# CHAPTER III
# EXPERIMENTAL

## 3.1 Materials and Equipment

### 3.1.1 Equipment and Apparatus

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

20. Silicone tube, Masterflex 96400-16 and 96400-17, Cole-Parmer instrument Co., Ltd., USA
21. Pinch clamp1/4 to 7/16 inch, Nalge Nunc international, USA
22. Glass tube cylinder (borosilicate)
23. Aeration pump, aquarium air pump
24. Solenoid valve
25. Thermometer
26. Relay

3.1.2 Chemicals and Solvents

- Poly(methyl methacrylate), or PMMA, (VH and MD grades, 99.5% purity)
- Palm oil, Morakot Industry, Co., Ltd., Thailand
- D-glucose anhydrous ($C_6H_{12}O_6$), analytical reagent grade, AJAX Finechem, Australia.
- Nutrient broth (NB), Difco, USA.
- Agar powder, bacteriological, Himedia laboratories Pvt. Ltd., India
- Sodium sulfate, $Na_2SO_4$, anhydrous crystal, Ajax Finechem, Australia
- Sodium nitrate, $NaNO_3$, Carlo Erba Reagenti, USA
- Potassium dihydrogen phosphate, $KH_2PO_4$, Ajax Finechem, Australia
- Dipotassium hydrogen phosphate, $K_2HPO_4$, Ajax Finechem, Australia
- Potassium chloride, KCl, Ajax Finechem, Australia
- Magnesium sulfate heptahydrate, $MgSO_4.7H_2O$, Ajax Finechem, Australia
- Iron (II) sulfate-7-hydrate, $FeSO_4.7H_2O$, Ajax Finechem, Australia
- Mercury (II) sulphate, $HgSO_4$, Ajax Finechem, Australia
- Total nitrogen HR hydroxide reagent test'N tube, 10-150 mg/L, HACH company world headquarters, Germany

- TN (Total Nitrogen) reagent A, HACH company world headquarters, Germany
- TN (Total Nitrogen) reagent B, HACH company world headquarters, Germany
- 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.
- Dichloromethane, $CH_2Cl_2$, Lab-Scan, Labsan Asia Co., Ltd.
- Ethanol, $C_2H_6O$, Lab-Scan, Labsan Asia Co., Ltd.
- Sulfuric acid, $H_2SO_4$ 98%, Lab-Scan, Labsan Asia Co., Ltd.

## 3.2 Methodology

### 3.2.1 Microorganisms

The rhamnolipids were produced by a strain of *Pseudomonas aeruginosa* SP 4 used throughout in this study was isolated from petroleum-contaminated soil, provided by Mr. Sarawut Paisanjit of 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 every two weeks.

### 3.2.2 Inoculum 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, 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. Then, 150 ml of these inoculums were transferred into the reactor in start-up period.

### 3.2.3 Carbon Sources and Nutrients

Palm oil and glucose used as the carbon sources and the mineral medium (MM) used as the nutrient source. In the experiments MM was composed of $NaNO_3$, $K_2HPO_4$, and $KH_2PO_4$, which an amount of each chemical was varied for

each oil-to-glucose ratios to keep C/N and C/P constant, $MgSO_4.7H_2O$ (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 (Pornsunthorntawee *et al.*, 2009). Mineral medium, palm oil, and glucose were autoclaved at 121°C for 15 min and cooled to 30°C before 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. The SBRs consist of rounded bottom-shaped vessel with the total volume of 3,000 ml and the working volume of 1,500 ml, operating with a foam collecting system. The SBRs were fabricated with an internal dimension 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 shown in table 3.1.

