CHAPTER III
RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

Trichloroethylene (TCE) (99.5 % purity) was purchased from BDH, England. Toluene (99.9 % purity) was purchased from Merck, Germany. R(+)-limonene (96.0 % purity) was purchased from Fluka, Germany. Iodonitrotetrazolium violet (INT) and iodonitrotetrazolium formazan (INTF) were purchased from Sigma-Aldrich, USA. Methanol (analytical grade) was purchased from BDH, Germany.

3.1.2 Culture Medium

Mineral Salt Media (MSM) consists of (in mg/L) K_2HPO_4, 1741.6; Na_2HPO_4, 359.94; $(NH_4)_2SO_4$, 1321.3; $MgSO_4$, 120.36; $Ca(NO_3)_2$, 16.409; $Fe(NO_3)_3$, 2.419; $MnSO_4$, 0.151; $ZnSO_4$, 0.161; $CuSO_4$, 0.160; $NiSO_4$, 0.015; $CoSO_4$, 0.016; Na_2MoO_4, 0.021. For MS agar (MSA), 15 g/L of bactoagar was added. Toluene was added into media at concentration of 172 mg/L as carbon and energy sources for microorganisms.

3.1.3 Bioaugmentation Cultures

3.1.3.1 TCE Degrader

TCE degrader, *Rhodococcus* sp. P3 (GenBank accession no. EF450777), a gram positive aerobic bacteria, isolated from petroleum-contaminated soil collected from Bangkok area is kindly provided by Dr.Ekawan Luepromchai, Department of Microbiology, Chulalongkorn University, Bangkok, Thailand. One loop-full of *Rhodococcus* sp. P3 from MSA was transferred into 30 mL sterile MSM containing 172 mg/L of toluene as a primary substrate, incubated at room temperature, shaken at 200 rpm on orbital shaker for 24 hrs and used as inoculum.

3.1.3.2 Aerobic Activated Sludge

Aerobic activated sludge was collected from wastewater treatment plant of Lardkrabang Industrial Sector, Bangkok, Thailand. Wastewater treated at this wastewater treatment plant is from electronic part industries where organic solvents such as TCE and toluene are used. Acclimatization of activated

sludge to toluene was conducted by adding activated sludge at 1:1 ratio (v/v) to MSM containing 172 mg/L of toluene as a primary substrate, incubated at room temperature and shaken at 200 rpm on orbital shaker for one week before used as inoculum. At this stage the acclimatized activated sludge would mostly contain TCE degrader.

3.1.4 Soil

Soil was collected from Tumbol Klang-Dong, Aumphur Pak-Chong, Nakonratchasima Province, Thailand. Soil was passed through 2 mm sieve and kept at 4°C prior the usage.

3.1.5 Support Materials

Corncob and coir were obtained from Faculty of Agriculture, Khon Kaen University, Thailand. Organic carbon and total nitrogen of corncob were 45.84 and 0.23 %, respectively. Organic carbon and total nitrogen of coir were 49.33 and 0.51%, respectively. They were shredded by knife into small pieces (approximately 0.5 x 0.5 x 0.5 cm) and passed through 0.5-1 cm sieve. After that, they were delignified by boiling in 1% NaOH for 3 hrs (Iconomou et al., 1995) to remove lignin which might be toxic to microorganisms and then thoroughly washed under tap water, and soaked in distilled water overnight. This process was done 2 times and kept at -20 °C prior the usage.

3.1.6 Potential Primary Substrate

Kaffir lime peel (*Citrus hystrix*) was obtained from local market in Khon Kaen Province, Thailand. This peel contains essential oil which consist of various plant terpene especially limonene. A concentration of limonene in kaffir lime peel used in this study was 940 mg/kg. Kaffir lime peel was air-dried at room temperature, chopped into pieces and passed through 2 mm sieve and kept at -20 °C to prevent the essential oil evaporation.

3.1.7 Biostimulation Material

Cassava pulp was used as organic amendment to biostimulate TCE biodegradation. Cassava pulp was obtained from Asian Citric Co.Ltd, Kalasin Province. It contains a high organic carbon which can serve as a carbon source for

microorganisms. This material was air-dried at room temperature, crushed into small pieces and passed through 2 mm sieve and kept at 4 °C prior the usage. Carbon and nitrogen contents of cassava pulp used in this study were 51.70 % and 0.25 % analyzed by Wakley-Black method (Wakley and Black, 1934) and Kjeldahl method (AOAC, 2000), respectively.

