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Original Article

Production and applications of biosurfactant from *Bacillus subtilis* MUV4

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Abstract

Bacillus subtilis MUV4 produced biosurfactant in shake-flask culture (200 rpm) at 30°C with modified Mckeen medium containing 1% glucose as a carbon source, 1% monosodium glutamate and 0.3% yeast extract as nitrogen sources. The supernatant of *B. subtilis* MUV4 reduced the surface tension of the medium from 53.50 mN/m to 33.50 mN/m after 48 h of cultivation. The yield of crude biosurfactant from *B. subtilis* MUV4 after precipitating the supernatant with 6N HCl was 0.652 g/L. Growth kinetics studies showed the specific growth rate (μ) of 0.14 h^{-1}, yield of biomass to substrate (Yx/s) of 0.713, yield of product to substrate (Yp/s) of 0.072 and yield of product to biomass (Yp/x) of 0.101. Moreover, *B. subtilis* MUV4 produced 0.30 g/L crude biosurfactant after 96 h of cultivation in the fermentor with agitation rate of 200 rpm without aeration and uncontrolled pH condition. The crude biosurfactant was dissolved in methanol and dried by vacuum evaporator (crude methanol). The supernatant, the crude biosurfactant and the crude methanol retained the biosurfactant activity over the pH range of 1-6, 7-10 and 4-10, respectively and the emulsion stability at 24 h (E_{24}) at pH 7 were 66.67%, 33.33% and 33.33%, respectively. The supernatant and the crude biosurfactant showed surface tension activity at 4°C, room temperature (30±2°C) and 50°C after incubation for 5 h. However, only crude methanol still retained surface tension activity after 100°C for 5 h. The surface tension activity of the supernatant and the crude biosurfactant was stable in 3-10% (w/v) NaCl while crude methanol showed stability in 3-20% (w/v) NaCl. However, all samples lost emulsion stability when NaCl concentration was higher than 5% (w/v). With sand pack column technique, crude methanol enhanced the recovery of crude oil and kerosene oil by 41.85% and 75.00%, respectively. In hydrocarbon degradation application study, the crude biosurfactant was added to the culture medium containing 0.3% crude oil as carbon source and the microorganism consortium from oil contaminated soil. The result showed that the saturated hydrocarbon was reduced by 96.63% after 7 day cultivation, while the control without crude biosurfactant showed only 19.96% reduction.

Keywords : biosurfactant, biodegradation kinetics, weathered crude oil, *Bacillus subtilis*

1. Introduction

Biosurfactants are surface-active substances derived from living organisms, especially microorganisms. Biosurfactants are amphiphilic compounds, containing hydrophobic and hydrophilic moieties. The hydrophilic moiety can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic moiety usually is a long chain fatty acid (Lang, 2002). Biosurfactants are being investigated as replacements for synthetic surfactants because they are biodegradable, less sensitive to extreme environments and can be produced on renewable substrates (Sullivan, 1998). The potential applications of biosurfactants in indus-

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trial include emulsification and foaming for food processing, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals (Lin, 1996). In addition, biosurfactants can be use in environmental applications such as bioremediation and dispersion of oil spills (Banat, 1995). Biosurfactants can be devided into 4 groups base on their overall structures. They are glycolipids, phospholipids, lipoproteins or lipopeptides and polymeric (Healy *et al*., 1996).

A large variety of *Bacillus subtilis* strains produce lipopeptide biosurfactants which possess a high surfactant activity such as surface-active properties and antibacterial activity. Surfactin is one of the most effective lipopeptide biosurfactants produced by *B. subtilis*. It reduced the surface tension of water from 72 mN/m to 27 mN/m (Cooper *et al*., 1981). Moreover, surfactin can be used for improving the treatment of residual hydrocarbon from ship bilge waste (Olivera *et al*., 2000). Addition of a non-sterile biosurfactant obtained from *B. subtilis* O9 could enhance biodegradation of aliphatic hydrocarbons from 20.9% to 35.5% and of aromatic hydrocarbon from nil to 41% (Moran *et al.,* 2000). The polycyclic aromatic hydrocarbons (PAHs) pose potential problem for bioremediation of contaminated sites because of their low water solubility. The surface-active agents like surfactin increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability (Ron and Rosenberg, 2002). *Bacillus subtilis* MUV4 is a rod shape bacterium, (0.5 x 2.0-3.0 mm) and motile by peritrichous flagella. It was the mutant of *Bacillus* KUBA 8601 after UV irradiation. The organisms produced antibiotics such as macrolactin, which inhibited *Aeromonas hydrophila, Bacillus anthracis* and *Salmonella* sp. (H-Kittikun, *et al*., 1993). Prommachan (2002) reported that *B. subtilis* MUV4 produced the lipopeptide biosurfactant 0.8 g/L in Mckeen Medium with 2.5% glucose. In this research, growth and biosurfactant production by *B. subtilis* MUV4 were studied. The applications of the crude biosurfactant after acid precipitation on oil recovery and enhancement of hydrocarbons degradation were also investigated.

