



CHAPTER III EXPERIMENTAL

3.1 Materials and Equipments

3.1.1 Equipments and Apparatus

- Autoclave KT-40D, Alp Co., Ltd., Japan
- Shaking Incubator, VS-8480SRN, SRN-L, Vision Scientific Co., Ltd., Korea
- Centrifugator, Hermle Z383K, Labnet International, Inc.,
- Data Physics, Germany laminar flow hoods, Pennyful Thailand, Co., Ltd., Germany
- Microprocessor pH meter 211, Hanna Instruments, USA
- Vacuum evaporator, Heidolph WB2001, Germany
- COD reactor, HACH 45600, Enviroscience Co., Ltd, USA
- COD spectrophotometer, HACH DR/2000, Enviroscience Co., Ltd, USA
- TOC analyzer, Shimadzu 500A, USA
- Filter papers, Whatman No.40 (110 mm. Dia), Whatman International Ltd., England
- Membrane filter, Pall (47 mm. Dia.), Pall Corporation, USA
- Membrane filter, Acro 50 Vent Devices with PTFE Membrane (7.3 cm. Dia), Pall Corporation, USA
- Contact angle measuring instrument, Kruss, DSA-10, Germany
- Diaphragm pump, Pulsatron Punta gorda, Fl., USA
- Peristaltic pump, EYELA microtube pump MP-3, Tokyo rikakikai Co., Ltd., Japan
- Water bath, Polyscience, USA
- Level controller, GEMS, Cole-Parmer instrument Co., Ltd., USA
- Timer, OMRON H3CR-F8-300 (12s – 300h), OMRON Corporation, Japan
- Vacuum pump, GAST, GAST manufacturing INC, USA

- Silicone tube, Masterflex 96400-16 and 96400-17, Cole-Parmer instrument Co., Ltd., USA
- Pinch clamp 1/4 to 7/16 inch, Nalge Nunc international, USA
- Aeration pump, aquarium air pump

3.1.2 Chemicals and Solvents

Carbon source used in this research is palm oil which is supplied from Morakot Industry, Co., Ltd., Thailand

Pseudomonas aeruginosa SP 4 obtained from previous work (Paisanjit, 2006) was used in present study and was maintained in nutrient agar (Agar powder, bacteriological, Himedia laboratories Pvt. Ltd., India). Nutrient broth (NB) supplied from Difco, USA was used to activate the culture.

Mineral medium (MM) was prepared from sodium nitrate (NaNO_3), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), potassium chloride (KCl), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and iron (II) sulfate-7-hydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) which were supplied from Ajax Finechem, Australia.

Mercury (II) sulphate, HgSO_4 (Ajax Finechem, Australia) and sulfuric acid, H_2SO_4 98% (Lab-Scan, Labsan Asia Co., Ltd) were used for preparing the reagent in COD test.

Total nitrogen was tested by using total nitrogen HR hydroxide reagent test N tube, 10-150 mg/L, TN (Total Nitrogen) reagent A and TN (Total Nitrogen) reagent B (HACH company world headquarters, Germany).

Total phosphorus was tested by using high rang total phosphate test N tube, 0-100 mg/L PO_4^{3-} (HACH company world headquarters, Germany).

Hydrochloric acid, HCl 37% (Lab-Scan, Labsan Asia Co., Ltd.) was used to acidify the sample for palm oil content testing.

Dichloromethane, CH_2Cl_2 (Lab-Scan, Labsan Asia Co., Ltd.) was used for extracting the palm oil remain in the sample.

Sodium sulfate, Na_2SO_4 , in the grade of anhydrous crystal was used to remove the remaining water in the step of palm oil content determination. (Ajax Finechem, Australia).

3.2 Methodology

3.2.1 Microorganism

The isolated strain of *Pseudomonas aeruginosa* SP 4 which is able to produce rhamnolipid-type surfactant was used throughout in this study. It was isolated from petroleum-contaminated soil (Paisanjit, 2006). It was maintained on nutrient agar slants at 4°C to stop the biological activity and subcultured every two weeks.

3.2.2 Inoculums preparation

Three series of 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth (Difco) were prepared. *Pseudomonas aeruginosa* SP 4 was activated in a nutrient agar slants medium and incubated at 37°C. One loop of the culture was then inoculated in each Erlenmeyer flask. These inoculums were incubated at 37°C, and 200 rpm in a shaking incubator for 22 h. Then, 150 ml of these inoculums were transferred into the reactor.

