CHAPTER III

METHODOLOGY

3.1 Laboratory equipments, chemicals

3.1.1 Laboratory equipments (screening and identification)

Equipment	Company	Country
Autoclave, MLS-3020	Sanyo Electric	Japan
Autoclave HV, 110	Hirayama	Japan
Biological safety cabinet, Forma Class II A2	Thermo Electron Corporation	USA
CentriVap® Concentrator, models 79700-01	Labconco Corporation	USA
Electroparation	Bio-rad Laboratories	USA
Fast Plasmid Mini, 0032 007 653	Eppendorf	USA
Finemixer, SH 2000	Bio-active Co., Ltd.	Thailand
Gel Document	Syngene	USA
Gene Amp® PCR system 2700	Applied Biosystems	USA
Incubator shaker, Innova 4000	New Brunswick Scientific	USA
Incubator shaker, Innova 4340	New Brunswick scientific	USA
Manual-Tensiometer, Krüss 8 tensiometer	Hamburg	Germany
Micropitte 20,100, 200, 1000 µl	Gilson	France
Plasmid DNA purification Miniprep kit (catalog number 74058850)	Macherey-Nagel	Germany

Equipment	Company	Country
Protector Laboratory Hood	Science Technology	USA
Refrigerated centrifuge, 5804R	Eppendorf	USA
Separatory funnel, 250, 1000 ml	Witeg Preciso	Germany
Spectrophotometer DU 650	Beckman	USA
Ultrasonic	Banderlin	Germany
Universalindikator, pH 0-14	Merck	Germany
UV transilluminater	BioDoc-It™ System	USA
96-microwell plate	Harward	USA

3.1.2 Laboratory equipments (characterization)

Equipment	Company	Country
Finningan TSQ-70 mass spectrometer (Department of Chemistry, Faculty of Science, Chulalongkorn University)	Finningan MAT	USA
Fourier-transform mass spectrometry, Model Apex II (Scientific and Technological Research Equipment Centre Chulalongkorn University)	Bruker Instrument	Germany
Nuclear magnetic resonance, AVANCE DRX- 500 model (Department of Chemistry, Faculty of Science, Chulalongkorn University)	Bruker Instrument	Germany

3.1.3 Laboratory chemical

Chemical		Company	Country
Acetonitrile (99.9% purity)	CAS No. 75-05-8, M.W. 41.05	LAB-SCAN Co. Ltd.	Thailand
Acetone (99.8% purity)	CAS No. 67-64-1, M.W. 58.08	Carlo Erba	France
Acetic acid glacial (99.7% purity)	CAS No. 64-19-7	LAB-SCAN Co. Ltd.	Thailand
Agar Agar		Scharlau Microbiology	Spain
Ammonium chloride		M&B laboratory chemicals	England
Ammonium oxalate		M&B laboratory chemicals	England
Ammonium sulphate	CAS No. 7783-20-2, M.W. 132.14	Carlo Erba	France
Bovine serum albumin (BSA)		Sigma	USA
Bromothymol blue		BDH	England
Calcium chloride		Merck	Germany
Chloroform		Merck	Germany
Chloroform-D		Merck	Germany
Copper sulphate		Carlo Erba	France
Crystal violet		BDH	England
Dichloromethane (99.5% purity)	CAS No. 75-09-2, M.W. 84.93	Carlo Erba	France

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Chemical		**Company**	**Country**
Diethyl ether (99.5% purity)	CAS No. 200-467-2, M.W. 74.12	BDH	England
Diesel oil, Power 1, 4T		Castrol	Thailand
Di-potassium hydrogen phosphate		Riedel	Germany
Di-sodium hydrogen phosphate		Fluka	Switzerland
Di-sodium thiosulfate		Carlo Erba	France
Ethelenediaminetetraacetic acid (EDTA)		Fluka	Switzerland
Ethylacetate (99.8% purity)	CAS No. 200-467-2, M.W. 74.12	LAB-SCAN Co. Ltd.	Thailand
Ethanol (99.8% purity)	CAS No. 64-17-5, M.W. 46.07	Carlo Erba	France
Ferric ammonium citrate		Sigma-Riedel	Germany
Ferrous sulphate		BDH	England
Folin-Ciocalteu's reagent (Code no. 463562)		Carlo Erba	France
Glucose monohydrate		Asia Pacific Specialty chemicals Limited	Australia
Glycerol		Univar	Australia
Hexane (95.0% purity)	CAS No.110-54-3, M.W. 86.18	Fisher Scientific	United Kingdom
Hydrochloric acid (99.5% purity)	CAS No.7647-01-0, M.W. 36.46	J.T. Baker	USA