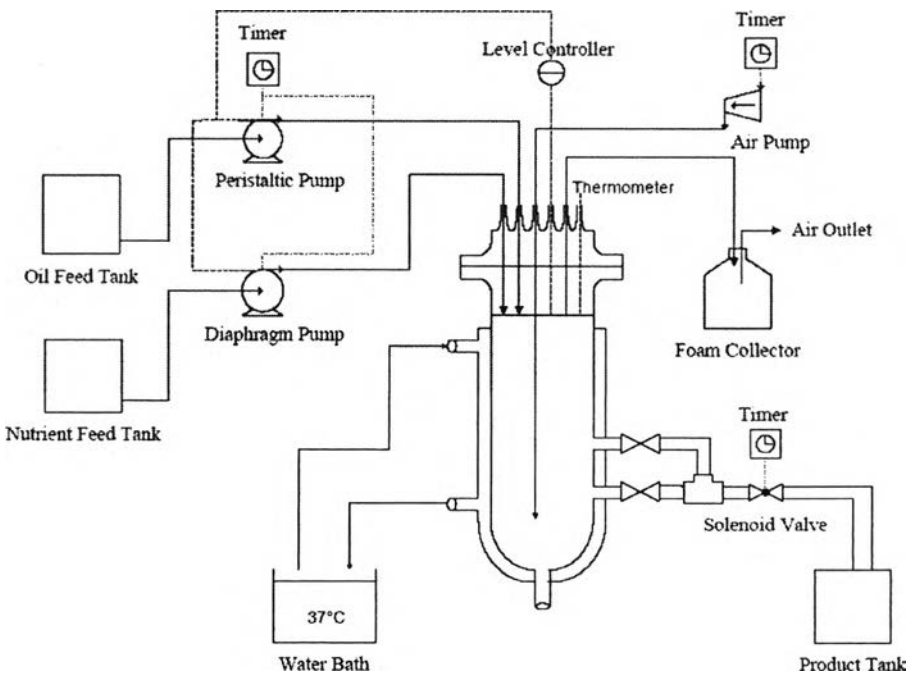


**Figure 3.1** Schematic diagram of SBR.

**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. |

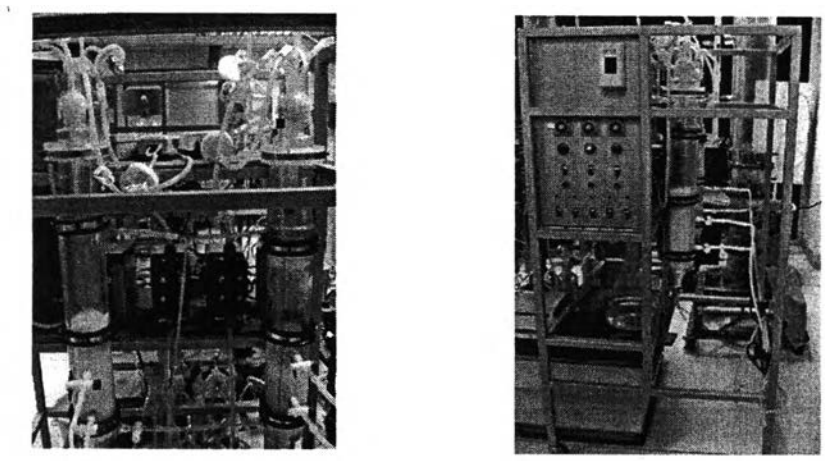

**Figure 3.2** Sequencing batch reactors (SBRs).

*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 shown in Figure 3.3. The longest and the most important cycle for experimental investigations is the react phase.

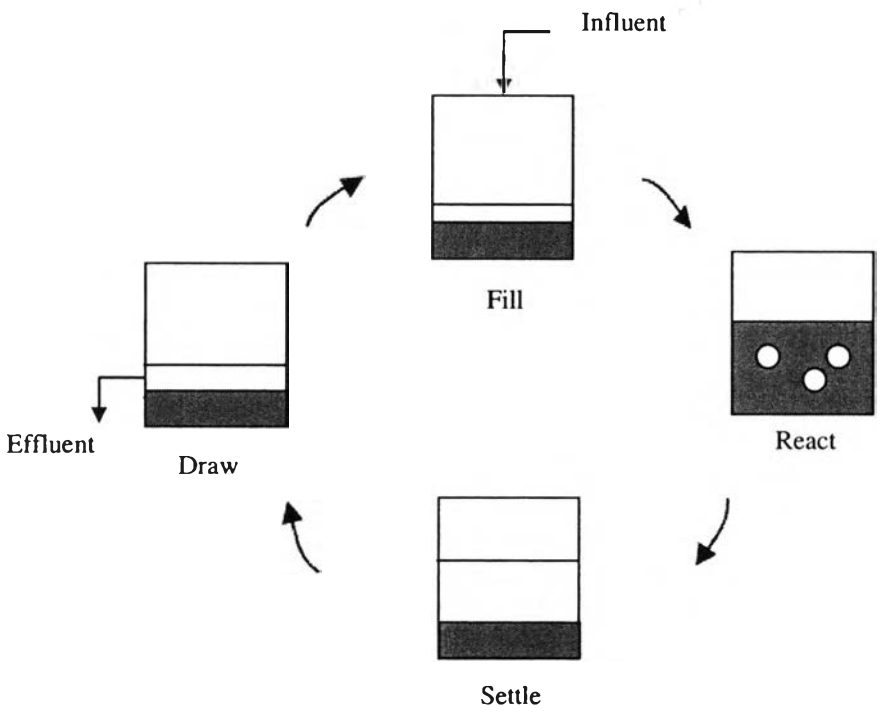


**Figure 3.3** SBR operation for each tank for one cycle for the four discrete time periods of Fill, React, Settle, and Draw.