3.2.3 Carbon Sources and Nutrients

Palm oil was used as a carbon sources and the mineral medium (MM) used in the experiments was composed of NaNO_3 (2 g), KH_2PO_4 (0.5 g), K_2HPO_4 (1 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), KCl (0.1 g), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (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 the maximum rhamnolipid production (Guerra-Santos *et al.*, 1984). Mineral medium and palm oil were autoclaved at 121°C for 15 min and cooled to 30°C before being used.

3.2.4 Sequencing Batch Reactors (SBRs) set-up and operation

3.2.4.1 Experimental set-up

A schematic diagram of the experimental setup is in Figure 3.1. Figure 3.2 illustrates the photographs of the two SBR units used in this study. The SBR unit consisted of a rounded bottom-shaped vessel the total volume of 3,000 ml and a working volume of 1,500 ml. The vessel was made of glass with an internal dimension of 7 cm and having a water jacket. The SBRs were operated under aseptic conditions and temperature was controlled at 37°C (± 1 °C) by circulating warm water through the bioreactor jacket. There were six ports in the reactor lid as summarized in table 3.1.

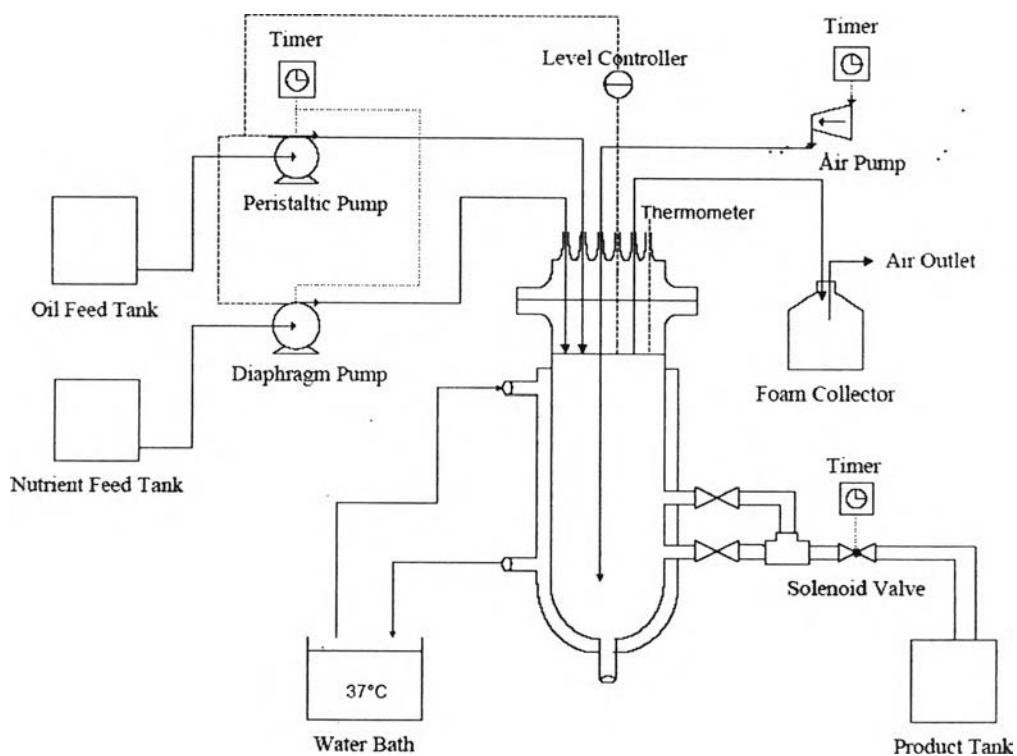


Figure 3.1 Schematic diagram of SBR.

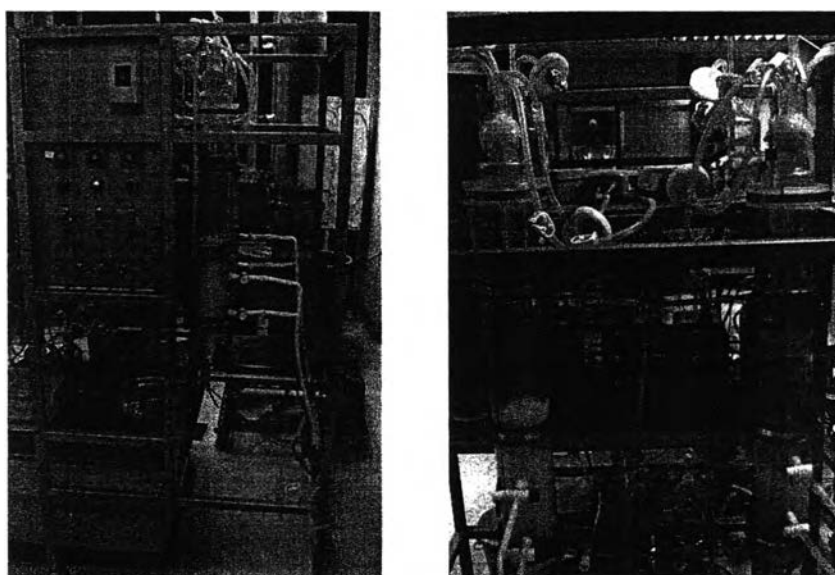


Figure 3.2 Sequencing batch reactors (SBRs).