(Continued)

Chemical		**Company**	**Country**
Iodine crystal		BDH	England
Lactose		Sigma	USA
Magnesium chloride		Merck	Germany
Magnesium sulphate		Carlo Erba	France
Mannose monohydrate		Fluka	Switzerland
Methanol (99.9% purity)	CAS No. 67-56-1, M.W. 32.04	Carlo Erba	France
Ninhydrin (Product No. 44060)		BDH	England
Nutrient Broth		Difco	USA
α-napthol	CAS No. 20470	Sigma	USA
Orcinol		Fluka	Switzerland
pGEM-T Easy vector		Promega	USA
Potassium dihydrogen phosphate		Carlo Erba	France
Potassium iodide		Merck	Germany
Potassium sodium tartrate		Carlo Erba	France
Primers: forward primer (63f) and reverse primer (1387r)		Pacific Science CO., LTD.	Thailand
Rhamnose (≥99.0% purity)	CAS No. 2227943, M.W. 182.18	Fluka	Switzerland
Safranin O		Fluka	Switzerland
Silica gel plates, F_{254} 1055540001		Merck	Germany
Sodium carbonate		BDH	England
Sodium chloride		BDH	England
Sodium disulphate		Carlo Erba	France

(Continued)

Chemical (continued)		Company	Country
Sodium hydroxide		Merck	Germany
Sodium nitrate		Carlo Erba	France
Sodium laulyl sulphate (SDS)		Sigma	USA
Sucrose		Fluka	Germany
Sulfuric acid (96.5% purity)	CAS No. 7664-93-9, M.W. 98.08	J.T. Baker	USA
1X Taq buffer with $(NH_4)_2SO_4$		Fermentas	USA
Taq polymerase (500 Unit)		Fermentas	USA
Tryptone		Himedia	India
Urea		Asia Pacific Specialty chemicals Limited	Australia
Yeast extract		Scharlau Microbiology	Spain

3.2 Culture medium and solution for determine of biosurfactant concentration

3.2.1 The mineral salt medium (MSM)

The mineral salt medium used for screening, isolation, cultivation and production as described (Sheppard and Cooper, 1991).

Media

Glucose	20.00 g
Na_2HPO_4	4.00 g
KH_2PO_4	1.50 g
NH_4Cl	1.00 g
$MgSO_4.7H_2O$	0.20 g
$CaCl_2.2H_2O$	0.01 g
$FeSO_4.7H_2O$	0.005 g

The component was dissolved in 1 liter of distilled water and adjusted pH to 6.8-6.9 by 1 M NaOH. The mineral salt medium was autoclaved at 110°C for 10 minutes.

3.2.2 Luria bertani medium (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

LB medium was dissolved in 1 liter of distilled water, adjusted pH to 7 and autoclaved at 121°C for 15 minutes.

3.2.3 Modified Hektoen Enteric agar

Tryptone	12.00 g
Lactose	12.00 g
Sucrose	12.00 g
Yeast extract	3.00 g
Ferric ammonium citrate	1.50 g
Na_2SO_3	5.00 g
NaCl	5.00 g
Bromthymol blue	0.065 g
Agar	14.00 g

HE medium was dissolved in 1 liter of distilled water, adjusted pH to 7.5 and autoclaved at 121°C for 15 minutes.

3.2.4 Orcinol solution (0.19% orcinol in 53%H_2SO_4)

Orcinol	0.19 g
H_2SO_4	53 ml

H_2SO_4 was dissolved in 100 ml of distilled water. Orcinol were dissolved in 100 ml of 53% vv^{-1} H_2SO_4 in distilled water. The orcinol solution can be kept in a dark area at 4°C for a period of approximately 7 days (Schnaar and Needham, 1994).

