CHAPTER III
EXPERIMENTAL

3.1 Materials and Equipment

3.1.1 Equipment and Apparatus

- Autoclave KT-40D.
- Shaking Incubator, VS-8480SRN, SRN-L, Vision Scientific CO., Ltd, Korea.
- Centrifugator, Hermle Z 383K, Diethelm Co., Ltd.
- Data Physics, Germany.Laminar flow hoods, Pennyful Thailand, Co., Ltd.
- Microprocessor, pH meter 211, Hanna Instruments.
- Vacuum evaporator, Heidolph WB2001.
- COD reactor, HACH 45600, Enviscience Co., Ltd
- COD spectrophotometer, HACH DR/2000, Enviscience Co., Ltd
- TOC analyzer, Sjimadzu 500A
- Filter paper, 0.45 μm, 0.2 μm, and Whatman No.40
- Distilling adapter with drip tip
- Du Nouy ring tensiometer
- Glass tube cylinder (borosilicate)
- Diaphragm pump
- Peristaltic pump
- Aeration pump
- Water bath
- Temperature controller
- Level controller
- Solenoid valve
- Relay
- Timer

3.1.2 Chemicals

- Palm oil, Morakot Industry, Co., Ltd.
- Fish steaming waste from canned fish processing, Thairuamsin Co., Ltd.
- Nutrient broth (NB), Difco, USA.
- Agar powder bacteriological
- Sodium hydroxide, NaOH
- Hydrochloric acid, HCl
- Dichloromethane, CH_2Cl_2
- Sodium sulfate, Na_2SO_4, anhydrous crystal
- Potassium dihydrogen phosphate, KH_2PO_4
- Dipotassium hydrogen phosphate, K_2HPO_4
- Potassium chloride, KCl
- Sodium nitrate, $NaNO_3$
- Magnesium sulfate heptahydrate, $MgSO_4.7H_2O$
- Iron (II) sulfate-7-hydrate, $FeSO_4.7H_2O$

3.2 Methodology

3.2.1 Microorganism

Pseudomonas aeuginisa SP 4 used throughout this work was kindly given by Mr. Sarawut Paisanjit (The Petroleum and Petrochemical College, Chulalongkorn University, Thailand). It was maintained on nutrient agar slants at 4 °C to stop the biological activity and subcultured at 1-month interval.

3.2.2 Inoculum

Three series of 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth (Difco) were prepared. One loop of culture from agar slant was inoculated in each Erlenmeyer flask (Figure 3.1). These inoculums were incubated for 22 h, 37 °C, and 200 rpm in a shaking incubator. Then, 150 ml of these inoculums was transferred into the reactor in start-up period.

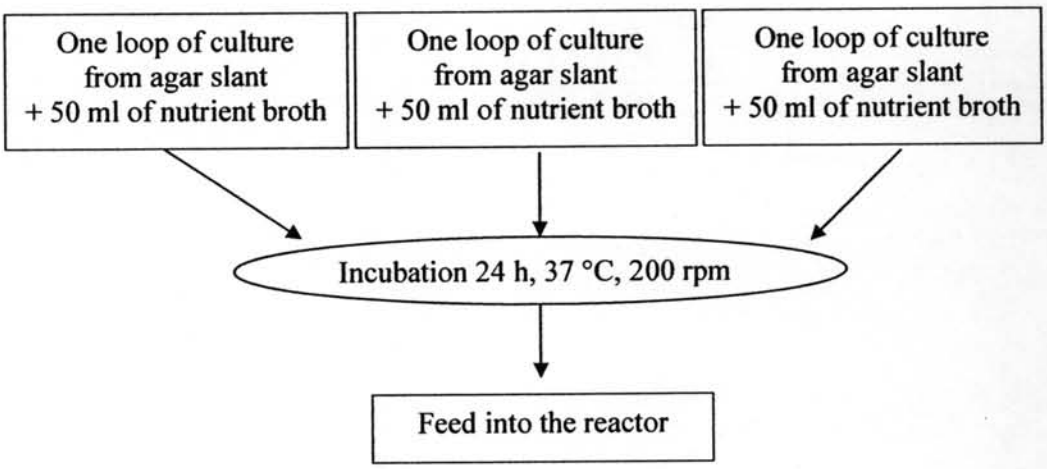

Figure 3.1 Incubation procedure flow diagram.

