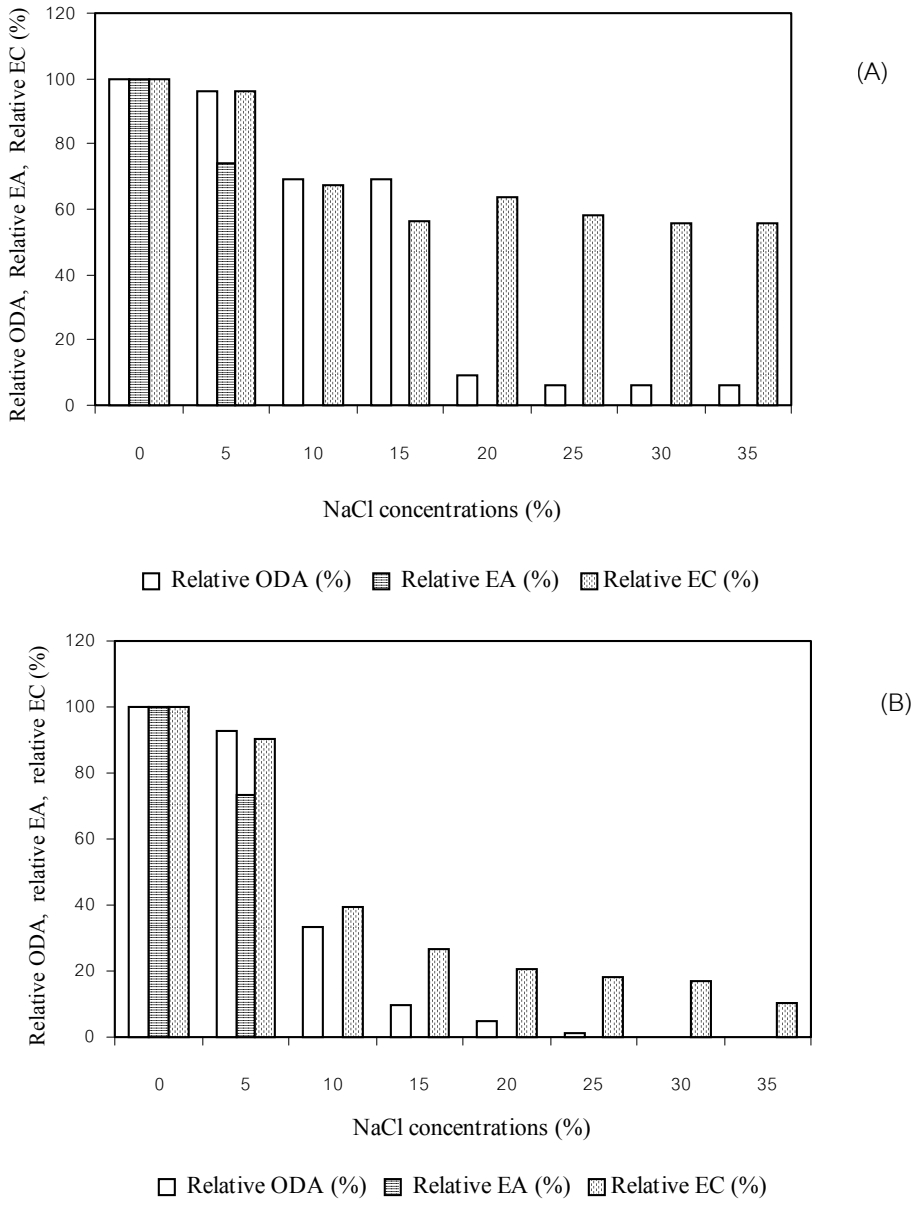


Figure 18 Effect of NaCl concentrations on stability of culture broth (A) and acid precipitated biosurfactant (B) from *Bacillus* MUV4 (NaCl was added in culture broth to final concentration 0-35% and allowed to stand for 20 min at 25 ^{o}C before measuring the ODA, EA and EC values)