2. Materials and Methods

2.1 Microorganisms

Bacillus subtilis MUV4 was a mutant of *Bacillus* KUBA 8061 isolated by Dr. Orapin and her group at Kasetsart University, Bangkok, Thailand (H-Kittikun *et al*., 1993). The strain was maintained on nutrient agar slant and stored at 4°C.

2.2 Growth and Biosurfactant Production

B. subtilis MUV4 was grown in nutrient broth at room temperature (30±2°C) with agitation speed of 200 rpm for 16 hours ($4.6x10^6$ CFU/ml) and was used as an inoculum at the concentration of 10%(v/v). For biosurfactant production, *B. subtilis* MUV4 was grown in 250 ml flask with 100 ml of Mckeen medium at the same conditions for 96 hours. The medium consisted of (w/v) 0.5-2.5% glucose, 1.0% monosodium glutamate, 0.3% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, 0.05% KCl and 0.1%v/v of trace element (0.64 g $MgSO_4.7H_2O$, 0.16 g $CuSO_4.5H_2O$ in 100 mL of distilled water) (Prommachan, 2002). The batch fermentation was conducted in a 2-L fermentor (Biostat B, B. Braun International) with 1 liter working volume. The culture conditions were uncontrolled pH at 30°C, 200 rpm with or without aeration at 1.0 vvm. These experiments were done by triplicate and used ANOVA for statistical test by SPSS program.

2.3 Analyses

Cell growth was determined by monitoring the optical density of culture broth at 660 nm. The biomass was determined from the cells after centrifugation of the culture broth at 10,000 rpm (6,700 g) 4°C for 10 min. The dry cell weight (DCW) was obtained from the cell pellets by washing twice with distilled water and drying in hot air oven at 105°C for 24 hours. The reducing sugar was determined from the culture supernatant using Nelson-Samogyi method (Samogyi, 1952). Biosurfactant activity was measured from the culture supernatant after cell separation by using a ring tensiometer (K6, Kruss, Germany) at room temperature (Yakimov *et al*., 1995). Emulsion Index (E_{24}) was performed by adding a sample and hexadecane at the 1:1 ratio and vortexing at high speed for 2 minutes. The emulsion stability was determined after 24 hours. The E_{24} was calculated by measuring the emulsion layer formed (Cooper and Goldenberg, 1987).

$$E_{24} = \frac{\text{Height of emulsion x 100}}{\text{Total height of the mixture}}$$

2.4 Isolation of Biosurfactant

After 36 hours of cultivation, the biosurfactant was isolated from the culture supernatant by precipitation with concentrated hydrochloric acid (6N HCl). After standing at 4°C overnight, the precipitate was centrifuged, dissolved in distilled water, neutralized with 2N NaOH and lyophilized (Yakimov *et al*., 1995). This product was named crude biosurfactant (crude BS). The crude BS was extracted with methanol 3 times and the solvent was removed by rotary evaporator under reduced pressure. The obtained product was named crude methanol (crude MeOH).

2.5 Applications of Biosurfactant

Enhancement of Oil Recovery : The application of the biosurfactant in oil recovery was evaluated using the sand pack column technique (Makkar and Cameotra, 1998). The glass column (13.0x0.5 cm) was packed with 1 g of acid-washed sand and was equilibrated with 0.5 mL oil

