CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Sources of Screening Samples

Petroleum-contaminated soil, deep sea water, and waste from food court were used for the screening of biosurfactant-producing bacteria in this study. For petroleum-contaminated soil samples were taken from areas just below the soil surface and stored at 4°C (Jennings *et al.*, 2000) until use (within 48 hours).

3.1.2 Media Used and Chemical Reagents

Agar powder bacteriological, Himedia, India

Ammonium dihydrogen orthphosphate, Univar, Australia.

Ammonium chloride, Univar, Australia.

Ammonium nitrate, Univar, Australia.

Chloroform (A.R.grade), Labscan Asia Co., Ltd.

Crude oil, PTTEP Co., Ltd.

Diesel motor oil complex, SAE 20W-50, Super Max Powerplus, Shell (Thailand) Co., Ltd.

Ethanol (A.R.grade), Labscan Asia Co., Ltd.

n-Hexane 99%, Labscan Asia Co., Ltd.

Iodine (fragment), Univar, Australia.

Iron(II) sulphate, Univar, Australia.

Magnesium sulphate, Univar, Australia.

Methanol (A.R.grade), Labscan Asia Co., Ltd.

Nutrient broth (NB), Difco, USA.

Ottawa sand, 20-30 mesh, Fisher Scientific, UK Limited

Palm oil, Morakot Industry, Co., Ltd.

Patassium chloride, Univar, Australia.

Sodium bicarbonate, Univar, Australia.

Thin Layer Chromatography (silica gel 60, 20×20 cm), Merck, Darmstadt, Germany.

3.1.3 Instrument and Apparatus

Autoclave KT-40D.

Centrifugator, Hermle Z 383K, Diethelm Co., Ltd.

Data Physics, Germany.Laminar flow hoods, Pennyful Thailand, Co., Ltd.

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.

TG-DTA, Pyris diamond, Perkin Elmer Co., Ltd.

Vacuum evaporator, Heidolph WB2001.

Wilhelmy plate DCAT II tensiometer, LMS Instruments Co., Ltd,

3.2 Methodology

3.2.1 Measurement of Surface Properties and Cell Growth

3.2.1.1 Surface Tension Measurement

Culture medium after incubation for 24 hours was centrifuged at 4°C and 8500 rpm for 20 minutes. Then, the surface tension of culture supernatant (free-cell culture broth) was measured by the Wilhelmy plate DCAT II tensiometer.

3.2.1.2 Oil Displacement Test

Fifteen micro liters of crude oil were put on to the surface of 40 ml of distilled water in a Petri dish (15 cm in diameter) with a scale paper under the Petri dish. Then, 10 µl of the culture supernatant (free-cell culture broth) was dropped on the center of the oil surface. A clear halo was visible under light; its diameter and area were then measured and calculated by the following equation.

Area of clear zone = πr^2 ; r = radius of clear zone (cm)

3.2.1.3 Dry Weight Cell Measurement

Cells in the culture medium, after incubation for 24 hours, were collected by centrifugation at 4°C and 8500 rpm for 20 minutes and then washed several times with distilled water. Dry cell weight was determined by weighing the cells after heating in an oven at 110°C for 12 hours.

3.2.1.4 pH Measurement

The culture supernatant (clear solution) after removal of the cells by centrifugation was measured for its pH by a pH meter.

3.2.1.5 Critical Micelle Concentration (CMC) Measurement

Supernatant from the culture medium was diluted with distilled water to obtain a range of 0-30 %v/v dilution. The surface tension of each dilution was then determined. A curve of surface tension versus concentration was plotted in order to determine CMC as described by Sheppard and Mulligan (1987).

3.2.2 Isolation of Biosurfactant Producing Microorganisms

One gram (wet weight) of petroleum-contaminated soil was serially diluted in 0.85% sterile saline. All dilutions were performed in triplicate.

Dilutions were spread-plated on nutrient agar plates (prepared one day prior to use) with final dilutions ranging from 10^{-1} through 10^{-3}. The nutrient agar plates were incubated at 37°C for 72 hours. After incubation, the colonies were isolated and cultured on nutrient agar plates.

The isolated bacterial colonies from the nutrient agar plates after incubation for 24 hours were spotted on nutrient agar plates covered with crude oil 30 µl. The plates were incubated at 37°C for 24 hours. The clear zone around the bacterial colonies was observed in order to select bacterial colonies with the ability to produce biosurfactants (Morikawa *et al.,* 1993).

3.2.3 Determination of Biosurfactant-Producing Bacteria Activity

Bacterial colonies in nutrient agar slants were transferred to nutrient broth containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for 24 hours. An oil displacement test was performed for selecting the potential biosurfactant-producing bacteria (Morikawa *et al.,* 1993).

3.2.4 Microorganism Identification

The bacterial culture was identified by using the API®20 NE system (Biomérieux, Inc., France) for a Gram-negative bacterium. The identification was referred to the Analytical Profile Index Software database in order to determine the coefficient of similarity of the genus. Then, comparable with Bergey's manual of determinative bacteriology (Holt *et al.*, 1994).

3.2.5 Optimization of Culture Medium for Biosurfactant Production

The bacterial colonies were transferred into nutrient broth (NB), basal medium (BM), and defined medium (DM). Each of them contained 2%, 4%, 6%, 8%, and 10% palm oil. The cultured mediums were incubated at 37°C in a shaking incubator at 200 rpm for 72 hours. After incubation, oil displacement, surface tension and dry cell weight were determined.