3.2 Methodology

3.2.1 Construction of Soil Microcosms

3.2.1.1 Soil Microcosms with Free Cells

This experiment was designed to examine the TCE degradation ability of mixed cultures obtained from activated sludge in comparison to pure culture in free cell form. The experiment was conducted under aerobic condition in the 50-mL serum bottle containing 10 grams dry wt of non-sterile soil and inoculated with acclimatized activated sludge or *Rhodococcus* sp. P3 to the final cell density of 10^6 CFU/g soil. Control was soil microcosm without inoculum which represented the TCE degradation ability of indigenous microorganisms. The initial moisture content of soil was adjusted to 18-20 %. TCE and toluene at concentration of 100 and 172 mg/kg soil, respectively, were added into serum bottle and immediately sealed with teflon-lined rubber septa and climped with aluminum cap. All treatments were triplicates. The bottle was incubated in the dark condition at room temperature. Samples were sacrificed at day 0, 3, 5, 7, 14, and 21 to determine the concentration of TCE remaining by GC-headspace technique and the numbers of microorganisms in soil by viable plate count technique.

3.2.1.2 Immobilization Method

Immobilized cells were prepared by putting 75 g dry wt of delignified corncob and coir into 300 ml MSM containing 4 g/L glucose as a carbon source and then autoclaved at 121 °C for 15 min for 2 times before inoculating with 10 % (v/v) of *Rhodococcus* sp. P3 or acclimatized activated sludge to the final cell density of 10^6 CFU/mL. Toluene at a concentration of 172 mg/L as a primary substrate was added into the bottle before incubating at room temperature and shaking at 200 rpm on orbital shaker for 24 hrs. After that, these support materials was harvested and transferred into fresh MSM containing 4 g/L glucose and 172 mg/L of toluene and incubated at the similar condition as described previously for 2 times before harvesting by washing with sterile distilled water. The numbers of

microorganisms in support materials were approximately 10^7 cells/g dry wt of support materials determined by viable plate count technique. These immobilized cells was used as inocula for soil microcosms study.

3.2.1.3 Soil Microcosms with Immobilized Cells

This experiment investigated an ability of TCE degrader in immobilized form to degrade TCE in comparison to free cell form. In addition, efficacy of support materials i.e., corncob and coir to immobilize mixed cultures and pure culture were evaluated. The experiment was conducted in a 50-mL serum bottle containing 10 grams dry wt of non-sterile soil and 10% (w/w) of immobilized acclimatized activated sludge on corncob and coir or immobilized *Rhodococcus* sp. P3 on corncob and coir at 10^6 CFU/g soil. Control was soil microcosm added with free cells of each acclimatized activated sludge or *Rhodococcus* sp. P3 at 10^6 CFU/g soil as inocula. The initial moisture content of soil was adjusted to 18-20 %. TCE and toluene at concentration of 100 and 172 mg/kg soil, respectively, were added into serum bottle and immediately sealed with teflon-lined rubber septa and climped with aluminum cap. All treatments were triplicates. This bottle was incubated in the dark condition at room temperature. Samples were sacrificed at day 0, 3, 5, 7, 14 and 21 to determine the concentration of TCE remaining by GC-headspace technique and the numbers of microorganisms in support materials and soil by viable plate count technique. The effectiveness of support materials for immobilization of inocula were evaluated. The relative percentage of TCE degradation was used to determine the amount of TCE dissipate overtime as following equation:

$$\frac{\text{Concentration of TCE at day 0- Concentration of TCE at day 21}}{\text{Concentration of TCE at day 0}} \times 100 \qquad (1)$$

The inocula that showed the highest degradation of TCE and the most efficient support material was further reused to evaluate the reusability of immobilized cell. This was conducted by harvesting, washing the most efficient immobilized cell with sterile 0.85% NaCl for two times and then re-inoculated into 10 g of non-sterile soil at 10^6 CFU/g soil. The initial moisture content of soil in microcosm was adjusted to 18-20 %. TCE and toluene at concentration of 100 and 172 mg/kg soil, respectively, were added into serum bottle and immediately sealed with teflon-lined rubber septa and climped with aluminum cap. All treatments were triplicates. This bottle was incubated in the dark condition at room temperature. Samples were sacrificed at day 0, 3, 5, 7, 14 and 21 to determine the concentration of

TCE remaining by GC-headspace technique and the numbers of microorganisms in support materials and soil by viable plate count technique.