3.3 Screening, isolation and identification of biosurfactant-producing bacteria

3.3.1 Screening and isolation of biosurfactant-producing bacteria

Bacteria were isolated from nineteen contaminated soils in Thailand with the history of hydrocarbon exposure or natural area. Soil samples collected from Burirum, Chanthaburi, Rachaburi, Rayong, Nakorn Patom and Tak provinces. All of soils collected from contaminated soils at the depth of 2-10 cm. Subsequently, ten grams (10 g) of contaminated soil transferred to 50 ml Erlenmeyer flasks containing 20 ml of distilled water. Flasks incubated in a rotary shaker at 30°C and 37°C at 250 rpm. After 24 hours, isolation used to select the bacteria. First, 0.1 ml of the culture grew on Modified Hektoen Enteric agar (As described in 3.2.3) plates and incubated at 30°C and 37°C for 24 hours.

Then, bacteria isolates were grown in mineral salt medium supplemented with 2% wv^{-1} glucose as a carbon source (As described in 3.2.1). Submerged microbial cultures incubated at 37°C on a rotary shaker at 250 rpm, 37°C for 3 days. Growth of isolates bacteria were monitored *via* the spectrophotometer as culture turbidity (OD_{600} nm). Cell growth in the presence of 2% wv^{-1} glucose were also monitored to distinguish bacteria of which mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source could served as a growth substrate (control). Finally, a drop-collapse method and emulsification index (E_{24}) have been refined for use to screen for biosurfactant-producing bacteria (As described in 3.3.2.2). Biosurfactant-producing bacteria maintained in LB agar (As described in 3.2.2) slants and subcultured every two weeks.

3.3.2 Preliminary screening of biosurfactant-producing bacteria

3.3.2.1 Preparation of cell-free supernatant for screening of bacteria

Then, microorganisms grew aerobically in 100 ml mineral salt medium supplemented with 2% wv^{-1} glucose as the carbon source in 250 ml Erlenmeyer flasks at 250 rpm at 37°C for 3 days. For isolation of biosurfactants, the collected culture supernatants were centrifuged at 9000×g for 15 min to remove cells.

3.3.2.2 Techniques of biosurfactant from biosurfactant-producing bacteria

a) Drop collapse method (Bodour and Miller-Maier, 1998)

For the drop collapse method, 200 µl of costal oil added to each well of a 96-well microtiter plate lid. The lid was equilibrated for 1 hour at room temperature, and then 100 µl of the culture added to the surface of oil (Bodour and Miller-Maier, 1998). The shape of the drop on the surface of oil inspected after 1 minute. Biosurfactant-producing cultures gave flat drops with scoring system '√' corresponding to complete spreading on the oil surface. Those cultures that gave rounded drops were scored as negative '_' indicative of the lack of biosurfactant production.

b) Emulsification index (E_{24}) (Cooper and Paddock, 1984)

A mixture of 1 ml supernatant and 1 ml diesel was mixed for 2 minuets using a vortex set on high speed with Finemixer, SH 2000 (Bio-active Co.Ltd., Thailand) at room temperature subsequently the height of emulsion layer was measured after 24 hours to determine the emulsion index (Cooper and Goldenberg, 1987). The E_{24} was calculated as the ratio of the height of the emulsified layer (cm.)

which gave white color and to the height of the total oil phase (cm.) gave to be nearer yellow color (As shown in the Figure 4.3). By repeating the reading after 24 hours, an indication of the stability of the emulsions was obtained. E_{24} = 0 indicates no emulsification and E_{24} = l, 100% emulsification. A good emulsifier-producing strain was designated as one having the $E_{24} > 0.5$ (equals 50% emulsification of the diesel oil layer) 2 hours after shaking (Bosch *et al.*, 1988). An emulsion was defined as stable if the E_{24} after 24 hours was 50% or better of the emulsion at 2 hours. The E_{24} was calculated using the following equation. The experiments were carried out in triplicate which distilled water and distinguished bacteria on mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source were used the control. (The calculation is shown in Appendix A).

$$\text{Emulsification index (\% } E_{24}) = \frac{\text{Height of emulsion layer}}{\text{Height of the oil plus emulsion layer}} \times 100$$

3.3.3 Identification of biosurfactant-producing bacteria

3.3.3.1 The morphology and characteristics of colonies

(1) Morphology of bacteria

Bacterial staining was used to study shape, size of bacteria and to classify bacteria into gram positive or gram negative. Bacteria were grown in LB agar medium (As shown in 3.2.2) for 24 hours, stained bacteria and visualized through a microscopy.