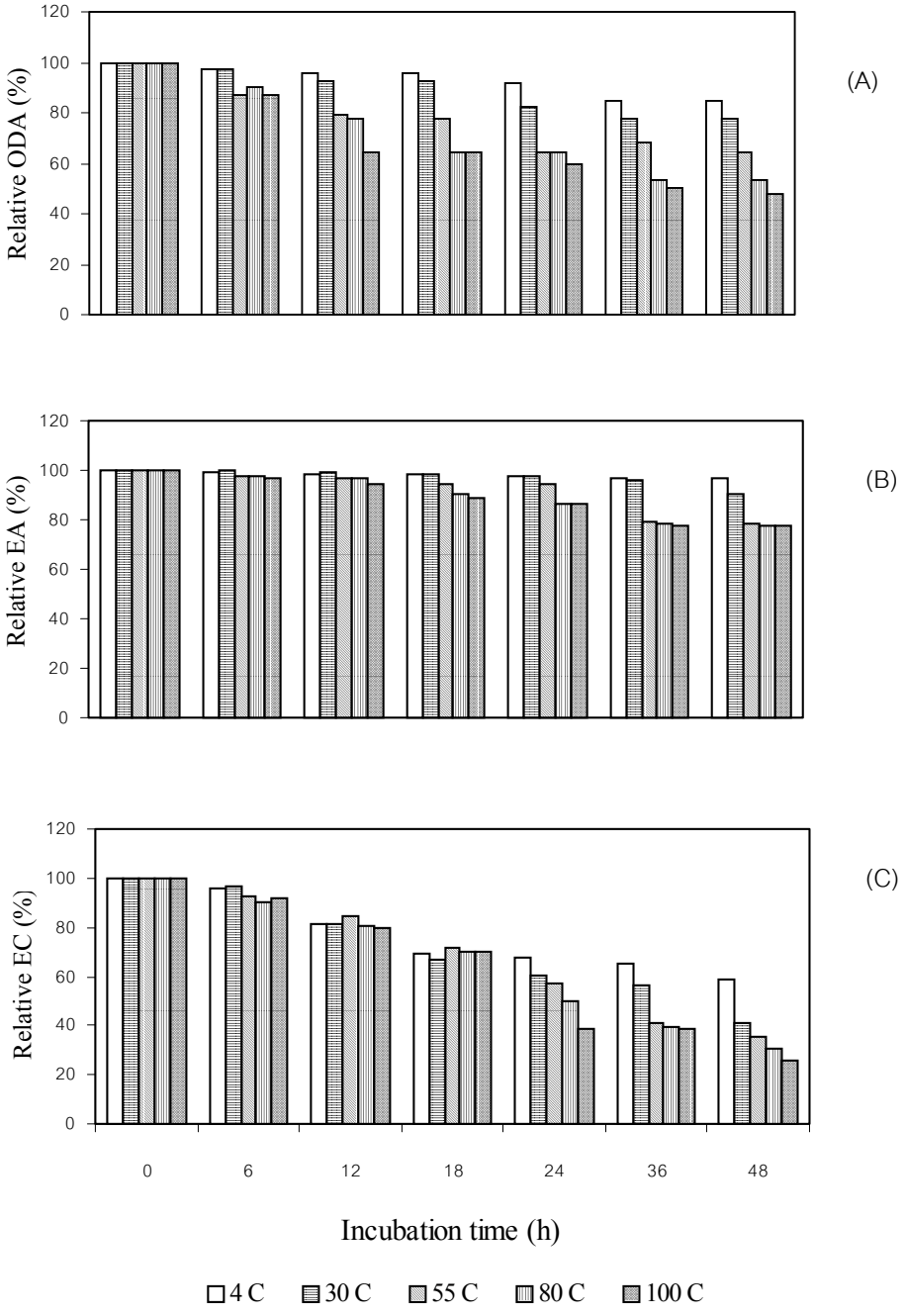


Figure 19 Effect of temperature on ODA (A), %EA (B) and %EC (C) stability of culture broth from *Bacillus* MUV4 (incubation the culture broth at various temperature for 48 h)