3.2.3 Nutrients and Carbon Sources

Fish steaming waste (FSW) or mineral medium was used as a nutrient source for the growth and proliferation of *Pseudomonas aeruginosa* SP 4 and palm oil was used as a carbon source. FSW obtained from the manufacturing of canned fish processing (Thairuamsin Co., Ltd.) was collected. Settleable solids and partial suspended solids were removed by passing fish steaming waste through the filter cloth. This pre-treated fish steaming waste was stored at 4 °C until needed for biosurfactant production.

The mineral medium (MM) used in the experiments was composed of $NaNO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$ (0.5 g), KCl (0.1 g), and $FeSO_4.7H_2O$ (0.01 g) in 1,000 ml distilled water (adapted from Dubey and Juwarkar, 2001). The C:N and C:P ratios in the MM feed were kept constant at 16:1 and 14:1, respectively, in which the optimum ratios were reported for maximum rhamnolipid production (Guerra-Santos *et al.*, 1984). Composition of MM with different oil loading rates is illustrated in Table 3.1.

Fish steaming waste, mineral medium and palm oil were autoclaved at 121 °C for 15 min and cooled to 30 °C before use.

Table 3.1 Composition of mineral medium with different oil loading rates

Oil loading rate (kg/m^3d)	$NaNO_3$ (g/l)	C:N ratio	KH_2PO_4 (mg/l)	K_2HPO_4 (mg/l)	C:P ratio
1	0.03	16:1	0.02	0.04	14:1
2	0.06	16:1	0.04	0.09	14:1
6	0.18	16:1	0.25	0.10	14:1
10	0.31	16:1	0.25	0.38	14:1

3.2.4 Sequencing Batch Reactors (SBRs)

Two identical sequencing batch reactors (SBRs) were maintained throughout the study. The SBRs consist of rounded bottom-shaped vessel with the total volume of 3000 ml and the working volume of 1500 ml, operating with a foam collecting system. The glass vessel (borosilicate) SBRs was fabricated with an internal diameter of 7 cm and a liquid height of 34 cm (26 cm of free-board). The SBRs were operated under aseptic conditions and temperature was controlled at 37 °C (±1 °C) by circulating hot water through the bioreactor jacket. There were six ports in the reactor lid. First and second ports were used to transfer carbon and nutrient source, respectively. The carbon and nutrient source addition were achieved using peristaltic pump and diaphragm pump, respectively. A third port was occupied by the air glass blower connected to an air pump. A fourth port was used for the level controller probe. The constant liquid level of 1500 ml in the reactor is controlled by the level controller. The solenoid valve was used to control the volume of the effluent flowing to the product tank. At a fifth port, a foam collector bottle was used to collect the overflowed foams and equipped with a filter paper (0.2 μm) to prevent microorganisms from the outside of reactor. A sixth port was used to measure temperature. Three electricity timers connected to the SBRs were used to automatically control feeding times, aeration or reaction time, and draining time, respectively (Figure 3.2 and 3.3).