Table 3.1 Six ports in the SBR lid

Port	Function/Their use
First port	Used to transfer carbon source by using peristaltic pump in order to addition into the reactor.
Second port	Used to transfer nutrient source by using diaphragm pump in order to addition into the reactor.
Third port	Occupied by the air glass blower connected to an air pump.
Fourth port	Used for the level controller probe. The constant liquid level of 1,500 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.
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.
Sixth port	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.

3.2.4.2 *SBRs operation*

The SBR has a cyclic character with following four steps per cycle which are fill, react, settle, and draw, as show in Figure 3.3. The longest and the most important cycle for experimental investigations is the react phase.

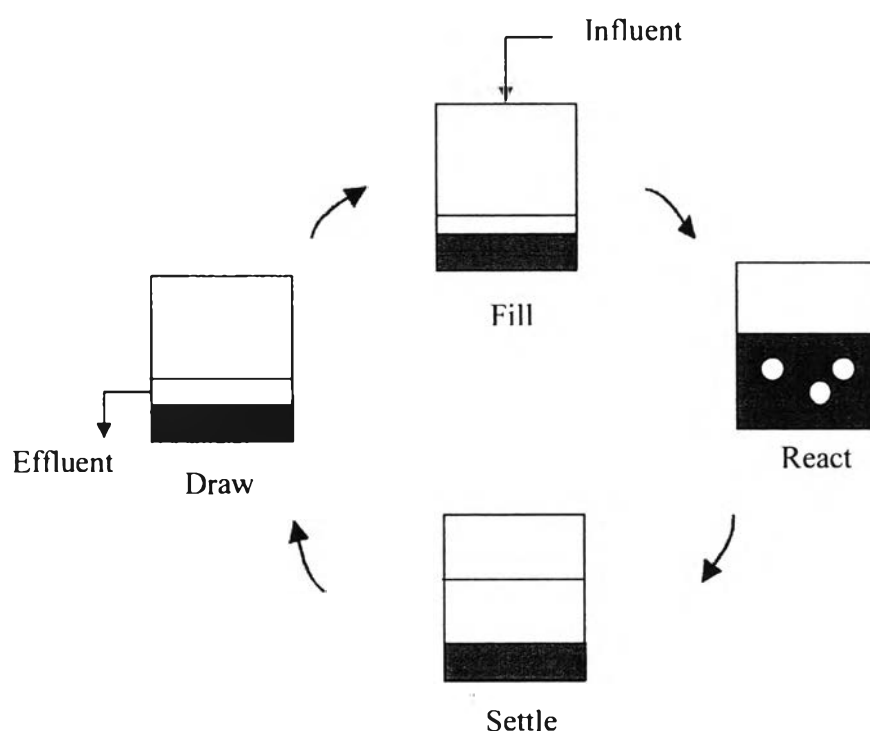


Figure 3.3 SBR operation for each tank for one cycle for the four discrete time periods of Fill, React, Settle, and Draw.

Each SBR was operated with four cycles per day. 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 1,000 ml to 1,500 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 reactor was decreased to 1,000 ml. The protocol for each cycle in the two reactors is summarized in Table 3.2.

Table 3.2 The operating conditions of SBRs

The operating conditions of SBRs	Condition 1	Condition 2	Condition 3
Cycle time (d/cycle)	1	2	3
- Fill (min)	5	5	5
- React (Aeration, hr)	23	47	71
- Settle (hr)	50	50	50
- Draw (min)	5	5	5
Hydraulic retention time (HRT, d) *	3	6	9
Working volume (ml)	1,500	1,500	1,500
Flow rates (ml/d)	500	250	166.667
Feeding (ml)	500	500	500
Decanting volume (ml/d)	500	250	166.667

*HRT is the time for which the influent resides in the bioreactor. HRT is related to the influent flow rate as follows: HRT (hydraulic retention time, days) = liquid volume in reactor / flow rate

For the start-up of reactor, 10% inoculums (150 ml) of working volume (1,500 ml) was fed into each reactor. *P. aeruginosa* SP4 was grown aerobically and allowed to accumulate in the SBRs. Each SBR was fed with palm oil and mineral medium at the beginning of each cycle until a working volume of 1,500 ml was reached. In order to distribute the feed uniformly and to 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. Steady-state operation was achieved when the effluent values for COD remained constant.

