CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Sources of Environment Sample

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

3.1.2 Chemicals

All chemicals used in this study are given as follows:

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.

3.2 Methodology

3.2.1 Isolation of Biosurfactant Producing Microorganisms

A quantity of 1 g (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 (NA) plates (prepared one day prior to use) with final dilutions ranging from 10^{-1} through 10^{-3}. The NA plates were incubated at a temperature of 37±2°C for 72 h. After incubation, the colonies were isolated and cultured on NA plates.

Each isolated bacterial colony from the nutrient agar plate after incubation for 24 h was spotted on a NA plate covered with crude oil 30 µl. The plates were incubated at 37°C for 24 h. The clear zone around each bacterial colony was used to select bacterial colonies with the ability to produce biosurfactants (Morikawa *et al.*, 1993).

3.2.2 Determination of Biosurfactant-Producing Bacteria Activity

Each bacterial colony in a NA slant was transferred to a nutrient broth containing 2% palm oil and incubated at 37°C in a shaking incubator at 200 rpm for 24 h. An oil displacement test was performed for selecting any potential biosurfactant-producing bacteria (Morikawa *et al.*, 1993).

3.2.3 Measurement of Effecting of Biosurfactant-Producing Bacteria in Flask Scale

3.2.3.1 Surface Tension Measurement

Culture medium after incubation for 24 h was centrifuged at 4°C and 8500 rpm for 20 min. Then, the culture supernatant (clear solution) was measured for surface tension by using a Wilhelmy-plate DCAT II tensiometer.

3.2.3.2 Oil Displacement Test

A quantity of 15 µl of crude oil was added onto 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 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 to indicate the surface activity of the tested culture solution.

3.2.3.3 Microbial Concentration Measurement

To measure the microbial concentrations in culture media, a culture broth was centrifuged at 8,500 rpm and 4°C for 20 min. The residue was then washed with distilled water and recentifuge for a few time. After that, the washed residue was placed in an oven at 110°C for 12 h and the dried weight was obtained to represent the microbial concentration in sample.

3.2.3.4 Critical Micelle Concentration (CMC) Measurement

The supernatant from a 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).

3.2.4 Nomenclature and Classification of Bacteria

3.2.5 Optimization of Culture Medium for Producing Biosurfactant

Any isolated bacterial colony was transferred into a nutrient broth (NB) containing different concentrations of palm oil. The cultured medium was incubated at a temperature (37±2°C) in a shaker at 200 rpm for 48 under aerobic condition. The oil displacement test and the surface tension measurement were used to determine the presence and the effectiveness of the biosurfactants produced by the microbes screened

and isolated from different sources. The most effective microbe in producing biosurfactants was then selected for further investigation.

3.2.6 Growth Curve Determination

The selected biosurfactant-producing bacteria were cultivated in NB and then incubated at a temperature of 37±2°C in a shaker at 200 rpm. The absorbance of culture media was determined every 3 h for 48 h.

3.2.7 Optimum Inoculums Determination

The selected 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 h and 48 h, surface tension, dry cell weight (g/l), and oil displacement tests were determined (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 Test of Biosurfactants in Free-Cell Broth for Oil Recovery from Ottawa Sand

The microorganism was cultivated in nutrient broth containing 2% palm oil at 37°C for 51 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).