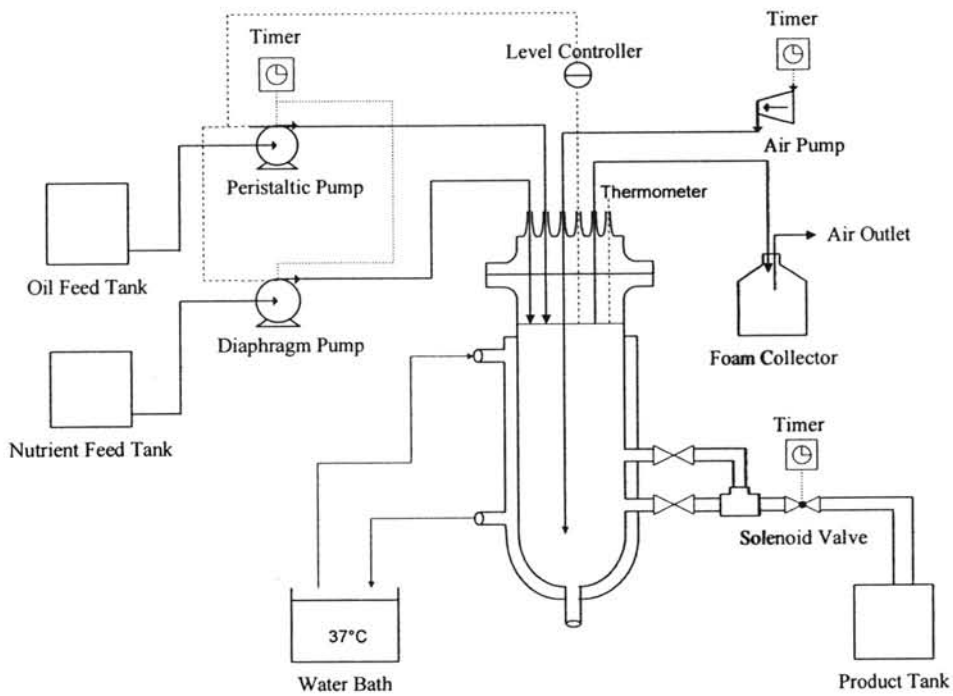

Figure 3.2 Schematic diagram of SBR.

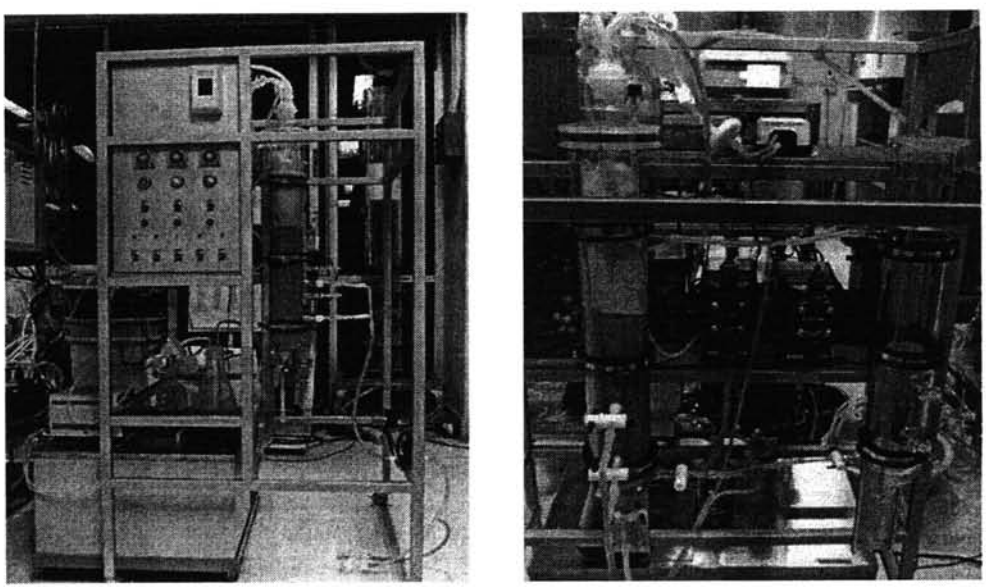

Figure 3.3 Sequencing batch reactors (SBRs).

3.2.5 Sequencing Batch Reactor (SBR) Operation

The SBR functioning cycle includes four temporal steps which are fill, react, settle, and draw step as shown in Figure 3.4.

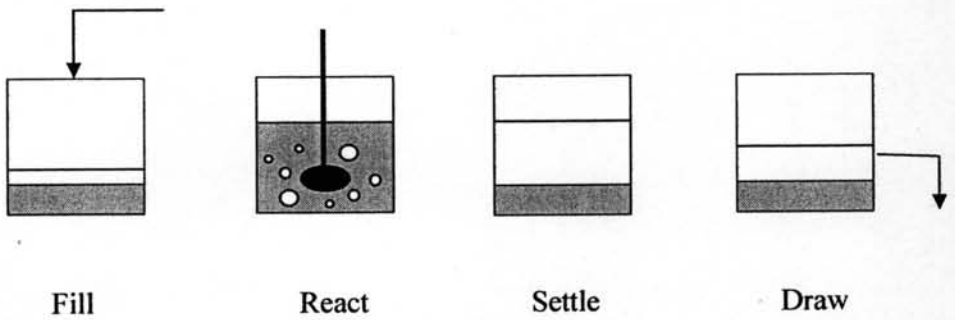

Figure 3.4 The operation cycle of the SBRs.