(2) Characteristics of colonies of bacteria

To determine the growth and of bacteria in mineral salt medium containing 11.1 mM (2% wv^{-1}) glucose as a carbon source, cells were grown for three days ,and then the physiological features were observed, for example, color, form, diameter, surface and edge.

3.3.3.2 Biochemical test

Biochemical characterizations of bacteria were analyzed by the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand.

3.3.3.3 16S ribosomal DNA gene sequencing

Genomic DNA from individual bacteria strains was extracted by a standard method. The 16S rDNA gene fragment was amplified from the genomic DNA of each bacteria by polymerase chain reaction (PCR) using the bacterium-specific primers: forward primer (63f) (5′ CAGGCCTAACACATGCAAGTC 3′) and a reverse primer 1387r (5′ GGGCGGWGTGTACAAGGC 3′) (Julian *et al.*, 1998). A reaction mixture (total volume of 25 µl) contained 1X Taq buffer with $(NH_4)_2SO_4$, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.4 µM of each primer, DNA sample 1 µl (500 ng DNA), Taq polymerase 5 units.μl^{-1} (Fermentas, USA). The reaction mixture was subjected to pre-denaturation at 94°C for 3 min, 30 cycles consisting of denaturation at 95°C for 1 min, 55°C and 1 min for annealing, chain extension at 72°C for 1.3 min with an additional extension time of 72°C for 5 min. The fragments (1,300 bps) was then cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) (3,015 base pairs) and transformed into competent *Escherichia coli* DH5α cells. Plasmid DNA

(DNA 650 ng.μl^{-1}) from the transformant was isolated using the Plasmid DNA purification Miniprep kit (catalog number 74058850) (Macherey-Nagel, Germany). The insert was then sequenced by the Macrogen Inc. (Seoul, Korea) and compared to the most similar sequences with Clustal W in NCBI database (http://www.ncbi.nlm.nih.gov).

3.3.4 Studies of biosurfactant-producing bacteria for activity

3.3.4.1 Growth condition

2% vv^{-1} inoculum was used to start the 100 ml bacterial culture grown in mineral salt medium in the presence of 2% wv^{-1} glucose as carbon source. Cell growth was determined by optical density (OD_{600}) and by the increase of cell protein concentration using modified Lowry method as described in 3.3.4.2. Cells were grown at 37°C for 3 days. Culture medium (100 ml) was collected to determine the biosurfactant production using orcinol method as described in 3.4.3.2.

3.3.4.2 Determination of protein

Protein estimation was done by the modified Lowry method with bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951; Dulley and Grieve, 1975). After 3 days of growth, cells-free supernatant were harvested by centrifugation at 9,000 rpm 15 minutes at 4°C. The supernatant fluid was used for partial purification of biosurfactant. The protein concentration was estimated by the method of modified Lowry method which to determine of protein must to use reagents such as:

Reagents

A solution: 2% Na_2CO_3 in 0.1 M NaOH containing 0.5% Sodium dodecyl sulfate (SDS)

B solution: 0.5% $CuSO_4.5H_2O$ in 1% Potassium Sodium tartrate

C solution: Phenol reagent

After preparation of enzyme solution (0.4 ml), 2 ml of mixed solution A and B (A: B, 50: 1) were added. Then, the mixture was incubated for 10 minutes at 30°C. Subsequently, 0.2 ml of C solution was added, rapidly mixed and then, incubated for 30 minutes at 30°C. Finally, to determine the quantity of protein, the clear color solution was detected by measuring the optical density at 750 nm.