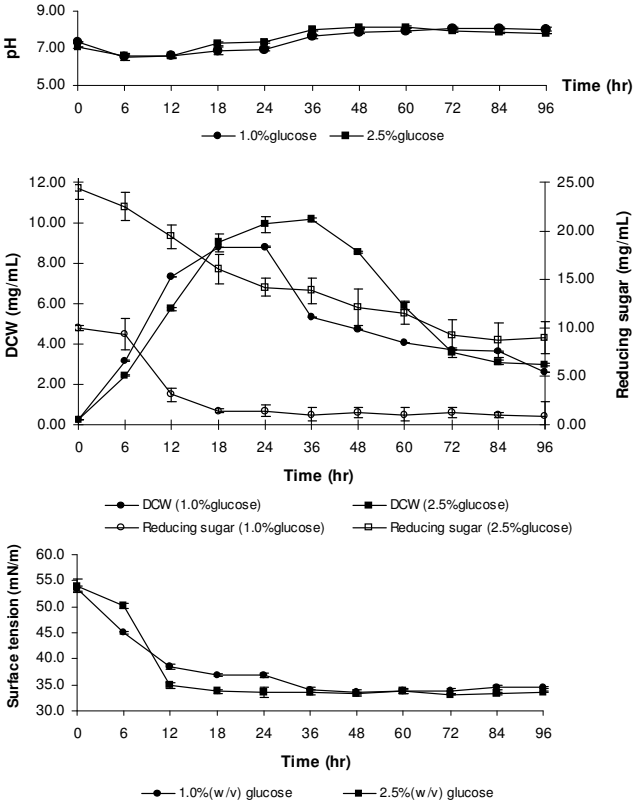

Figure 1. Time course of growth and surface tension activity of *Bacillus subtilis* MUV4 in shake-flask culture at 200 rpm and 30°C in Mckeen medium with 1.0% and 2.5%(w/v) glucose. (DCW = Dried Cell Weight)

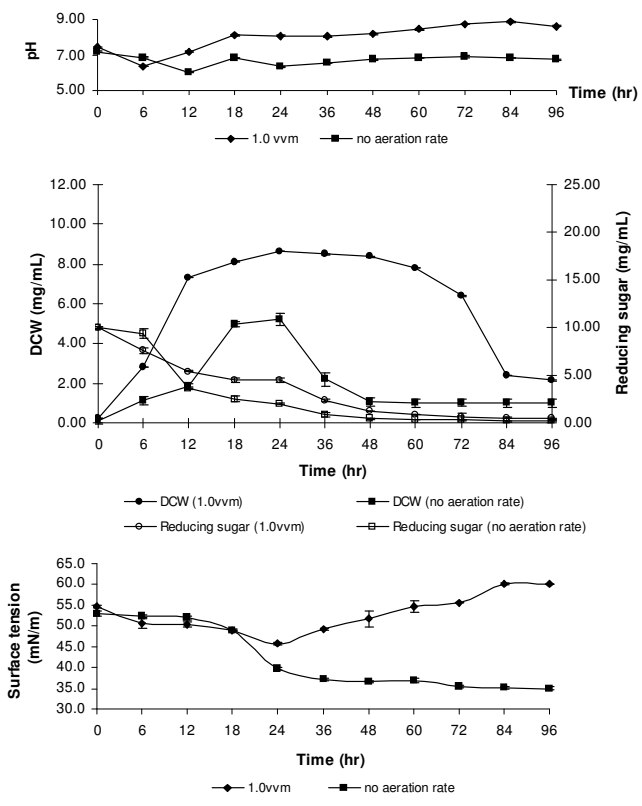

Figure 2. Time course of growth and surface tension activity of *Bacillus subtilis* MUV4 in Mckeen medium (1.0%(w/v)glucose) in fermentor with aeration rate at 200 rpm, with and without aeration at 30°C and uncontrolled pH. (DCW = Dried Cell Weight)

Table 1. Kinetics of growth and biosurfactant production by *Bacillus subtilis* MUV4 in Mckeen medium with different glucose concentrations (in shake-flask culture at 30°C and 200 rpm).

%w/v glucose	Crude biosurfactant (mg/mL)	DCW (mg/mL)	Specific growth rate (μ) (h^{-1})	Sugar Utilization (q_s)	Yx/s	Yp/x	Yp/s
0.5	0.436	6.538	0.14	0.053	1.413	0.077	0.110
1.0	0.652	8.804	0.14	0.065	0.713	0.101	0.072
1.5	0.778	8.883	0.11	0.053	0.697	0.085	0.059
2.5	0.332	10.154	0.11	0.029	0.941	0.023	0.022

(weathered crude oil or kerosene oil). Supernatant, crude BS or crude MeOH at concentration 1 mg/mL (3 mL) was used to elute the oil in the column comparing to distilled water or 1 mg/mL sodium dodecyl sulfate (SDS). The released oil was determined by extracting the eluting components with chloroform and weighing the oil after evaporation.

