CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Microorganisms

Bacterial strain *Pseudomonas aeruginosa* strain SP4 was isolated from petroleum contaminated soil in Thailand. The isolated strain was maintained on nutrient agar slants at 37 °C and was subcultured every 2 weeks.

3.1.2 Media Used and Chemical Reagents

- Agar powder bacteriological, Himedia, India
- Nutrient broth (NB), Difco, France.
- Palm oil, Morakot Industry, Co., Ltd.
- Sodium bicarbonate, Labscan Asia Co., Ltd.
- Chloroform (A.R.grade), Labscan Asia Co., Ltd.
- Ethanol (A.R.grade), JT Baker, Malaysia.
- Antrone reagent (A.R. grade), Fluka, India.
- Sulfuric acid (A.R.grade), JT Baker, Thailand.

3.1.3 Instrument and Apparatus

- Autoclave KT-40D, Alp Co., Ltd., Japan
- Centrifugator, Tomy MX-301.
- Microprocessor, pH meter 211, Hanna Instruments.
- Shaking Incubator, VS-8480SRN, SRN-L, Vision Scientific Co., Ltd, Korea.
- Shimadzu UV-VIS spectrometer 2550, Barawindsor Co., Ltd.
- Vacuum evaporator, Heidolph WB2001.
- Hot plate, Heidolph MRHei-standard
- Foam fractionation apparatus

- Video camera (Sony, SSC-DC58AP/1) connected to the optical lens (OPTEM)
- HPLC-ELSD (Alltech 580 autosampler, an Alltech HPLC pump, model 626, and an Inertsil® ODS-3 column)
- Mass spectrometry

3.2 Methodology

3.2.1 Inoculums Preparation

Three series of 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth (Difco) was prepared at a fixed concentration of 8 g/l, as recommended by the supplier. *Pseudomonas aeruginosa* SP4 was activated in a nutrient agar medium, incubated in a convention incubator at 37°C. One loop of these culture was inoculated in each Erlenmeyer flask. These inoculums were incubated for 22 h, 37°C, and 200 rpm in a shaking incubator.

3.2.2 Preparation of Biosurfactant Solution

To produce biosurfactant solution, Then a nutrient broth containing 2% inoculums and 2% palm oil was incubated at 37°C under aerobic condition in a shaking incubator at 200 rpm for 48 h to obtain the highest microbial and surfactant concentration. After that, the solution was centrifuged at 4°C and 8500 rpm for 20 min in order to remove the bacterial cells. The obtained supernatant will be further studied.

3.2.3 Foam Fractionation Apparatus

The single stage foam fractionation apparatus used in this study is shown in Figure 3.1 The column was built by using glass, inner and outer diameters of the column are 3 cm and 3.5 cm, respectively. The sinter glass disk was added at the bottom of the column in order to generate bubbles to the top of the column.