According to the previous work (Maksung, 2007), an optimum oil loading rate of $2 \text{ kg/m}^3\text{d}$ was used as a base condition to determine the effect of cycle time in this study. The SBRs were operated at an oil concentration of 0.6, 1.2 and 1.8 %w/v with a 1 d/cycle, a 2 d/cycle and 3 d/cycle, respectively (Table 3.3). The optimum cycle time was determined based on the reduction of surface tension of mineral medium, and the COD and oil removal in the effluent, as well as the effluent suspended solids (SS) and mixed liquor suspended solid (MLSS).

Table 3.3 SBRs operating conditions with the effect of cycle time

Parameters	1 d/cycle	2 d/cycle	3 d/cycle
Hydraulic retention time (days)	3	6	9
Oil loading rate ($\text{kg/m}^3\text{d}$)	2	2	2
Oil concentration (%w/v per cycle)	0.6	1.2	1.8

3.3 Chemical Analysis and Measurement Methods

3.3.1 pH measurement

The pH of the culture supernatant of influent and effluent obtained after removal of the cells by centrifugation (8,500 rpm for 20 min) was measured by a pH meter. (Microprocessor pH meter 211)

3.3.2 Suspended Solids (SS) Measurement

The suspended solids (SS) are used to indicate both the cell loss and the microbial concentration in this study. A volume of 5 ml of an effluent taken during the decanting step was filtered by using a magnetic filter funnel through $0.45 \mu\text{m}$ of filter paper and washed with distilled water. The residues retained on the filter were dried at $103\text{-}105^\circ\text{C}$ at the least 24 h before weighing (APHA *et al.*, 1992). Samples were analyzed in triplicates.

3.3.3 Mixed Liquor Suspended Solids (MLSS) Measurement

Mixed Liquor Suspended Solids (MLSS) was assayed in the reaction step (aeration period) during steady state operation to represent the microbial concentration in the reactor. The MLSS was followed the standard methods (APHA *et al.*, 1992) A quantity of 5 ml of a well-mixed sample was filtered through a 0.45 μm filter paper and the residue retained on the filter was dried at 103-105°C for at least 24 h before weighing. Samples were analyzed in triplicate.

3.3.4 Biosurfactant Productivity Measurement

An effluent sample was centrifuged at 8,500 rpm for 20 min at 4°C to remove the microbial cells. The supernatant was taken for the determination of surface tension, critical micelle concentration (CMC), and biosurfactant concentration.

3.3.4.1 Surface Tension Measurement The surface tension of the biosurfactant was measured by the pendant drop method using a contact angle measuring instrument (Kruss, DSA-10, Germany) at room temperature. The contact angle measuring instrument was calibrated with distilled water.

3.3.4.2 Biosurfactant Concentration Measurement The critical micelle dilution (CMD), a parameter used as an indirect measure of surfactant concentration, is determined by measuring the surface tension of serial dilutions with distilled water (Haba *et al.*, 2000). For the critical micelle dilution (CMD) method, the supernatant sample taken from the bioreactor was diluted several fold (10 to 100-folds) until the surface tension increased above the minimum surface tension, and the inverse of this dilution factor is the CMD.

3.3.5 Chemical Oxygen Demand (COD) Analysis

To quantify total organic carbon (e.g., biosurfactant, metabolites and remaining, palm oil), chemical oxygen demand (COD) was employed by using a COD reactor (HACH, 45600) and a HACH DR/2000 spectrophotometer. Each sample was analyzed for COD at least twice.

3.3.6 Total Organic Carbon (TOC) Analysis

Total organic carbon (TOC) content was assayed with a TOC analyzer (Shimadzu 500A). The measurement was performed in duplicates.

3.3.7 Total Nitrogen (TN) and Total Phosphorous (TP) Measurements

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

3.3.8 Palm Oil Quantification

A sample was analyzed for the total oil content by the partition-gravimetric method with dichloromethane as a 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 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 Na₂SO₄, both of which have been solvent-rinsed, into a clean distilling flask. Extraction should be done 3-5 times to completely extract all the oil in the samples. Finally, the solvent was evaporated at 40 °C to obtain the constant weight of the extracted oil. The results are expressed as milligram of oil and grease per liter of sample.