Table 3.2 Operating conditions of SBRs during sequence steps

Cycle time (days)	HRT (days)	Flow rate (ml/days)	Step	Time	Aeration
1	3	500.00	Fill	5 min	Off
			React	23 h	On
			Settle	50 min	Off
			Draw	5 min	Off
3	9	166.67	Fill	5 min	Off
			React	71 h	On
			Settle	50 min	Off
			Draw	5 min	Off

During the fill step, 500 ml of the influent (palm oil and nutrient source) was introduced to the reactors and the liquid level was brought from 1000 ml to 1500 ml. Aeration was provided throughout the reaction step, and was shut-off during the settle step to allow the sedimentation of bacterial cells and a clarified supernatant was observed on the top of liquid. In the draw step, 500 ml of the supernatant phase was decanted off and the liquid volume in the reactors was decreased to 1000 ml.

Pseudomonas aeruginosa SP 4 was grown aerobically and allowed to accumulate in the SBRs. In these experiments, fish steaming waste from canned fish processing or mineral medium was added as a nutrient source in the SBRs. For reactor start up, inoculum 10% (150 ml) of working volume (1500 ml) was fed into each reactor. Initial mixed liquor suspended solids (MLSS) concentration was 10 mg/l, which is initial cells. Two SBRs were fed with palm oil and nutrient until a working volume of 1500 ml was reached. Nutrient and palm oil were fed at the beginning of each cycle. In order to distribute the feed uniformly and enhance oxygen transfer, the reactors were aerated with 3 l min^{-1} by the air blower. The SBRs system was automatically controlled by using three electricity timers and done in cycle to find the best condition for the biosurfactant production. Three retention times are typically sufficient to reach steady state conditions in biological reactors (Cassidy *et al.*, 2002). Steady-state operation was achieved when the effluent values for COD remained constant during 9 days (3 retention times). The definition of the HRT is the time for which the influent resides in the bioreactor. HRT is related to the influent flow rate as follows:

$$\text{HRT (hydraulic retention time)} = \frac{\text{net volume of reactor}}{\text{influent flowrate}}$$

Operating conditions of SBRs during sequence steps are listed in Table 3.2.

3.2.6 Effect of Nutrient Source on Biosurfactant Production

At a given of oil loading rate (2 kg/m^3d), fish steaming waste or diluted FSW (1:60 times diluted) or mineral medium was used as a nutrient source and the SBRs had a 1-day cycle time and a 3-day hydraulic retention time (HRT). Suitable nutrient sources were screened on the basis of reduction of surface tension, and COD and oil removal in the effluent, as well as the effluent total suspended solids (TSS) and mixed liquor suspend solid (MLSS).

3.2.7 Effect of Oil Loading Rate on Biosurfactant Production

The optimized nutrient source with respect to biosurfactant production was used in order to achieve an effective optimum oil loading rate (OLR) for

biosurfactant production. The SBRs were operated at a 1-day cycle time and a 3-day hydraulic retention time (HRT). The OLR was varied as follow; 1, 2, 6, and 10 kg/m^3d. Oil loading rate was calculated by the following equation.

$$\text{Oil loading rate } (kg/m^3d) = \frac{\text{concentration of oil} \times \text{feed flow rate}}{\text{working volume of reactor}}$$

Concentrations of biosurfactant and palm oil were measured for each OLR, with surface tension, effluent COD, effluent TSS, and MLSS as described in the next section.

Table 3.3 SBRs operating conditions with four different oil loading rates during the SBR cycle

Oil loading rate (kg/m^3d)	Oil concentration (%w/v)	Oil concentration (%v/v)
1	0.30	0.35
2	0.60	0.70
6	1.80	2.11
10	3.00	3.52

3.2.8 Effect of Cycle Time on Biosurfactant Production

After optimizing the oil loading rate, the SBRs were operated at an oil concentration of 0.6 %w/v with a 1-d cycle or a 3-d cycle (Table 3.4). The optimum cycle time was selected base on reduction of surface tension of mineral medium, and COD and oil removal in the effluent, as well as the effluent total suspended solids (TSS) and mixed liquor suspend solid (MLSS).

Table 3.4 SBRs operating conditions with 1-d cycle and 3-d cycle during the SBR cycle

Parameters	1-d cycle	3-d cycle
Hydraulic retention time (days)	3	9
Oil loading rate (kg/m^3d)	2	0.67
Oil concentration (%w/v per cycle)	0.6	0.6

3.2.9 pH Measurement

The pH was measured in the influent and effluent by a pH meter.

