# CHAPTER III
# EXPERIMENTAL

## 3.1 Materials

### 3.1.1 Nonionic Surfactant

The nonionic surfactant used in this study was Tween 80 (100% solution). It was obtained from Fluka (Milwaukee, WI, USA).

### 3.1.2 Anionic Surfactant

The anionic surfactant used in this study was Sodium Dodecyl Sulfate or SDS (100% solution). It was obtained from Sigma (St.Louis, MO, USA). The properties of the two surfactants are described in Table 3.1.

**Table 3.1** The characteristics of surfactants used in this study

| Surfactant | Molecular formula | Molecular weight (g/mol) | HLB | CMC(M) |
|---|---|---|---|---|
| SDS | $C_{12}H_{25}SO_4Na$ | 288.4 | 40.0 | $8.4 \times 10^{-4}$ |
| Tween 80 | POE(20)sorbitan monooleate | 1310 | 15.0 | $1.2 \times 10^{-5}$ |

POE: polyoxyethylene, HLB: hydrophilic-lipophilic balance.

### 3.1.3 Oil Sludge

Oil sludge was kindly provided by PTT Public Company Limited, Thailand. The water portion of the sludge was separated by decantation and the excess moisture was removed by drying the oil sludge at open atmosphere for 120 hours or 5 days in a petri dish.

### 3.1.4 Media

Mineral salts medium (MSM) used in this study consists of 1.8 g $K_2HPO_4$, 1.2 g $KH_2PO_4$, 4.0 g $NH_4Cl$, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, and 0.01 g $FeSO_4.7H_2O$ in 1,000 mL distilled water (Ijah and Upke, 1992). The final pH of the

MSM was adjusted to pH 7.4 using 0.1 N NaOH and 10% HCl. MSM was autoclaved at 110°C, 15 psi for 15 min before used in the biodegradation experiment.

The medium composition used for culturing the oil sludge degrader was the same as that used in the biodegradation experiment. The agar medium consisted of 0.1 g of Bacto peptone (Difco), 0.5 g of yeast extract (Difco), 1 g of Glucose (Difco) and 1.8 g of agar (Difco) in 100 mL of MSM.

3.1.5 Bacteria and Cultivation

The bacteria strain, *Pseudomonas aeruginosa*, was kindly provided by Dr. Prayad Pokethitiyook at Mahidol University, which isolated from contaminated soil (Sungpeth, 1998). *Pseudomonas aeruginosa* is a gram-negative bacterium; these are prokaryotes that have a complex cell-wall profile consisting of an outer membrane and an inner, that is noted for its environmental versatility. Straight or slightly curved rods, but not helical, 0.5-1.0 x 1.5-5.0 μm. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur an aerobically. Most, if not all, species fail to grow under acidic conditions (pH 4.5) and widely distributed in nature. The bacteria culture was cultivated in 250 mL Erlenmeyer flask containing 100 mL of nutrient in doubly distilled water, followed by incubation. Inoculation was made by using 10 g of autoclaved oil sludge were added to 250 mL Erlenmeryer flask containing 100 mL of sterilized MSM. The mixture was incubated and shaken on an orbital shaker (Daigger, OR-200, 3141) at 150 rpm at 30 °C. Transfer was performed every 7 days by taken 1 mL of culture solution as inoculum into the fresh sterilized MSM. The microorganisms grown at this step were used as an oil sludge degrader in the biodegradation study.

## 3.2 Method

3.2.1 Effect of Surfactant on Solubilization of Hydrocarbons in Oil Sludge

An air-dried sludge (1000±0.5 mg) and 50 mL of MSM were mixed in a 250-mL glass stoppered Erlenmeyer flask. The required amount of surfactant was added on a weight by volume basis. For single surfactant systems, the surfactant

was added to the flask prior to adding the medium. For mixed surfactant systems, the first surfactant was added prior to adding the second surfactant, and the mixture was then mixed with the medium. Two controls were performed. The first control received oil sludge but no surfactant whereas the second control received only surfactant. The flasks were agitated on an orbital shaker (150 rpm) at room temperature. The dispersing power of surfactant was characterized by determining the total organic carbon of the aqueous phase. The sludge samples were filtered through filter paper (Whatman 42, size 11.5 cm.) and filtrates were injected into a TOC analyzer (Shimadsu, TOC-$V_{CSH}$). The samples were also analyzed for Chemical Oxygen Demand (COD) by COD reactor (HACH, 45600). The surfactants were added at various concentrations to evaluate their effect on the solubilization of hydrocarbons from oil sludge. The contact time of surfactants to hydrocarbons in oil sludge was measured thus indicating the formation of a stable emulsion of each type of surfactants. The estimation of hydrocarbons in oil sludge was done by the method of oil extraction as described in the next section.