3.4 Characterization of biosurfactant for biosurfactant-producing bacteria

3.4.1 Partial purification of biosurfactant production (Wei *et al.*, 2005)

Biosurfactant-producing bacteria grown aerobically in 100 ml mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source (As described in 3.2.1) in 250 ml Erlenmeyer flasks at 250 rpm at 37°C for 72 hours. After method in 3.3.2.1 which prepared of cell-free supernatant for screening of bacteria and kept cell-free supernatant was precipitated by acidification at pH 2.0 with 1M HCl which to use the universalindikator, pH 0-14 (Merck, Germany) the determination. Biosurfactants were grouped as glycolipid (Chen and Zhu, 2005), lipopeptide (Lee *et al.*, 2006), phospholipid (Yaguëe *et al.*, 1997) and fatty acids and neutral lipids (Kim *et al.*, 1997). After centrifugation at 9000×g for 20 minutes, the precipitate was discarded and removed to keep the supernatant following to extract in 250 ml separated funnel with ethyl acetate also the ratio 1:1 then gentle mix and to stand

30 minutes. The biosurfactants such as glycolipid, phospholipid and fatty acids and neutral lipids were extracted with ethyl acetate to separate organic and solvent phase at room temperature for 3 hours. The organic phase was then transferred to a round-bottom flask connected to a rotary evaporator at 50°C for 1 hour (Buchi, rotovapor R-200, Germany), allowing to remove solvent for the yield biosurfactant products after collected to determine by silica gel plates (F_{254} 1055540001). In the part, phospholipid and fatty acids and neutral lipids were determined ninhydrin for amino acids, amines, phosphates and amino-sugars (Makula and Finnerty, 1974). Another biosurfactant type, glycolipids were examined by analytical TLC employing silica gel plates (F_{254} 1055540001) and spray α-napthol solution. For lipopeptide, biosurfactant was extracted three times with pure methanol (100%) for 3 hours. The crude biosurfactant was obtained as a brown-colored material using a rotary evaporator, and then purified by silica gel plates (F_{254} 1055540001). Finally, the methods for purification of lipopolysaccharides (e.g. emulsan) were described earlier (Gorvenko *et al.*, 1997). Cell cultures were harvested by centrifugation (30 min and 10,000 rpm) and the polymer was precipitated by the addition of ammonium sulfate to approximately 40% wv^{-1} saturation while the solution was maintained at 4°C. The precipitated product was isolated by centrifugation, desalted by dialysis (Spectrum, MW cut-off 6,000–8,000 Da), and lyophilized. Residual aliphatic impurities were removed by Separatory funnel extraction with ether. Associated proteins were removed from the polymer by hot phenol extraction (Zuckerberg *et al.*, 1979).