Enhancement of Oil Degradation : The culture medium was mineral salt yeast extract medium (MSYM) consisted of (g/L) 4.0 Na_2NO_3, 0.5 Na_2HPO_4, 1.5 KH_2PO_4, 0.01 $CaCl_2$, 0.2 $MgSO_4$, 0.0005 $FeCl_3$, 0.1 yeast extract and 0.3 mL of weathered crude oil as a carbon source. Ten grams of the oil contaminated soil which comprised of the indigenous microbial community was added into the medium and cultivated by shaking (200 rpm) at room temperature (30± 2°C) for 3 days and was subcultured 3 times on the same medium. This culture was used as an inoculum (10%v/v) to degrade weathered crude oil in MSYM with or without 10 mg of crude BS. After cultivation the crude oil component was analyzed by TLC/FID (Sharma *et al*., 1998).

3. Results and Discussion

3.1 Growth and Biosurfactant Production

The growth kinetics and biosurfactant production of *B. subtilis* MUV4 in Mckeen medium containing 0.5-2.5%

(w/v) glucose are shown in Table 1. The results showed that glucose had a profound effect on growth as well as biosurfactant production. Maximum growth was found in the medium with 2.5% glucose but maximum biosurfactant production was obtained in the medium with 1.5% glucose. However, the medium with 1.0% glucose showed the highest specific biosurfactant production rate (g-biosurfactant/g-cells). Although yield of product to substrate in the medium with 0.5% glucose was higher than 1.0% glucose, the yield of product to biomass was lower. The surface tension of the culture supernatants in Mckeen medium with 1.0, 1.5 and 2.5 % glucose was not significant different ($p \leq 0.05$).

The growths of *B. subtilis* MUV4 in the Mckeen medium with 1.0 and 2.5% glucose in shake-flask culture were characterized by a short lag phase, a log phase after 6 hours. The highest cell density in the medium with 1.0%(w/v) glucose occurred at 24 hours (Figure 2) while the highest cell density in the medium with 2.5%(w/v) glucose occurred at 24 hours (Figure 2). The pH of the medium decreased during the lag phase and then slightly increased during the early log phase and was stable after 30 hours. The surface tension of the culture supernatants drastically decreased from 53.5 mN/m at 0 hour to 37.0 and 33.5 mN/m at 12 hours of cultivation in the medium with 1.0% and 2.5% (w/v) glucose, respectively. However, a reduction in the surface tension of a medium as a result of biosurfactant production and accumulation during exponential and stationary phases has already been reported (Sandrin *et al.*,1990 ; Roongsawang *et al.*, 2003).

When cultivated in fermentor, *B. subtilis* MUV4 also entered log phase after 6 hours. Aeration stimulated the growth of *B. subtilis* MUV4 but inhibited the biosurfactant production (Figure 2). Cell growth was maximized at 24 hours with 9.80 g/L DCW and the surface tension of the culture supernatant was gradually increased after 24 hours. On the contrary, in the fermentor without aeration, *B. subtilis* MUV4 produced the highest DCW of 5.22 g/L at 24 hours. The lowest surface tension was obtained at 96 hours of cultivation. The yield of crude biosurfactant was 0.30 g/L after lyophilization. The specific growth rate (h^{-1}), yield biomass to substrate (Yx/s), yield product to biomass (Yp/x) and yield product to substrate (Yp/s) in fermentor at 1.0% glucose were 1.51, 0.644, 0.049 and 0.032, respectively. Kim *et al.* (1997) reported that under O_2-limited condition, the biosurfactant was produced by *Bacillus subtilis* C9 at a rate 3 times higher than under O_2-sufficient condition. Lin *et al.* (1994) found that *Bacillus licheniformis* JF-2 produced the biosurfactant at 30% dissolved oxygen (DO) better than at 85% DO.

3.2 Properties of crude biosurfactants

The pH of supernatant, crude BS and crude MeOH were adjusted in the range of 2-12 with either NaOH or HCl and kept for 24 hours before measurement. The surface tension activities of the supernatant and crude BS were maintained over a pH range of 6-12 and pH 7-10, respectively, whereas crude MeOH showed pH stability in the range of pH 4-10 (Figure 3). Roongsawang *et al.* (2003) found that crude MeOH from *B. subtilis* BKK-1 retained activity over a wide range of pH from 5-10. Loss of its activity was observed at pH below 4.0 due to precipitation. At pH 7 the supernatant showed the emulsion index E_{24} of 66.67% whereas crude BS and crude MeOH showed the E_{24} of 33.33% (Figure 4).

