CHAPTER III
EXPERIMENTAL

3.1 Materials

Nonionic surfactants

Three nonionic surfactants used in this study were Brij 30, Triton X-100, and Tween 80. Brij 30 and Triton X-100 (100% solution) were obtained from Aldrich Chemical Co., Ltd. (St. Louis, MO, USA), whereas Tween 80 (100% solution) was obtained from Fluka (Milwaukee, WI, USA). The three surfactants were used as received from the suppliers without further purification. The properties of the 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)
Triton X-100	POE(10)octylphenol	625	13.5	1.7×10^{-4}
Brij 30	POE(4)lauryether	363	9.7	5.5×10^{-5}
Tween 80	POE(20)sorbitan monooleate	1310	15.0	1.2×10^{-5}

POE: polyoxyethylene, HLB: hydrophilic-lipophilic balance.

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 in a petri dish.

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 autoclave at 121°C, 15 psi for 15 min before used in the experiment.

The medium composition used for culturing the oil sludge degrader was the same as that used in the 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.

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 170 rpm at 30 ^{o}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

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. 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 a rotary shaker (170 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 (Shimadzu, 500A). The samples were also analyzed for Chemical Oxygen Demand (COD) by COD reactor (HACH, 45600). The surfactants as added at various concentration of surfactants for evaluating 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 nonionic surfactants. The estimation of hydrocarbons in oil sludge was done by the method of oil extraction as described in the next section.

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 Brij 30, Triton X-100 and Tween-80, surfactants were added to the flask prior to adding the medium. Following inoculation, the flasks were incubated at room temperature on an orbital shaker set at 170 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 (Perkin Elmer, model AV-200). All experiments were done in triplicate.

Determination of total petroleum hydrocarbons (TPH) in oil sludge by oil extraction

Following incubation, 20 mL of dichloromethane (DCM) was added to flasks which were mixing 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 rinsed three more times 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.