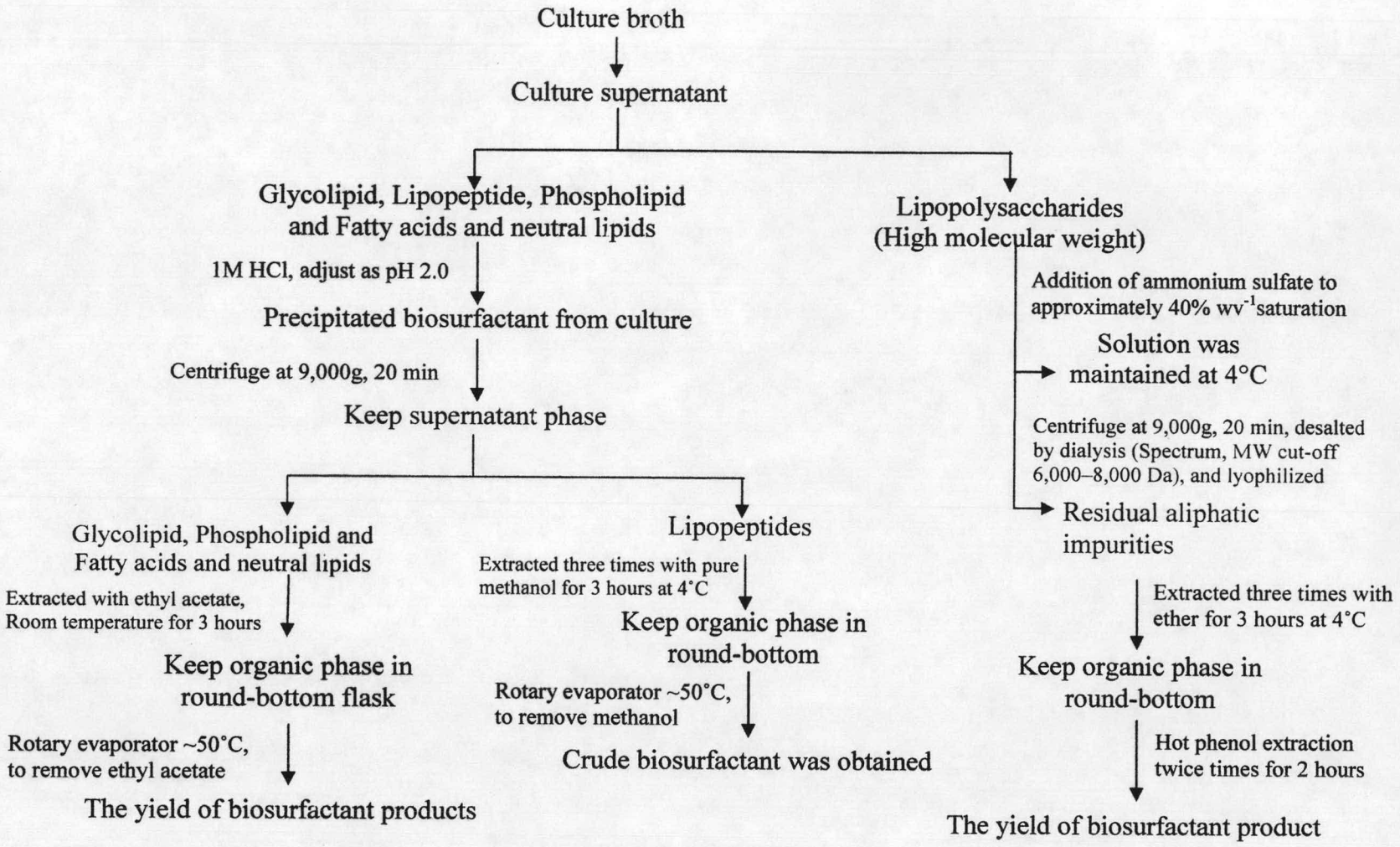

Figure 3.1 The experimental flow chart of partially purification of biosurfactant.

3.4.2 Measurement of the surface tension of partial purified biosurfactant

Surface tension was measured using a Du Nouy ring tensiometer (McInerney *et al.*, 1990). First, twenty milliliters of partial purified biosurfactant was put into a glass beaker (50 ml) and placed onto the tensiometer platform. A platinum plate was slowly touched the liquid air interface, to measure the surface tension ($mN.m^{-1}$). Between each measurement, the platinum plate was rinsed three times with water, three times with acetone, and was then allowed to dry. Pure water was used as standard. Two milliliters of the culture were equilibrated for 15 minutes in a small weighing dish prior to measuring the surface tension. The surface tension value shown was the average of three readings from the same culture.

3.4.3 Determination of biosurfactant concentration

3.4.3.1 Preparation of sugar part on biosurfactant for orcinol assay

For analysis of the sugar composition, glycolipids (1 ml) were hydrolyzed with 1 M HCl in water for 10 hours at 100°C under Protector Laboratory Hood (Science Technology). The reaction mixture was washed 2 times with 3 ml hexane to remove fatty acids. Then, aqueous solution was kept warm (room temperature) and the sugar part was detected with thin-layer chromatography (As described in method 3.5.1) using a commercial silica gel plates F_{254} 1055540001.

3.4.3.2 Orcinol method

Glycolipids were quantified in triplicate by weight and by the colorimetric determination of sugars with orcinol method (Chandrasekaran and Berniller, 1980). A modified orcinol method was used to assess the amount of glycolipids in the sample. The culture supernatant (333 µl) was extracted twice with

1 ml of diethyl ether which was mixed for 10 minuets using a vortex in microtube at 9,000 rpm for 15 minutes with Finemixer, SH 2000 (Bio-active Co.Ltd., Thailand) at room temperature. The ether fractions (upper phase) were pooled and evaporated at 35°C until dried, and 0.5 ml of H_2O was added in order to determine concentration of biosurfactant.