For thermal stability (Figure 5), supernatant, crude BS and crude MeOH were incubated for 5 hours at different temperature (4-100°C). Supernatant and crude BS were stable in the range of temperature 4-50°C while crude MeOH still retained its surface tension activity at 100°C. However, supernatant showed maximum E_{24} at room temperature and still had high activity at 50 and 100°C. It is noted that the crude BS had higher emulsion stability at 4°C than at room temperature. The crude MeOH had much less emulsion stability at 4°C and at room temperature but no emulsion stability at 50 and 100°C (Figure 6). The result indicates that the preparation process of crude BS and crude MeOH might have some effect on the property of the biosurfactant.

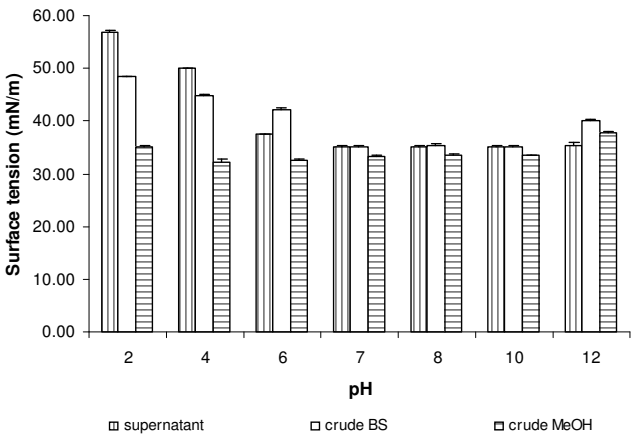

Figure 3. Effect of pH on the surface tension of biosurfactant from *Bacillus subtilis* MUV4.

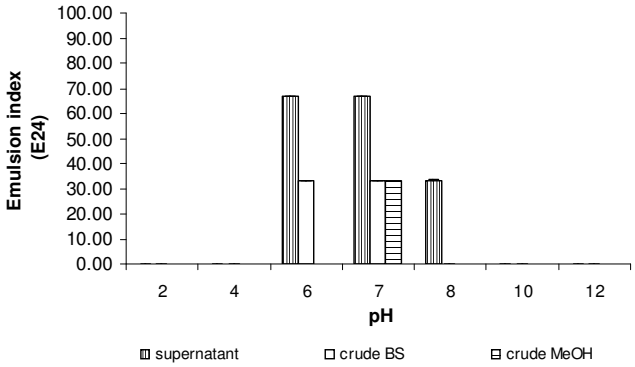

Figure 4. Effect of pH on emulsion index of biosurfactant from *Bacillus subtilis* MUV4.

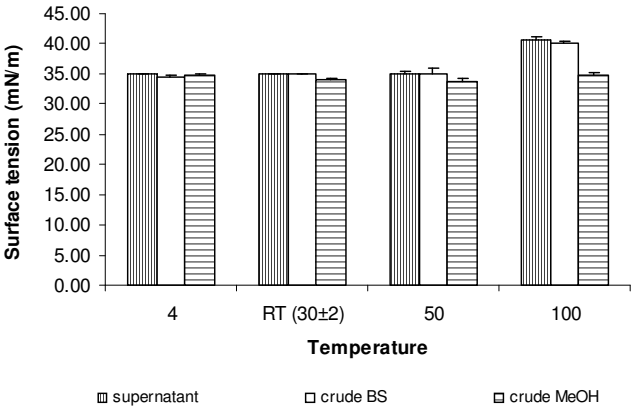

Figure 5. Effect of temperature on the surface tension of biosurfactant from *Bacillus subtilis* MUV4.

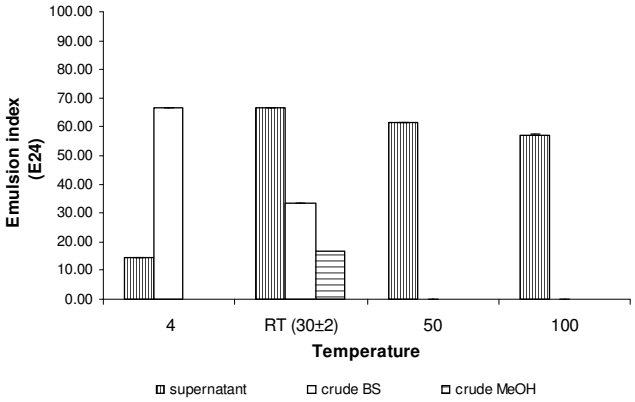

Figure 6. Effect of temperature on emulsion index of biosurfactant from *Bacillus subtilis* MUV4.