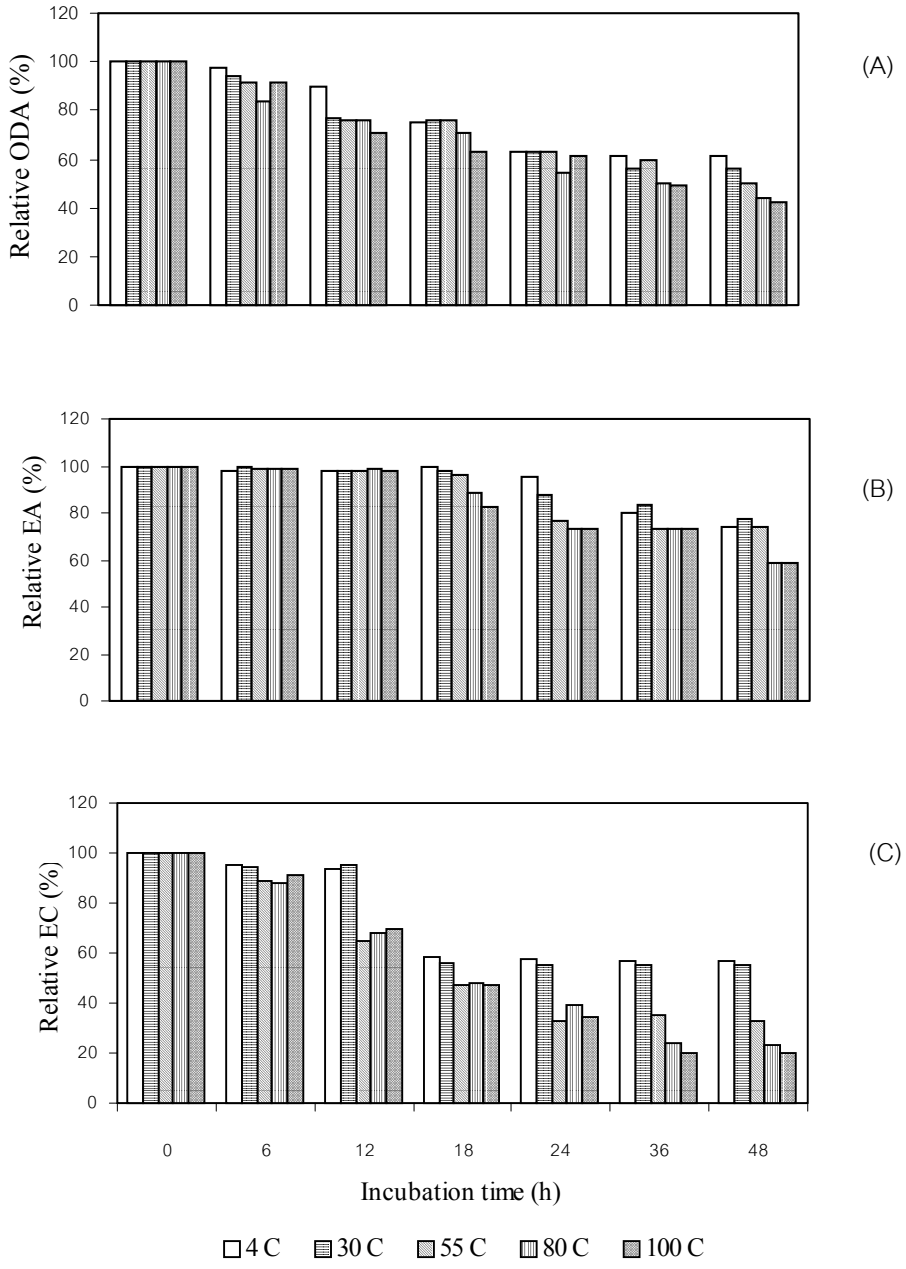


Figure 20 Effect of temperature on ODA (A), %EA (B), %EC (C) stability of acid precipitated biosurfactant from *Bacillus* MUV4 (incubation the of acid precipitated biosurfactant solution at various temperature for 48 h)

4. Analysis of the components of biosurfactant

Biosurfactant from *Bacillus* MUV4 cultivated in the optimal Mckeen medium showed the high ODA, %EA and %EC. In addition, the biosurfactant from *Bacillus* MUV4 was stable over a wide range of temperature (4-100 ^{o}C), pH (6-12) and 5% NaCl.

The biosurfactant was obtained by acid precipitation with 6.0 N HCl of 1.0 litter of cell free broth after 60 h cultivation. The acid precipitate biosurfactant that formed was collected by centrifugation. The acid precipitate was dissolved in distilled water and adjusted to pH 7.0 with 2.0 N NaOH and the solution was lyophilized. The acid precipitated biosurfactant yield was 0.8 g/l with yellow brown color. It was dissolved in 0.02 M Tris-HCl pH 7.3 and measured the ODA, %EA and %EC. The ODA, %EA and %EC values of the acid precipitated biosurfactant were shown in Table 4 compared to those of culture broth. The results showed the biosurfactant in acid precipitate was lower ODA, %EA and %EC value than those of culture broth these may be due to its losing in the step of extraction and lyophilization.