3.2.1.4 Soil Microcosm with Potential Primary Substrate

This experiment was conducted to examine the effect of different concentrations of potential primary substrate i.e., kaffir lime peel on TCE biodegradation in soil. The experiment was conducted in a 50-mL serum bottle containing 10 grams dry wt of non-sterile soil, 10% (w/w) of immobilized acclimatized activated sludge on corncob containing 10^6 CFU/g soil of TCE degrader and 0.53, 1.06, 1.59, 2.65 g of kaffir lime peel which equivalent to 50, 100, 150 and 250 mg/kg soil of limonene. Toluene was used as a positive control at various concentrations of 50, 100, 150 and 250 mg/kg soil. Control soil microcosm was conducted in a similar manner but without inoculum. In addition, abiotic control soil microcosm was also constructed in a similar manner except all materials were sterilized and 1.06 g of kaffir lime peel which equivalent to 100 mg/kg soil of limonene was added. The initial moisture content of soil was adjusted to 18-20 %. Thereafter, TCE at the initial concentration of 100 mg/kg soil was spiked into soil microcosm and immediately sealed with teflon-lined rubber septa and climped with aluminum cap. All treatments were triplicates. Soil microcosms were incubated in the dark condition at room temperature. Samples were sacrificed at Day 0, 3, 6, 10 and 14 to determine the concentration of TCE remaining by GC-headspace technique and the numbers of microorganisms in soil by viable plate count technique.

3.2.1.6 Soil Microcosm with Cassava Pulp

This experiment was designed to examine the effect of different C:N ratios of cassava pulp amended in soil to stimulate TCE biodegradation. The experiment was conducted in a 50-mL serum bottle containing 10 grams dry wt of non-sterile soil, 10% (w/w) of immobilized acclimatized activated sludge on corncob containing 10^6 CFU/g soil of TCE degrader and 0.53 g kaffir lime peel which equivalent to limonene of 50 mg/kg soil. This concentration was an optimal concentration obtained from 3.2.1.4. C:N ratios in microcosm were adjusted to 20:1, 30:1 and 40: 1 by adding with 0.09, 0.32 and 0.60 g of cassava pulp, respectively. Control was soil microcosm without cassava pulp. The initial moisture content of soil was adjusted to 18-20 %. Thereafter, TCE at the initial concentration of 100 mg/kg soil was spiked into soil with microcosm and the bottle was immediately sealed with teflon-lined rubber septa and climped with aluminum cap. All treatments were

triplicates. Soil microcosms were incubated in the dark condition at room temperature. Samples were sacrificed at Day 0, 3, 6, 10 and 14 to determine for the concentration of TCE remaining by GC-headspace technique and the numbers of toluene degrading bacteria, total bacteria, actenomycetes and fungi in soil by viable plate count technique and microbial activity by dehydrogenase assay.

3.3 Analytical Method

3.3.1 Analysis of TCE Concentration Using Gas Chromatography-Head Space Technique

TCE concentration in soil microcosms was analyzed by GC-head space technique. Serum bottle containing soil sample was heated in heat box at 90°C for 30 min. Fifty µl of head space sample were taken by gas tight syringe and analyzed for TCE concentration using GC-17A Shimadzu-Flame Ionization Detector. The capillary column is 30-m Rtx-VGC with the inner diameter of 0.45-mm (Restex Inc., USA). Helium was used as a carrier gas. Splittless mode was used. The injection and detector temperature were maintained at 200 ° C. The column temperature retained at 60 ° C for 5 min and was then increased at 8 ° C/min until reach 180 ° C then hold for 2 min.

3.3.2 Enumeration of Microorganisms in Soil and Support Materials

For enumeration of microorganisms in support materials, one g wet wt of support materials were washed with sterile 0.85% NaCl for 2 times to remove soil and then blended by blender into small pieces. For enumeration of microorganisms in soil, one g dry wt of soil was mixed with 9 ml of 0.85% NaCl to make soil dilution. Then, a serial 10-folds dilution of each suspension was plated on MS agar and incubated in the box fumigated with toluene as a primary substrate to enumerate toluene degrading bacteria. Bacteria and actenomycetes were enumerated on Plate Count Agar (PCA) (Difco, US). Fungi was enumerated on Rose Bengal Agar (RBA) (HiMedia, India) containing 50 ug/ml of streptomycin and incubated at room temperature for one week for toluene degrading bacteria, 2 days for bacteria and actinomycetes and 3-5 days for fungi. The number of colony forming units (CFU) between 30-300 colony in each plate were counted.

3.3.3 Microbial Activity

Microbial activity of microorganisms were measured using intracellular dehydrogenase enzyme (DHA). DHA is an intracellular enzyme involved in microbial respiratory metabolism indicating microbial activity (Von Mersi and Schinner, 1991). The DHA is determined by reducing INT to INTF. Soil, 2.5 g dry weight, collected from each experiment was added into a 20-ml vial, mixed with 1.25 ml of DI water and 0.5 ml of INT solution (5 g /L). The vials were sealed with septum and incubated for 22 h at 27° C in dark. After that, the metabolic product i.e., INTF was extracted by adding 12.5 ml methanol, then mix and filtrate the extract through a Whatman paper No 42 and measured the absorbance of the metabolic product at λ_{max}= 428 nm using a spectrophotometer. The INTF standard was prepared in methanol. DHA was expressed as mg INTF/kg dry soil/h (Dungan et al., 2006).