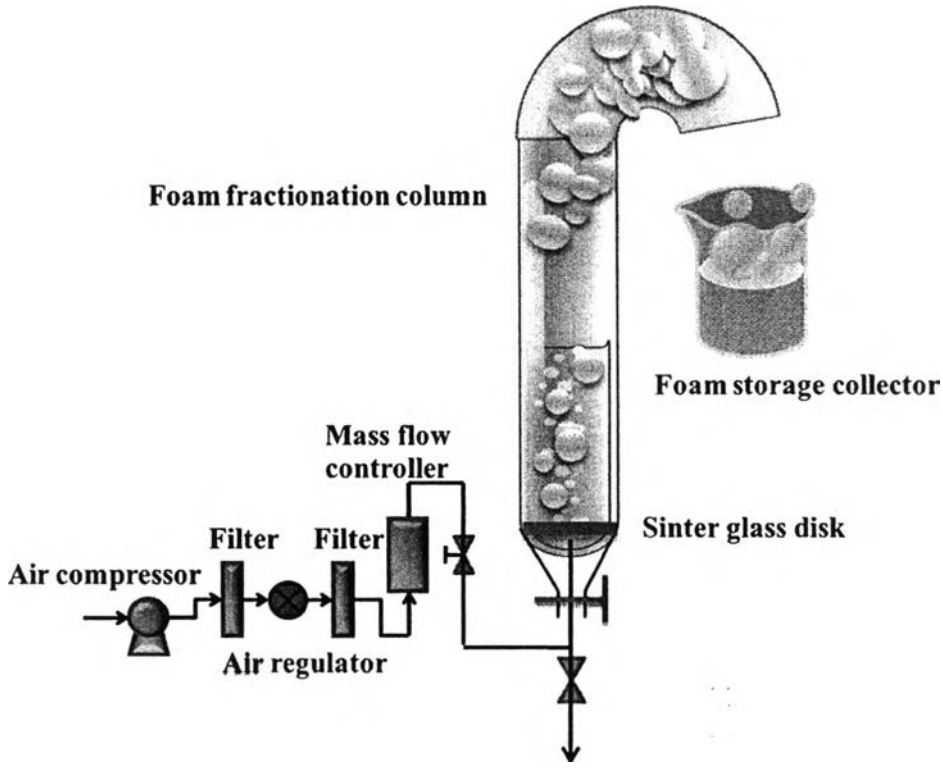

Figure 3.1 Foam fractionation apparatus

3.2.4 Experimental Methods

The surfactant solution was feed to the top of column. The air pressure from a compressor was maintained to have the flow rate of 30-110 ml/min by a mass flow controller. The compressed air was introduced to the bottom of column through sintered-glass diffuser. Pore size of Sintered-glasses No. 0, 1 and 3 were 160-250, 100-160 and 16-40 microns, respectively. The foam at the top of the column was collected at different heights of the column by beaker. Then, it was frozen, thawed and then weighted to get the collapsed foamate volumn for study foam wetness. Moreover, the solution volume was studied at several volunm of solution from 25-125 ml and the foam collecting time was investigated from 1.5-4 hour.

3.2.4.1 Effect of Operating Parameters

The effect of operating parameters on foam fractionation technique was studied by varying the air flow rate, column height, pore size of sinter glass disk, the solution volume and repeated foam fractionation cycle to find the optimum condition for separating biosurfactant from *Pseudomonas aeruginosa* SP4.

Effects of several parameters on the separation efficiency of the single stage foam fractionator operated in a batch mode were studied and evaluated using the %biosurfactant removal and the enrichment ratio as shown below:

$$\text{Biosurfactant removal (\%)} = \frac{(C_i V_i - C_l V_l)}{C_i V_i} * 100 \qquad (1)$$

$$\text{Enrichment ratio} = \frac{C_f}{C_i} \qquad (2)$$

C_i: the concentration of an initial biosurfactant solution(μl/ml)

C_l: the concentration of remain biosurfactant concentration(μl/ml)

C_f: the concentration of biosurfactant in the collapse foam(μl/ml)

V_i: the initial volumn of biosurfactant solution(ml)

V_l: the remain volumn of biosurfactant solution(ml)

In each experiment, foam wetness (grams of foam solution/l of foam), and the biosurfactant concentration (μg/l) in the collapsed foam solution were measured. The concentration of biosurfactant were determined using colorimetric assay (Anthrone reaction) for rhamnose identification (Bailey, 1958)

3.2.4.2 *Measurement of Size of Air Bubbles in foam fractionation column*

The measurement of froth sizes of the air bubbles was performed at different position of column. Images of the air bubbles were taken by using a color video camera (Sony, SSC-DC58AP/1) connected to the optical lens (OPTEM). A light generation unit (Photonic, PL 3000) was used as a light source to obtain clear-cut images. One hundred images were taken at a time interval of 1 s. and then the average bubble diameter was calculated using Image-Pro Plus software (Version 5.1). Fig. 4.33 shows images of the air bubbles in the foam fractionation column.