The acid precipitated biosurfactant was twice extracted with equal volume of ethyl acetate. The insoluble materials (precipitate) and ethyl acetate extract were dissolved in 0.02 M Tris-HCl pH 7.3 and measured the ODA and %EC. It was found that it did not have the activity of biosurfactant in the residual precipitate, but in the ethyl acetate extract had the ODA and %EC were 49.1 cm^2 and 4.0%. The activity of solvent extract was lower than those of acid precipitated biosufactant. These may be due to its losing in the step of ethyl acetate extraction and evaporation.

Table 4 The activity (ODA (cm^2) ,%EA and %EC) of biosurfactant in culture broth and acid precipitated biosurfactant

Fraction	ODA (cm^2)	%EA	%EC
Culture broth	80.0	81.82	4.80
Acid precipitate	72.35	80.0	4.21

Adsorption chromatography of the crude biosurfactant on a silica gel column by stepwise elution using solvent with increasing polarities (hexane > acetone > chloroform > chloroform : methanol (2:1 v/v) > methanol). After elution each fractions was evaporated to removed solvent and measured the ODA value. It was found that the biosurfactant was eluted by chloroform and methanol but the chloroform eluated fraction gave higher ODA value than methanol eluated fraction. The compounds eluted by chloroform was analyzed by thin layer chromatography (TLC) using different solvent systems. This result showed that different solvent systems gave the different pattern of each spot separation. The chloroform eluated fraction was separated well when using the following solvent system: chloroform/methanol/acetic acid/water (25/15/4/2 v/v/v/v). When it was tested with TLC reagents, it showed the different R_f values. While the methanol eluated fraction was not good separated by different solvent systems.

The components of crude biosurfactant were tested by spraying the TLC plates with ninhydrin, rhodamine 6G and alkaline potassium permaganate and visualized under UV light (280 nm) and iodine vapour. The result was shown in Figure 21 and the chemicaltesting of each spots from TLC plate was shown in Table 5. They were positive detection with UV light (spot A, C with R_f of 0.90 and 0.67, respectively) with blue spots. When the TLC plate was tested with iodine vapour, it gave the brown spots (spot A, C, D) indicated that it was carbohydrate compounds such as sugar mercaptals, alcohols, glycosides, N-acylamino sugar or non-reducing sugar but when the TLC plate tested by alkaline potassium permanganate and anisaldehyde, it showed negative detection indicated that it was not sugar, alcohol, reducing sugar and non-reducing sugar. However, iodine vapour are used for detecting lipid, which brown spots are given by all ipids (Dowson *et a.,* 1968) and when the TLC plate was sprayed with rhodamine 6G for detecting lipid it was showed positive detection on spot A and C indicated that they were lipid compounds (Table 5). The TLC plate was tested with ninhydrin reagents, the violet color spots were observed on TLC

plate (spot A, B, C, D, E with R_f of 0.90, 0.70, 0.67, 0.29 and 0.09, respectively) indicated that it was amino acid compounds. As a result for analyzing the components of biosurfactant from *Bacillus* MUV4 suggested that it was lipopeptide surfactants, which consisted of lipid moiety containing peptide groups in the molecules. These results similar to another biosurfactant produced by *Bacillus* species, which usually produced lipopeptide or lipoprotein surfactant such as lipopeptide from *Bacillus licheniformis* JF-2 (Lin *et al*., 1994) and *B. licheniformis* (Jenny *et al*., 1991), which decreased the surface tension of water from 72 mN/m to 27 mN/m. The lipopeptide biosurfactant BL-86 produced by *B. licheniformis* 86 is capable of lowering the surface tension of water to 27 mN/m (Horowitz *et al*., 1990)

Each spots from TLC plate were scrapped off and extracted with chloroform. The solvent was removed by evaporation to dryness. The dry sample was dissolved in 0.02 M Tris-HCl and tested for emulsification activity and antimicrobial activity. The results showed that spot A, C and D was positive detection on emulsification activity and spots A, B, C and D showed antimicrobial activity against the growth of *B. anthacis*, *B. subtilis*, *Shigella* sp. and *S. faecalis* ATCC 29212 (Table 6).