Each SBR was operated with four cycles per day. During the fill step, 500 ml of the influent (palm oil, glucose, 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 reactors was decreased to 1,000 ml. The protocols for all oil-to-glucose ratios in the two reactors are as shown in Table 3.2.

**Table 3.2** The operating conditions of SBRs

| **The operating conditions of SBRs** | |
|---|---|
| Oil-to-glucose ratios | All ratios were performed with the same conditions |
| Cycle time (d/cycle) | 2 |
| - Fill (min) | 5 |
| - React (Aeration, hr) | 47 |
| - Settle (hr) | 50 |
| - Draw (min) | 5 |
| Hydraulic retention time (HRT, days) * | 6 |
| Working volume (ml) | 1,500 |
| Flow rates (ml/day) | 250 |
| Feeding (ml) | 500 |
| Decanting volume (ml/day) | 250 |

*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) = V/Q

where: V is the net volume of reactor (ml)

Q is the flow rate of the system (ml/day)

For reactor start up, 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 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.

3.2.5 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.

3.2.6 Suspended Solids (SS) Measurement

Suspended solids (SS) were measured in the settle step. Five ml of samples were filtered by magnetic filter funnel through 0.45 μm of filter paper and washed with distilled water. The residues retained on the filter were dried to constant weight at 103-105°C at least 24 h before weighing. This measurement was according to standard methods 2540-D (APHA *et al.*, 1992). The increase in weight of the filter represents the suspended solids (SS). SS was measured on triplicates.

3.2.7 Mixed Liquor Suspended Solids (MLSS) Measurement

Mixed Liquor Suspended Solids (MLSS) were assayed in the reaction step (aeration period) during steady state operation in order to represent the microbial concentration in the reactors. MLSS was followed procedures in standard methods 2540-D by filtered 5 ml of a well-mixed sample through 0.45 μm filter paper and the residue retained on the filter were dried to a constant weight at 103-105°C at least 24 h before weighting. Samples were analyzed in triplicates.

3.2.8 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.

*3.2.8.1 Surface Tension Measurement*

A 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 samples were measured the surface tension in mN/m. The instrument was calibrated with distilled water (72 mN/m) before the samples were measured,

*3.2.8.2 Biosurfactant Concentration Measurement*

Critical micelle concentration ($CMC^{-1}$), a parameter used as an indirect measure of surfactant concentration, was determined by measuring the surface tension of serial dilutions of crude extract in distilled water at pH 7 (Haba *et al.,* 2000). Critical micelle dilution (CMD) method, the supernatant were diluted several fold (10- to 100-fold) with distilled water until a surface tension increased above the minimum surface tension, and the inverse of this dilution factor is the CMD.

*3.2.8.3 Foam Layer Thickness Measurement*

Foam layer thickness was measured visually.

*3.2.8.4 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).

3.2.9 Chemical Oxygen Demand (COD) Measurement

To quantify total organic carbon (e.g., biosurfactant, glucose, metabolites, palm oil), chemical oxygen demand (COD) was measured in the influent and effluent by COD reactor (HACH, 45600) and a HACH DR/2700 spectrophotometer. Measurement was performed in duplicates.

3.2.10 Total Organic Carbon (TOC) Measurement

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

3.2.11 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/2700 spectrophotometer. Measurements were performed in triplicates.

3.2.12 Palm Oil and Glucose 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 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 room temperature for one day. Results are expressed as mg oil and grease/l sample.

The glucose concentration in the effluent was quantified by Glucose (HK) Assay Kit (GAHK-20, Sigma Aldrich) to determine the consumption of glucose by microorganisms. Briefly, samples were centrifuged at 8,500 rpm for 20 min. Then, the centrifuged sample was diluted with deionized water to 0.05-5 mg of glucose/ml (10x dilution for product) following by TECHNICAL BULLETIN from the supplier. The tubes were shaken and incubated for 15 min at room temperature, and then cuvette was filled with each sample for measuring absorbance at 340 nm versus deionized water by UV-VIS spectrometer (2550, Shimadzu). After that, measured absorbance was calculated to express as the glucose concentration.

$$\frac{\text{mg glucose}}{\text{ml}} = \frac{(\Delta A)(TV)(F)(0.029)}{(SV)} \qquad (3.1)$$

$A_{\text{Total Blank}} = A_{\text{Sample Blank}} + A_{\text{Reagent Blank}}$

$\Delta A = A_{\text{Test}} - A_{\text{Total Blank}}$

TV = Total assay volume (ml)

SV = Sample volume (ml)

F = Dilution factor from sample preparation