Chapter 2

Materials and Methods

Materials

1. Microorganisms and media

Bacillus sp. MUV4 was used in this study. It was obtained from the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University. The organism was maintained on slant of the nutrient agar at 4 ^{o}C and subcultured every month. The Mckeen medium contained 20 g glucose, 5.0 g DL-glutamic acid, 1.0 g K_2HPO_4, 1.02 g $MgSO_4.7H_2O$, 0.5 g KCl and 1 ml trace element in distilled water 1000 ml. The trace elements solution composed of 0.5 g $MnSO_4.7\ H_2O$, 0.16 g $CuSO_4.5H_2O$ and 0.015 g $Fe_2SO_4.7H_2O$ in distilled water 100 ml. The pH of medium was adjusted to 7.0. Cultivation was performed on rotary shaker with agitation speed of 200 rpm at 30^{o} C.

Microorganisms used in the antibiotic assay such as *Bacillus anthracis*, *Bacillus subtilis* and *Salmonella* sp. were obtained from the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginos, Shigella* sp., *Staphylococcus aureus* ATCC 25923 and *Streptococcus faecalis* ATCC 29212 were obtained from Department of Microbiology, Faculty of Science, Prince of Songkla University. The cultures were kept in the slant of nutrient agar.

2. Weathered oil

Weathered oil was the by product obtained from Oman crude oil distillation at the temperature of 350 ^{o}C. It was obtained from the Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University.

3. Chemicals

Chemicals used in the analysis were analytical grade. Thin layer chromatography (TLC) aluminium sheets F_{254}, layer thickness 0.2 mm used for analysis the components of biosurfactant.

4. Instruments

Instruments	Model	Company
Double-beam Spectrophotometer	U-2000	Hitachi Koki Co., Ltd., Japan
Fermentor	Biostat B	B.Braun Biotech.International,Germany
Hot air oven	MOV.212	Sanyo Electric Co., Ltd., Japan
Incubator	200	Memmert GmbH+Co.KG., Germany
Incubator shaker	3525-ICC	Lab-line Instrument. Inc., Japan
pH meter	HM-7E	Tokyo TOA Electronic Co.,Ltd., Japan
Refrigerated centrifuge	SCR 20B	Hitachi Koki Co., Ltd., Japan
	Centurion 8000	Dynostic Biotechnology. Ltd., UK
Rotary evaporator	ELELA, SB-651	Tokyo Rikakikai Co., Ltd., Japan
Water bath	W350	Memmert GmbH+Co.KG.,Germany

Analytical methods

1. Cell growth measurement

Culture broth was collected and diluted with medium broth to obtained optimum dilution. After mixing, the absorbance was measured at 660 nm (OD_{660}) by spectrophotometer (Kim *et al*., 1997).

1. Cell dry weight determination

Cell dry weight was determined in triplicate by centrifugation of the culture broth (20 ml) at 10,000 ×g, 4 ^{o}C for 10 min to remove the cells. The cells pellet was resuspended in distilled water (20 ml) and centrifuged again to wash the cells. The washed cells were dried for 24 h at 105 ^{o}C in hot air oven, and then weighed to constant weight after cooling in a desiccator (Cooper and Goldenberg, 1987).

3. Emulsifying capacity (EC) test

The test tube (1.5×15 cm) containing 1 ml of supernatant and 1 ml of 0.02 M Tris-HCl buffer pH 7.3 supplemented with 2 drops of weathered oil was weighed (W1). Then the mixture was vortexed for 30 s and allowed to stand for 5 min. After each addition by 2 drops of weathered oil, the mixture was vortexed for 30 s and allowed to stand for 5 min. This procedure was repeated by added weathered oil until the emulsion collapsed. The test tube was finally weighed (W2) and calculated for percentage of emulsification capacity using the following equation. This method was modified from Ghurye and Vipulanandan (1994) by using weathered oil instead of n-hexadecane.

$$\% \text{ EC} = \frac{W2-W1}{W1} \times 100$$

4. Oil displacement test

Weathered oil (15 µl) was put onto the surface of 40 ml of distilled water in a petri dish (150 mm in diameter). A thin membrane of oil formed immediately. Then, 10 µl of sample solution (supernatant) was gently dropped on the center of the oil membrane. A clear halo was visible under light. The area of this circle was measured and calculated for oil displacement area (ODA) using the following equation (Morikawa *et al*., 1993).