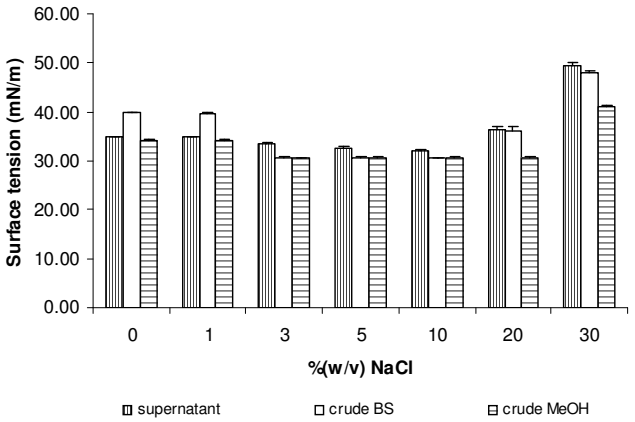

Figure 7. Effect of NaCl on surface tension of biosurfactant from *Bacillus subtilis* MUV4.

The salt resistance of the biosurfactant from *B. subtilis* MUV4 was studied by adding 0-30% (w/v) NaCl into the supernatant, crude BS or crude MeOH before allowing them to stand at 30°C for 24 hours. The supernatant and the crude BS were able to reduce the surface tension in 3-10% (w/v) NaCl while the crude MeOH was stable in 3-20%(w/v) NaCl concentrations (Figure 7).

3.3 Enhancement of Oil Recovery

The supernatant, crude BS and crude MeOH were used to elute weathered crude oil and kerosene oil in the sand packed column (Table 2) compared with SDS and distilled water. The crude MeOH showed better oil recovery than crude BS and the supernatants. However, SDS could elute 100% of both crude oil and kerosene oil while distilled water showed the lowest oil recovery. Percent recovery of kerosene oil was higher than percent recovery of crude oil because the kerosene oil was less viscous and not as complex as the weathered crude oil which composed of the aliphatic hydrocarbon, aromatic hydrocarbon and non-hydrocarbons (Tong *et al*., 1999).

3.4 Enhancement of Oil Degradation

When crude oil was analyzed by TLC/FID, there were 4 major components ; saturated hydrocarbons (SA), aromatic hydrocarbons (AR), resin (RE) and asphalthene (AS) at the retention time of 0.15, 0.29, 0.39 and 0.46, respectively. The SA was reduced rapidly in 3 days when added crude BS in the medium (Figure 8). The reduction of SA was 66.43% and 96.63% after 3 and 7 days of cultivation, respectively, while in the medium without biosurfactant, SA reduction was 3.91% and 19.96% in 3 and 7 days of cultivation, respectively (Figure 9). Aromatic components reduced slightly at 27.54% and 33.78% in 7 days for the medium without and added biosurfactant, respectively. This result may be due to the aliphatic hydrocarbons being more easily to degraded than the aromatic hydrocarbons. Palittapongarnpim *et al.* (1998) found that n-alkane was reduced 99.6% in 7 days of soil microoreganisms when using Tapis crude oil as carbon source in the presence of biosurfactant.

Acknowledgments

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Table 2. Oil recovery from sand pack column by supernatant, crude BS, crude MeOH of biosurfactant from *Bacillus subtilis* MUV4.

Sample	% crude oil recovery ± S.D.	% kerosene oil recovery ± S.D.
Supernatant	25.11±0.75	42.85±1.75
Crude BS	28.56±1.08	50.00±0.34
Crude MeOH	28.56±1.08	75.00±0.43
SDS	100.00±0.58	100.00±0.81
Distilled water	12.64±0.90	18.56±1.10