3.2.6 Microbial Growth Determination

The biosurfactant-producing bacteria were cultivated in nutrient broth and nutrient broth containing 0.02% glucose and then incubated at 37°C in a shaking incubator at 200 rpm. The absorbances at 600 nm of both culture mediums were determined every 3 hours for 48 hours.

3.2.7 Determination of Optimum Inoculums

Biosurfactant-producing bacteria were cultured in culture medium using various amounts of inoculums (2%, 4%, 6%, and 8%). After incubation at 37°C in a shaking incubator at 200 rpm for 24 hours and 48 hours, surface tension, dry cell weight (g/l), and oil displacement tests were performed (Morikawa *et al.*, 1993).

3.2.8 Growth Curve of Biosurfactant-Producing Bacteria

2% Inoculums of biosurfactant-producing bacteria were transferred from nutrient broth after incubation at 37°C in a shaking incubator at 200 rpm for 22 hours to nutrient broth containing 2% palm oil. The culture mediums were incubated

at 37°C and 200 rpm. Then, surface tension, dry cell weight (g/l), and oil displacement (Morikawa *et al.,* 1993) were determined every 6 hours for 5 days.

3.2.9 Extraction of Biosurfactants and Biosurfactant Analysis by Thin Layer Chromatography (TLC)

After cultivation the strain SP4 in nutrient broth at 37°C for 48 hours, culture broth was centrifuged at 4°C and 8500 rpm for 20 minutes. 6M HCl solution was gradually added into the supernatant until pH equal to 2 in order to precipitate biosurfactant. The suspension was left overnight at 4°C for complete precipitation. Then, the suspension was centrifuged at 4°C and agitated 8500 rpm for 20 minutes. The precipitate was washed with HCl solution pH 2.

It was then dissolved in 0.1 M sodium bicarbonate (0.1 M $NaHCO_3$, pH 8.6) followed by the extraction of biosurfactants by 2:1 chloroform to ethanol (Zhang and Miller, 1992). Next, the solvent was vacuum dried at 40°C. The processes of precipitation and evaporation were repeated three times. The precipitate was analyzed by the TLC technique. Twenty-five µl of biosurfactants was dissolved in 50 µl Tris-HCl pH 8.0 and spotted on TLC plate (Silica gel 60, 20×20 cm). TLC plate was immersed in a tank consisting of 65% chloroform, 25% methanol, and 4% water (Morikawa *et al.,* 1993). When the moving phase moved on the TLC near the top edge of the plate (2.5cm from top edge), the TLC plate was kept in a box that contained iodine for 20 minutes. Then, the rate of flow (Rf) value was determined by the following equation.

Rf = Distance of moving substance on TLC plate / Distance of moving solvent

3.2.10 Preliminary Test of Biosurfactants in Free-Cell Broth for Oil Recovery from Ottawa Sand

The microorganism SP4 was cultivated in nutrient broth containing 2% palm oil at 37°C for 48 hours. After separation of cells by centrifugation at 8500 rpm for 20 minutes, the free-cell broth was used to test for oil recovery from Ottawa sand. A cylinder was packed with Ottawa sand for the height of 15 centimeters and flooded with D.I. water times of 20 pore volumes. Then, the column was flooded with a motor oil complex times of 1.5 pore volumes (the motor oil was held in the

pores of the sand). Next, a column was flooded with D.I. water again for 10 times of pore volumes. Then, the free-cell broth was flushed through the column for recovering the motor oil from the Ottawa sand. The recovered oil was collected every 11 minutes by using fraction collector. Finally, the recovered oil in each fraction was dissolved with hexane 5 ml and then analyzed the carbon content by a total organic carbon analyzer (TOC).

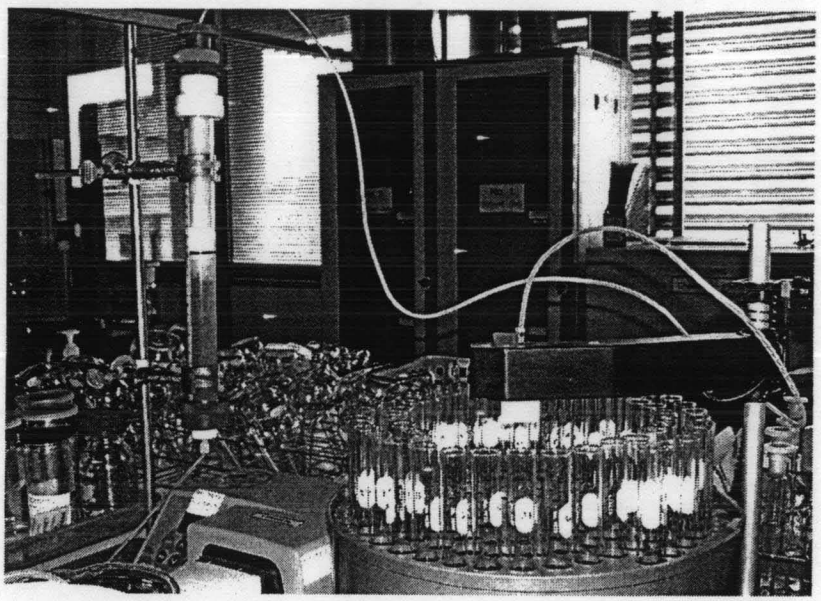

Figure 3.1 A cylinder packed with Ottawa sand and all equipments.