$$\text{ODA} = 22/7 \ (\text{radius})^2 \quad \text{cm}^2$$

5. **Emulsification activity (EA)**

To measure emulsification activity, 4 ml of kerosene oil was added to 4 ml of culture broth in test tube (1.5×15 cm), vortexed at high speed for 2 min and stood for 10 min. The emulsion stability was recorded in terms of percent emulsion. The percent emulsification activity calculated by using the following equation (Cooper and Goldenberg, 1987).

$$\%EA = \frac{\text{height of emulsion formed}}{\text{total height of solution}} \times 100$$

6. Solubility test

Ten mg of the acid precipitated biosurfactant was tested its solubility in several solvents (10.0 ml) such as distilled water, alkaline water, methanol, ethanol, acetone, ethyl acetate, acetonitrile, chloroform and hexane. The biosurfactant-solvent mixture was allowed to stand for 24 h and the solubility was observed. The biosurfactant-solvent mixture was centrifuged and then the solution was evaporated to remove the solvent. The crude samples were dissolved in 0.02 M Tris-HCl buffer pH 7.3 and tested for the oil displacement activity (Kim *et al*., 1997).

7. Sand pack test

A glass column (40×2.5 cm) was packed with 100 g of acid-washed sand (0.2 mm^3). The column was then saturated with 100 ml of kerosene oil. The activity of the isolated surfactant in oil recovery was estimated by pouring 100 ml of aqueous solution of biosurfactant in to the column. The amount of oil released was measured. The samples in this experiment were crude culture broth, culture broth with cell and the partially purified biosurfactant (Makkar and Cameotra, 1997a).

8. Antimicrobial activity

Antibiotic activity of biosurfactant was tested against different microorganisms with the agar diffusion method (modified from Barry and Thornsberry, 1980). The tested microorganisms were cultured in Mueller Hilton medium on rotary shaker (200 rpm) for 16 h. The cultures were diluted with medium broth to obtain the viable cell counts of 10^5 CFU/ml (OD_{660}= 0.5). The 0.1ml of cultures were dropped and spread on the Mueller Hilton agar. A sterile filter paper disc (6 mm) soaked with the biosurfactant solution (10µl) until saturation and put down on the surface of containing the culture of the tested microorganisms. Plates were incubated at 37 ^oC for 24 h. The clear zone around the paper-discs were measured, indicated inhibitory activity against microorganisms.

Methods

1. Effects of carbon and nitrogen sources on growth and biosurfactant production

Starter preparation

One loop of *Bacillus* MUV4 from slant was inoculated into 100 ml of nutrient broth in 250-ml flask and incubated with shaking at 200 rpm at 30 ^oC for 16 h. The culture was diluted with the medium to obtain proper dilution with OD_{660} = 0.7 and used as starter culture.

1.1 Time course of biosurfactant producing by *Bacillus* MUV4 in shak flask

Starter culture (2.5%) was transferred into the Mckeen medium (50 ml) in a 250-ml flask and cultivated on a rotary shaker (200 rpm) at 30 ^oC for 3 days. Triplicate samples were taken at 0, 6, 12, 18, 24, 36, 48, 60 and 72 h to measure cell growth (OD_{660}), pH, %EC and ODA.

1.2 Effect of carbon sources

1.2.1 Types of carbon source

The effects of various carbon sources : glucose, sucrose, glutamate and molasses at 2.0% and n-hexadecane, weathered oil and palm oil at 0.1% concentration were studied.

1.2.2 Concentration of carbon source

Cultivation was performed in Mckeen medium containing a suitable carbon source (from section 1.2.1) with varying concentrations at 1.0, 2.0, 2.5, 3.0, 3.5 and 4.0% for glucose, sucrose, glutamate and molasses or with varying concentration at 0.1, 0.3 and 0.5% for n-hexadecane, weathered oil and palm oil.

1.3 Effect of nitrogen sources

1.3.1 Types of nitrogen source

Cultivation was performed in the culture medium containing a suitable carbon source, suitable concentration of carbon source (from section 1.2.2) and different kinds of inorganic nitrogen sources such as $(NH_4)_2SO_4$, KNO_3, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)H_2PO_4$, $NaNO_3$ (0.5%) compared with organic nitrogen sources such as DL-glutamic acid, L-glutamic acid and monsodium glutamate (0.5%).