Determinations of concentration of partial purified biosurfactants were done. To 100 µl of each sample, 900 µl of a solution containing 0.19% orcinol (In 53% vv^{-1} H_2SO_4) was added (As described in method 3.2.4). After heating at 80°C for 30 minutes, the samples were cooled at room temperature for 15 min and the A_{421} was measured by UV spectrophotometer (The calculation shown in Appendix C).

The concentration of glycolipids was calculated by comparing the data with those of glucose standards between 0 and 50 $\mu g.ml^{-1}$ (Appendix C). Standards, blanks, samples as glucose were analyzed in triplicate, and the linear correlation was demonstrated between the quantity of glycolipid and optical density (The calculation shown in Appendix C).

3.4.4 Physiochemical properties and activity of biosurfactant

3.4.4.1 Critical micelle concentration (CMC)

The surface tension of each sample was determined to evaluate CMC of partial purified biosurfactants were performed at 31±1°C with Manual-Tensiometer, Krüss 8 tensiometer (Hamburg, Germany). From more hydrophilic glycolipids like yielded CMC values as high as 200 $mg.l^{-1}$ (Syldatk and Wagner, 1987), whereas lower values of 5–60 $mg.l^{-1}$ have been reported for mixtures containing mainly $RhC_{10}C_{10}$ monorhamnolipid (Van Dyke *et al.*, 1993; Sreekala and Shreve, 1994). The dirhamnolipid $Rh_2C_{10}C_{10}$ showed intermediate CMC values of

40–65 $mg.l^{-1}$. Thus, samples were prepared in flasks which using the range of concentrations of partial purified biosurfactants (0-100 $mg.l^{-1}$) by mineral salt medium as control. Consequently, partial purified biosurfactants was prepared for examined the CMC using 250 ml of solution on beaker (A manual of Tensiometer, model Krüss 8 tensiometer). Surface tensions were plotted against the logarithm of the biosurfactant concentration. The biosurfactant concentration at which the surface tension no longer decreases significantly is the CMC (Kim *et al.*, 2001). Measurements were done in triplicate.

3.4.4.2 Stability of biosurfactant at various temperatures and pH

The biosurfactant was also maintained at a constant temperature range of 30–75°C and used for the emulsification assay (As described in 3.3.2.2). To determine the effected of pH on activity, the pH of the biosurfactant was adjusted (2.0–12.0) and thereafter used in emulsification assay.

3.4.5 Effect of biosurfactant-producing bacteria in Luria Bertani broth (LB) and nutrient broth (NB)

All the strains were grown at 37°C aerobically in LB (As described in 3.2.2) or NB (Difco, USA) for the activities analyses which compared biosurfactant production in mineral salt medium (MSM) using 2% wv^{-1} glucose as the carbon source (As described in 3.2.1). Samples were initially grown in 250 ml Erlenmeyer flasks, each containing 100 ml LB, NB and MSM. The flasks were incubated at 37°C in the shaker incubator at 250 rpm. Samples were withdrawn every 24 hours to analyze the emulsification index (E_{24}) and therefore to select the best time or medium which gave for biosurfactant production.

3.5 Identification of the biosurfactant types

3.5.1 Thin-layer chromatography (Longas and Meyer, 1981)

Following in the 3.4.3.1 was analyzed with thin-layer chromatography, which separated of sugar part on biosurfactant. TLC was carried out on silica gel 60 F_{254} 1055540001 (Merck, Germany). Samples (From method in 3.4.1) were developed with ethyl acetate/acetic acid/water (6:3:2). After air-drying, the chromatogram was sprayed with 0.2% wv^{-1} ninhydrin in acetone, followed by heating for detecting protein compounds (Mangold, 1965). Carbohydrate components were located by the 5% vv^{-1} H_2SO_4 charring technique. Glucose, mannose and rhamnose were used as standards (R_f 0.38, 0.41 and 0.61 respectively).