3.2.4.3 Oil Removal

The residue palm oil in sample was quantified in the collapsed foam fractionation and the initial biosurfactant solution. The residue palm oil were determined by the partition-gravimetric method with dichloromethane as solvent (Feng and Aldrich, 2000). Samples were acidified with 1:1 HCl to pH 2 or lower. The acidified samples were transferred to centrifuge tubes, and centrifuged for 10 min at 12,000 rpm to break oil-in-water emulsions. The centrifuged samples were transferred to a separatory funnel. The centrifuge tubes of sample were rinse with 30 ml of extracting solvent and solvent washings then were added to a separatory funnel. A reparatory funnel, which contained centrifuged samples and solvent washings, was shaken for 2 min. The lower layer (solvent layer) was subsequently drained through a funnel containing a filter paper (Whatman No.40) and 10 g Na_2SO_4, both of which have been solvent-rinsed, into a clean distilling flask. Extraction should be done 3-5 times to certainly extract all the extent of oil in the samples. Finally, solvent was evaporated at 40 °C. Results are expressed as mg oil.

The oil removal can be calculated by the following equation:

$$\text{Oil removal (\%)} = \frac{(C_i V_i - C_f V_f)}{C_i V_i} * 100 \qquad (5)$$

where C_i: the concentration of oil in an initial solution(mg/ml)

C_f: the concentration of oil in the collapsed foam(mg/ml)

V_f: the volume of the collapsed foam(ml)

V_i: the initial volume of the feed solution(ml)

3.2.4.3 Anthrone Reaction

Anthrone reagent: anthrone (20 mg) was dissolved in 70% H_2SO_4. All anthrone solutions were discarded after 24 h and kept at 0°C. Briefly procedure: Sample 1 ml were pipetted into large boiling tubes (20 cm x 25 mm). Each tube was placed in a bath of cold water and agitated whilst anthrone reagent (10 ml) was slowly added. The tubes were stoppered and, after their contents were thoroughly mixed, transferred to a vigorously boiling water bath for the specified time. They were then placed in a cold-water bath and stored in the dark for 30 min.

The optical densities of the coloured solutions were read on a UV visible spectrophotometer (Shimadzu UV-VIS spectrometer 2550, Barawindsor Co., Ltd.) with cells of 1 cm. thickness. The bluish-green solutions produced by rhamnose an absorption maximum at 631 nm, therefore, this wavelength was used.

3.3 Characteristics of crude biosurfactant

3.3.1 High Performance Liquid Chromatography with Evaporative Light Scattering Detector

The components of the crude biosurfactant were fractionated by using HPLC-ELSD technique, and the structure of biosurfactant will be characterized by MS analyses.

The components of the crude biosurfactant were fractionated using a high performance liquid chromatography (HPLC) (an Alltech 580 autosampler, an Alltech HPLC pump, model 626, and an Inertsil® ODS-3 column) equipped with an evaporative light scattering detector (ELSD) (Alltech, 2000ES). The mobile phase solutions were an aqueous solution of 10% acetonitrile (A) and pure acetonitrile (B). Both eluents contained 0.1% trifluoroacetic acid. The gradient system was used, starting with B from 30% to 70% in 5 min and then from 70% to 90% in 15 min. After that, the gradient of B was raised again to 100% at the end of the process. The flow rate of the mobile phase was set constant at 0.5 ml/min and the sample injection volume was 50 ll. The ELSD drift tube temperature was maintained at 100 °C while the nebulizer flow rate was kept constant at 1.5 l/min (Noordman et al., 2000). The gradual change in the affinity of the mobile phase resulted in the fractionation of the components in the crude biosurfactant. All fractions eluted from the HPLC column at different retention times were collected. Each fraction was then evaporated to remove all of the eluents to obtain a high-purity biosurfactant-containing material.

3.3.2 Mass spectrometry

The isolated fractions eluted from the HPLC column were directly subjected to analysis with mass spectrometry. The experiments were performed with a Waters mass spectrometer using electrospray in the positive mode and a scanning mass range of 300–900 Da.