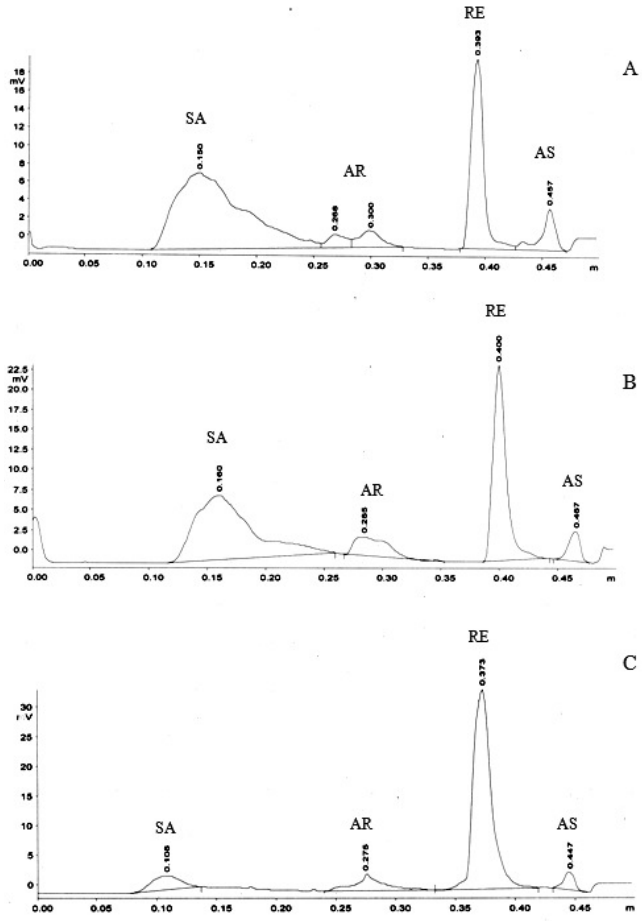

Figure 8. TLC/FID chromatogram of crude oil when cultured in MSYM and added biosurfactant.
A = 0 day of cultivation
B = 3 days of cultivation
C = 7 days of cultivation
Note : Saturated hydrocarbon (RT=0.150±0.01), Aromatic hydrocarbon (RT=0.288±0.01), Resin (RT=0.403±0.00) and Asphaltene (RT= 0.472±0.01) analyzed by TLC/FID method.

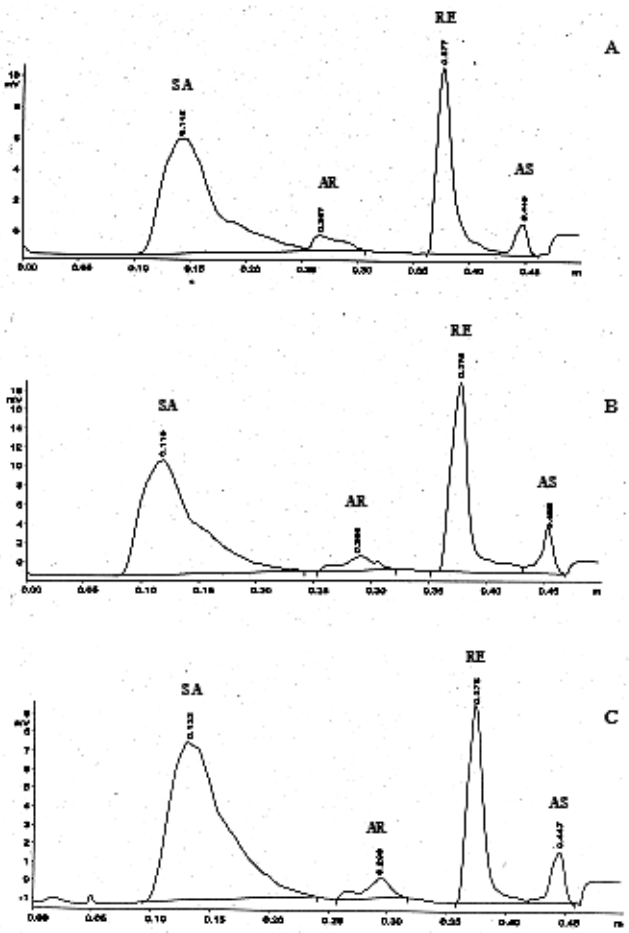

Figure 9. TLC/FID chromatogram of crude oil when cultured in MSYM and no biosurfactant.
A = 0 day of cultivation
B = 3 days of cultivation
C = 7 days of cultivation
Note : Saturated hydrocarbon (RT=0.154±0.02), Aromatic hydrocarbon (RT=0.291±0.01), Resin (RT=0.386±0.00) and Asphaltene (RT= 0.463±0.00) analyzed by TLC/FID method.

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