3.5.2 Nuclear magnetic resonance (NMR)

Samples were prepared for nuclear magnetic resonance (NMR) as follows. Partial purified biosurfactant (about 1 g) dissolved in $CDCl_3$ at room temperature (Li *et al.*, 1984). Spectra were acquired on the following Bruker instruments (Bruker Biospin Corporation, Billerica, Mass.): an AVANCE DRX- 500 model instrument, operating at a proton frequency of 400 MHz with a 5 mm triple-resonance triple-axis gradient probe (Nalorac Corporation, Martinez, Calif.). Partial purified biosurfactant were analyzed by Department of Chemistry, Faculty of Science, Chulalongkorn University in Thailand.

3.5.3 Fourier-transformed infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture (Rodrigues *et al.*, 2006). This

technique measured the absorption of various infrared light wavelengths by the material of interest. The 1 mg of freeze-dried partial purified biosurfactant were extracted from the supernatant fluid at pH 2.0 (2 ml) with ethyl acetate (2 ml), dried by evaporation on a rotary evaporator. The IR spectra were recorded on the Perkin Elmer (Spectrum One) Fourier-transformed infrared spectroscopy, in the 4,000-400 cm^{-1} spectral region at a resolution 2 cm^{-1}, 100 scans for each spectrum, using a 0.23 mm potassium bromide (KBr) liquid cell. (Scientific and Technological Research Equipment Centre Chulalongkorn University)

3.5.4 Mass spectrometry

MS used to characterize the glycolipid biosurfactants. Partial purified biosurfactant was extracted with an equal volume of 2:1 chloroform/methanol solvent mixture and mixing thoroughly. The organic layer was separated using a separating funnel, air-dried and dissolved in 1 ml methanol for 1 hour. The MS characterization and detection of the glycolipid fractions under investigation were carried out using quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) utilizing electrospray ionization. Standard solutions and samples under investigation were infused into the mass spectrometer at a flow rate of 10 $\mu l.min^{-1}$. In the electrospray ionization source nitrogen sheath and auxiliary gas, flows were maintained at 50 and 5, respectively, and refer to arbitrary values set by the software. The heated capillary temperature was 250°C, and the spray voltage was set to 5 kV. Partial purified biosurfactant were analyzed by Department of Chemistry, Faculty of Science and Chulalongkorn University in Thailand. The results showed in Figure 4.24-4.25.

3.6 Effect of carbon or/and nitrogen source of biosurfactant production

According to previous method (3.4), 11.1 mM glucose for carbon source, additional carbon or/and nitrogen source were supplemented as follow:

1) Carbon source: glucose, maltose and sucrose which varies concentration followed 11.1, 44.4, 85.5 mM, respectively

2) Nitrogen source: $NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O which varies concentration followed 15, 75, 150 mM, respectively

3) Carbon and nitrogen source: 11.1 mM glucose and 75 mM $NaNO_3$

After additional carbon or/and nitrogen source was supplemented, bacterial culture placed on the rotary shaker at 250 rpm, at 37°C. Culture medium was collected on in the range 3 days. From this experiment, suitable conditions contained high percentage of total production or growth of bacteria were selected and then various concentration of additional carbon or/and nitrogen source were used. Furthermore, the conditions contained high percentage of total biosurfactant production or growth of bacteria for the best of either carbon or nitrogen source, which gave the highest production, were mixed. Besides this report was studied to affect of supplemented carbon source on biosurfactant production which used various types oil (2% vv^{-1}) such as olive oil, sunflower oil, soybean oil and diesel oil. Biosurfactant-producing bacteria grew in mineral salt medium (2% wv^{-1} glucose) with oil as supplemented carbon source and distinguish bacteria no oil which mineral salt medium could serve as a growth substrate (control).

3.7 Determination of the effect of NaCl, temperature and pH on the production of the biosurfactant

For growth studies and biosurfactant production at different NaCl concentrations and pH, the NaCl concentration and pH of the medium were adjusted accordingly. Growth studied in mineral salt medium using 2% wv^{-1} glucose as the carbon source. Experiments were done in triplicate and the results reported were averages of three independent experiments. The organism grew and produced biosurfactant at different NaCl concentrations (0.1, 0.5, 1.0 and 2.0% wv^{-1} or 17.1, 85.5, 171.0 and 342.0 mM) and pH values (2.0–12.0).