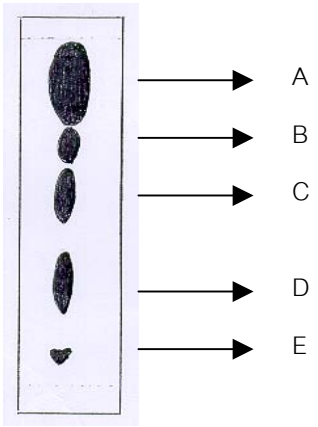


Figure 21 Thin-layer chromatography of biosurfactant from *Bacillus* MUV4 when developed in solvent system :chloroform/methanol/acetic acid/water (25/15/4/2 v/v/v/v)

* R_f of each spots : A=0.90, B=0.70, C=0.67, D=0.29 and E=0.09

Table 5 The chemical testing of each spots from TLC plate

Each spot from TLC plate	Chemical testing				
	UV light	Iodine vapour	Ninhydrin	Rhodamine 6G	Anisaldehyde
A	+	+	+	+	-
B	-	-	+	-	-
C	+	+	+	+	-
D	+	+	+	-	-
E	-	-	+	-	-

Table 6 Emulsification activity and antimicrobial activity of each spots from TLC plate

Each spots from TLC plate	Emulsification activity	Antimicrobial activity
A	+	+
B	-	+
C	+	+
D	+	+
E	-	-

5. Application of biosurfactant

5.1 Antimicrobial activity

Antibiotic activity of the acid precipitated biosurfactant was tested in the concentration 5 mg/ml. A survey of the result was given in Table 7.

Table 7 Antibiotic activity of the biosurfactant from *Bacillus* MUV4 against microorganisms in agar-diffusion test

Microorganisms	Inhibition zone (mm)
Bacillus anthracis	10.0
Bacillus subtilis	10.0
Escherichia coli ATCC 25922	-
Pseudomonas aeruginosa	-
Salmonella sp.	9.0
Shigella sp.	-
Staphylococcus aureus ATCC 25923	-
Streptococcus faecalis ATCC 29212	12.0

[a] Media composition in g/l : NA, bacto peptone, 5 ; beef extract, 3 ; bacto agar, 15.

The biosurfactant showed activity against the growth of *B. anthracis*, *B. subtilis*, *Shigella* sp. and *Streptococcus faecalis* 29212 but was not against the growth of *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa, Salmonella sp*. and *Staphylococcus aureus* ATCC 25923. As a result, it was shown that the biosurfactant from *Bacillus* MUV4 inhibited the growth of Gram-positive bacteria greater than Gram-negative due to may some antimicrobial agent concluded in the culture broth since the *Bacillus* MUV4 could produce antibiotic agent, which inhibited the growth of *B. anthracis*, *Salmonelar* sp. and *A. hydrophila* (H-Kittikun *et al*., 1993). Jenny *et al*. (1991) reported that lipopeptides from *B. licheniformis* inhibited the growth of Gram-negative

bacteria *P. aeruginosa* and *E. coli* and showed activity against a variety of yeast strains such as *Saccharomyces cerevisiae*, *Trichosporon cutaneum*. Yakimov *et al*. (1995) reported that lichenycin A inhibited growth of *Bacillus subtilis*, *E. coli*, *P. fluorescens* and *Staphylococcus aureus*. No growth inhibition by lichenycinA was detected for *B. licheniformis* BAS50, *B. cereus* and *Rhodococus gluberulus*. Since these tests were not standardized, the results are only qualitative factor such as medium composition, density and age of innoculum, which could all affected the determination of antibiotic activity were not investigated.

5.2 Sandpack column test

To study the possible commercial application the precipitated biosurfactant in microbially enhanced oil recovery (MEOR), a sandpack column was prepared with acid washed sand. The acid precipitated biosurfactant (1.0 g/l) aqueous solution) was effective in recovery of oil from sandpack column saturated with known amounts of kerosene oil. When the sandpack column was washed with the distilled water or medium broth, 35.34% and 30.51%, respectively of kerosene oil was recovered (Table 6). However, on adding the acid precipitated biosurfactant solution (1.0 g/l), cell-free broth and culture broth with cell to the column, 50.04, 44.41 and 37.34% of kerosene oil could be recovered as a result of biosurfactant efficacy. Compared with water, the acid precipitated biosurfactant (1.0 g/l) could be recovered kerosene oil from sandpack column about 1.4 folds. This result compared to the other biosurfactant from *Serratia marcescens* and *Arthrobacter protophormiae* (Pruthi and Cameotra, 1997a; Pruthi and Cameotra, 1997b). They were demonstrated that as a result of biosurfactant action 80-90% of oil could be efficiently recovered from the column.

Table 8 Oil released from sandpack column test with various samples

Samples	% oil released
Water	35.34
Medium broth	30.51
Culture broth with cell	37.34
Culture broth	44.41
Acid precipitated biosurfactant	50.04