1.3.2 Concentration of nitrogen source

Cultivation was performed in the culture medium containing a suitable carbon source, concentration of carbon source and nitrogen source (from section 1.3.1) with varying concentration at 0.1, 0.3, 0.5, 1.0 and 1.5%.

1.4 Effect of yeast extract and bacto peptone

Cultivation was performed in the culture medium containing a suitable nutrient (from section 1.3.2) with varying concentration of yeast extract and bacto peptone at 0.1, 0.3 and 0.5%.

1.5 Time course of biosurfactant production under optimal medium in shake-flask cultivation

Starter culture (2.5%) was transferred into the optimized medium from section 1.4 (50 ml) in a 250-ml flask and cultivated on a rotary shaker (200 rpm) at 30^{o}C for 3 days. Triplicate samples were taken at 0, 6, 12, 18, 24, 36, 48, 60 and 72 h to measure growth (OD_{660}), pH, %EC, %EA and ODA.

2. Effect of environmental condition on growth and biosurfactant production

Starter culture (2.5%) was transferred into 2.0-l fermentor containing 1.5-l of optimal medium with agitation rate of 0.5 vvm, agitation speed of 200 rpm and the temperature was controlled at 30 ^{o}C. Samples were taken triplication at 0, 6, 12, 18, 24, 36, 48, 60 and 72 h and all parameters were analyzed as mentioned in the shake-flask cultivation. The effects of the following conditions were investigated.

2.1 Effect of pH

Cultivation under uncontrolled and controlled pH (pH 7.0) was compared. The agitation speed of fermentor was 200 rpm with aeration rate 0.5 vvm.

2.2 Effect of aeration

The aeration (compressed air) was varied from 0, 0.1, 0.5, 1.0 and 1.5 vvm at agitation speed of 200 rpm.

2.3 Time course of biosurfactant production under optimal condition

Bacillus MUV4 was cultivated under optimal cultivation condition (from section 2.2). Triplicate samples were taken at 0, 6, 12, 18, 24, 36, 48, 60 and 72 h. The parameters were determined and compared to those from shake-flask cultivation.

3. Properties of the partially purified biosurfactant

Preparation of partially purified biosurfactant

Bacillus MUV4 was cultivated at optimal condition (from section 2.3). A crude biosurfactant was obtained by centrifuging (10,000×g, 10 min, 4 ^{o}C) to remove the cells. The supernatant was subjected to acid precipitation by adding 6 N HCl to a final pH of 2.0 and allowing the precipitate to form at 4^{o}C overnight. The pellet was collected by centrifugation (10,000×g, 30 min, 4 ^{o}C) and washed three times with acidic water (pH 2.0), dissolved in distilled water and adjusted pH to 7.0 with 2.0 N NaOH and solution was lyophilized (Yakimov *et al*., 1995).

3.1 Solubility of the acid precipitated biosurfactant

Ten mg of the acid precipitated biosurfactant was tested its solubility in several solvents (10.0 ml) such as distilled water, alkaline water, methanol, ethanol, acetone, ethyl acetate, acetonitrile, chloroform and hexane. The biosurfactant-solvent mixture was allowed to stand for 24 h and the solubility was observed. The biosurfactant-solvent mixture was centrifuged and then the solution was evaporated to remove the solvent. The crude samples was dissolved in 0.02 M Tris-HCl buffer pH 7.3 and tested the oil displacement activity. (Kim *et al*., 1997).

3.2 pH stability of biosurfactant

The culture broth and the partially purified biosurfactant was adjusted with 1 M HCl or 1 M NaOH to various pHs ranging from 2 to 14 and stored for 12 h at 4 ^{o}C before measuring the %EC, %EA and ODA (Roongsawang *et al*., 1999).

3.3 NaCl concentration stability of biosurfactant

NaCl was added to the culture broth and the partially purified biosurfactant solution to final concentration of 0-35% and stood for 20 min at 25 ^{o}C before measuring the %EC, %EA and ODA (Roongsawang *et al*., 1999).