3.2.10 Microorganism Quantification

The number of live cell was measured using spread plate method (APHA *et al.*, 1992). Samples were serially diluted with distilled water and prepared on duplicates for each dilution. 0.5 ml of the dilution was distributed onto surface of predried agar plate using a sterile bent glass rod, and then incubated at 20°C for 7 day. After incubation, the colony-forming units (CFUs) on each plate were counted and results are reported as CFU/ml.

3.2.11 Total Suspended Solids (TSS) Measurement

Total suspended solids (TSS) were measured in the settle step. Samples were filtered (0.2 μm) and washed with distilled water. The residues retained on the filter were dried to constant weight at 105 °C at least 24 h before weighing (APHA *et al.*, 1992). The increase in weight of the filter represents total suspended solids (TSS). TSS was measured on triplicates.

3.2.12 Mixed Liquor Suspended Solids (MLSS) Measurement

Mixed Liquor Suspended Solids (MLSS) were assayed in the reaction step (aeration period) during steady state operation, representing the microbial concentration in the reactor, and by filtering the samples through 0.2 μm

filter paper. The residues retained on the filter were dried at 105 °C at least 24 h to constant weight before weighting. Samples were analyzed in triplicates.

3.2.13 Biosurfactant Productivity Measurement

The supernatants obtained after removal of cells by centrifugation at 8000 rpm for 20 min at 4 °C. The supernatants were used for the determination of surface and minimum surface tensions, critical micelle concentration (CMC), and biosurfactant concentration.

3.2.13.1 Surface Tension Measurement

Surface tension was measured using a Du Nouy Ring Tensiometer calibrated with distilled water (72 dynes/cm). Surface tension was measured on duplicate 30 ml samples.

3.2.13.2 Biosurfactant Concentration Measurement

The biosurfactant concentration was measured on duplicate 30 ml samples using the critical micelle dilution (CMD) method. When the surfactant concentration is above the critical micelle concentration (CMC), the surface tension remains constant at some minimum value. The supernatant was diluted several fold (10- to 100-fold) with distilled water until the surface tension increased above the minimum surface tension, and the inverse of this dilution factor is the CMD.

3.2.13.3 Foam Layer Thickness Measurement

Foam layer thickness was measured visually.

3.2.13.4 Critical Micelle Concentration (CMC) and Minimum Surface Tension Measurement

The supernatant was serially diluted with distilled water. Surface tension was measured for each dilution. CMC and minimum surface tension were determined from the curve of surface tension versus concentration as described by Marikawa *et al.* (2000).

3.2.13.5 Emulsification Capacity (EC) Measurement

A practical measure of the utility of a biosurfactant solution is its ability to emulsify nonaqueous liquids. Emulsification capacity (EC) was measured on duplicate 15 ml filtrate (0.45 µm) samples by the addition of n-hexadecane in 0.5 ml increments. The mixture was vortexed 15 s and allowed to

stand for 2 min. This was repeated until the separate phase developed on top of the liquid. The maximum volume of n-hexadecane emulsified per initial volume of filtrate samples was defined as EC.

3.2.14 Chemical Oxygen Demand (COD) Measurement

To quantify total organic carbon (e.g., biosurfactant, metabolites, palm oil), chemical oxygen demand (COD) was measured in the influent and effluent by COD reactor (HACH, 45600) and a HACH DR/2000 spectrophotometer. Measurement was performed in duplicates.

3.2.15 Total Organic Carbon (TOC) Measurement

Total organic carbon (TOC) content was assayed in the influent with a TOC analyzer (Sjimadzu 500A). Measurement was performed in duplicates.

3.2.16 Total Nitrogen (TN) and Total Phosphorous (TP) Measurement

Total nitrogen (TN) and total phosphorous (TP) were measured in the influent by COD reactor (HACH, 45600) and a HACH DR/2000 spectrophotometer. Measurements were performed in triplicates.

3.2.17 Palm Oil Quantification

The palm oil was quantified in the whole samples. The whole samples estimate the palm oil present in all phases (i.e., aqueous phase, non-aqueous phase). The oil and grease were determined by the partition-gravimetric method with dichloromethane as solvent (APHA *et al.*, 1992). 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 separatory 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 and grease/l sample.