### 3.2.2 Biodegradation Studies

For all experiments, the medium was always prepared fresh and 50 mL of medium was added directly to 250-mL Erlenmeyer flasks, followed by the addition of oil sludge (1000±0.5 mg) and surfactant if necessary. In the case of Tween-80, surfactant was added to the flask prior to adding the medium. Following inoculation, the flasks were incubated at room temperature on an orbital shaker set at 150 rpm and pH was maintained at 7.0 with 1 N NaOH as required. The extent of the biodegradation of the nonvolatile fractions of oil sludge was determined by comparing to control flask at a time zero and autoclaved control. The growth of the microorganism as a result of biodegradation of petroleum components can be measured by dry weight cell method. The growth of the microorganism as a result of biodegradation of surfactants can also be measured by optical density at 600 nm using spectrophotometer (UV-VIS Spectrophotometer, Shimadsu). All experiments were done in duplicated.

3.2.3 Determination of Total Petroleum Hydrocarbons (TPH) in Oil Sludge by Oil Extraction (Modified from Tiehm, 1993)

Following incubation, 20 mL of dichloromethane (DCM) and 30 mL of the sample were added to flasks which were mixed 15 minutes by sonicator (Crest, 575D) into obtain total petroleum hydrocarbons extract. The aqueous and solvent phases were transferred to four 50 mL centrifuge tubes, and flasks were added with 30 mL of DCM to bring the total volume of DCM used to 50 mL. Tubes were centrifuged at 12,000 x g (Hermile, Z383K) to break oil in water emulsions. The upper layer was discarded and the lower oil containing phase was filtered into a round-bottom flask through sodium sulfate to remove residual water. The majority of the solvent was removed under vacuum with an Evapotec Rotary Evaporator (Heidolph, VV2011) and allowed to dry to a constant weight on a fume hood prior to a gravimetrical measurement of the TPH extract. Asphaltenes were precipitated by adding 5 mL of n-hexane to the TPH extract, mixing with a glass rod. The contents of the beaker were then filtered through Whatman GF/A glass microfiber filter (Whatman International Ltd., Maidstone, England). The concentrated residue of hydrocarbons was diluted to 10 mL with n-hexane and this was injected into the GC/MS.

3.2.4 Bioreactor

The semi-batch reactor was made from a 1 liter beaker equipped with an air pump for aeration. Oil sludge (2%w/v) and the surfactant mixture were mixed with 500 mL of MSM. In this part of the study, mixed surfactants of SDS and Tween 80 was used at the concentration of 2%w/v and 0.1%w/v, respectively.The operation was done by aeration every day and this operation used fill and draw process. In each day, the aeration was stopped for 30 minutes and then the sample (50 mL) was collected to determine the TPH extraction, the growth of bacteria and Total Organic Carbon (TOC). After that, mixture of oil sludge, surfactant and MSM (50 mL) was filled into the reactor and aeration was continued. For *Pseudomonas aeruginosa* reactors, the microorganism was added after the oil sludge, surfactants and MSM were put in the reactor. In this experiment, ten bioreactors were operated of which five reactors for Indigenous Bacteria, four reactors for *Pseudomonas aeruginosa* and

one reactor for the control process (no added surfactants). Table 3.2 shows the conditions of each bioreactor.

**Table 3.2** The conditions of each bioreactor in fill and draw process

| Reactor | Total volume of 50 mL filled each day | | Total volume drawn in each day (mL) | Bacteria |
|---|---|---|---|---|
| | oil sludge + mix surfactant MSM at same ratio(mL) | MSM (mL) | | |
| Reactor1 (In) | 5 | 45 | 50 | Indigenous |
| Reactor2 (In) | 10 | 40 | 50 | Indigenous |
| Reactor3 (In) | 20 | 30 | 50 | Indigenous |
| Reactor4 (In) | 30 | 20 | 50 | Indigenous |
| Reactor5* (In) | 30 | 20 | 50 | Indigenous |
| Reactor1 (P) | 5 | 45 | 50 | Pseudomonas a. |
| Reactor2 (P) | 10 | 40 | 50 | Pseudomonas a. |
| Reactor3 (P) | 20 | 30 | 50 | Pseudomonas a. |
| Reactor4 (P) | 30 | 20 | 50 | Pseudomonas a. |
| Control | 0 | 50 | 50 | Autoclaved |

Reactor5* = Add Glucose