3.4 Temperature stability of biosurfactant

The culture broth and acid precipitated biosurfactant solution were incubated at different temperatures : 4, 30, 55, 80 and 100 ^{o}C for 48 h and then cooled to room temperature. The %EC, %EA and ODA were measured (Kim *et al*., 1997).

4. Extraction and partial purification of biosurfactant

4.1 Extraction of biosurfactant

After cultivation, a crude biosurfactant was obtained by centrifugation (10,000×g, 10 min, 4 ^{o}C) to remove the cell. The supernatant was adjusted to pH 2.0 with 6 N HCl. The acidified liquid was kept at 4 ^{o}C overnight. The precipitate was collected by centrifugation (10,000×g, 30 min, 4 ^{o}C) and dissolved in distilled water. The pH of the solution was adjusted to 7.0 with 2 N NaOH, and the solution was lyophilized. The lyophilized material was designated the acid precipitate (Yakimov *et al*., 1995)

The acid precipitated surfactant was extracted with equal volume of chloroform and ethyl acetate for 3 times. Solvents were removed by a rotary evaporation under reduced pressure. The crude material was collected for further purification by the chromatographic procedures describes below.

4.2 Adsorption chromatography

Sample preparation

Sample (freeze-dried biosurfactant) was dissolved in chloroform, mixed with a small amount of silica gel and dried with rotary evaporation.

Silica gel column

Silica gel was dried in hot air oven (100 ^{o}C) overnight and cooled to until 45 ^{o}C. The silica gel was flooded with hexane and mixed well until no air bubble was observed. The column (1.5 × 35 cm) was packed with silica gel in hexane and do not let column dry. The sample was applied on the surface of the column and

eluted with hexane first after that eluted with solvent of gradually increasing polarity : hexane > acetone > chloroform > chloroform : methanol (2:1 v/v) > methanol. Each fractions were evaporated with rotary evaporator at 40^{o}C. The oil displacement of each fractions were measured after dried and redissolved in 0.02 M Tris-HCl buffer pH 7.3. The fractions that demonstrated the oil displacement test were further separated by thin-layer chromatography.

4.3 Analysis the components of the partially purified biosurfactant by thin-layer chromatography (TLC)

The crude biosurfactant from section 4.2 was separated by TLC using aluminium sheets silica gel 60 F_{254} plates with various solvent systems.

$CHCl_3$: CH_3OH: CH_3COOH	80: 15: 5
$CHCl_3$: CH_3OH: CH_3COOH: H_2O	50: 5: 4: 1
	40: 10: 2: 1
	25: 15: 4: 2
$CHCl_3$: CH_3OH: H_2O	65: 25: 4
	40: 25: 2
$CHCl_3$: CH_3OH: CH_3COCH_3	90: 10: 6
$CHCl_3$: CH_3OH	95: 5
$CHCl_3$: C_6H_{14}: CH_3COOH	45: 25: 1
$CHCl_3$: C_6H_{14}: CH_3COOH: H_2O	60: 30: 1: 1
CH_3OH: H_2O	90: 10

The components were observed under UV light (wavelength of 280 nm). The best separated solvent system was selected for separation of biosurfactant by TLC. The spots from TLC plate were visualized by staining with ninhydrin, rhodamine 6G, alkaline potassium permanganate, anisaldehyde and iodine vapor. The plates stained with ninhydrin were placed at 100 ^{o}C for 4-5 min and then viewed directly for presence of amino acids. The plates sprayed with rhodamine 6G were dried and observed under ultraviolet light for the presence of lipids.

The plates sprayed with alkaline potassium permanganate were viewed directly for the presence of organic compounds, and plates sprayed with anisaldehyde were observed directly for sugar (Appendex A) (Dawson *et al*., 1986). Each spots from TLC plate were scrapped off and then extracted with chloroform. The extracted compounds were evaporated to dryness, then dissolved in 0.02 M Tris-HCl pH 7.3 and tested for emulsification activity and antimicrobial activity.

5. Application of biosurfactant

5.1 Applied for oil recovery

The application of the biosurfactant in oil recovery was evaluated using the sandpack technique described by Makkar and Cameotra (1997a).

5.2 Applied for antimicrobial activity

Antibiotic activity of biosurfactant was tested against different microorganisms with the agar diffusion method (modified from Barry and Thornsberry, 1980).