CHAPTER III

METHODOLOGY AND MATERIALS

3.1 Experimental Framework

The main part of this study concerns the degradation of MT by microorganisms from wastewater treatment systems and sediment from masculinization ponds of Nile tilapia fry. Degradation studies were conducted under aerobic and anaerobic conditions. Experiments are divided into 3 parts: 1) biodegradation of MT by microorganisms from wastewater treatment systems and sediment under aerobic and anaerobic conditions, 2) isolation of MT-degrading bacteria from aerobic sludge of a wastewater treatment system, and 3) identification and characterization of the isolated MT degrading bacteria. The experimental framework is shown in Figure 3.1.

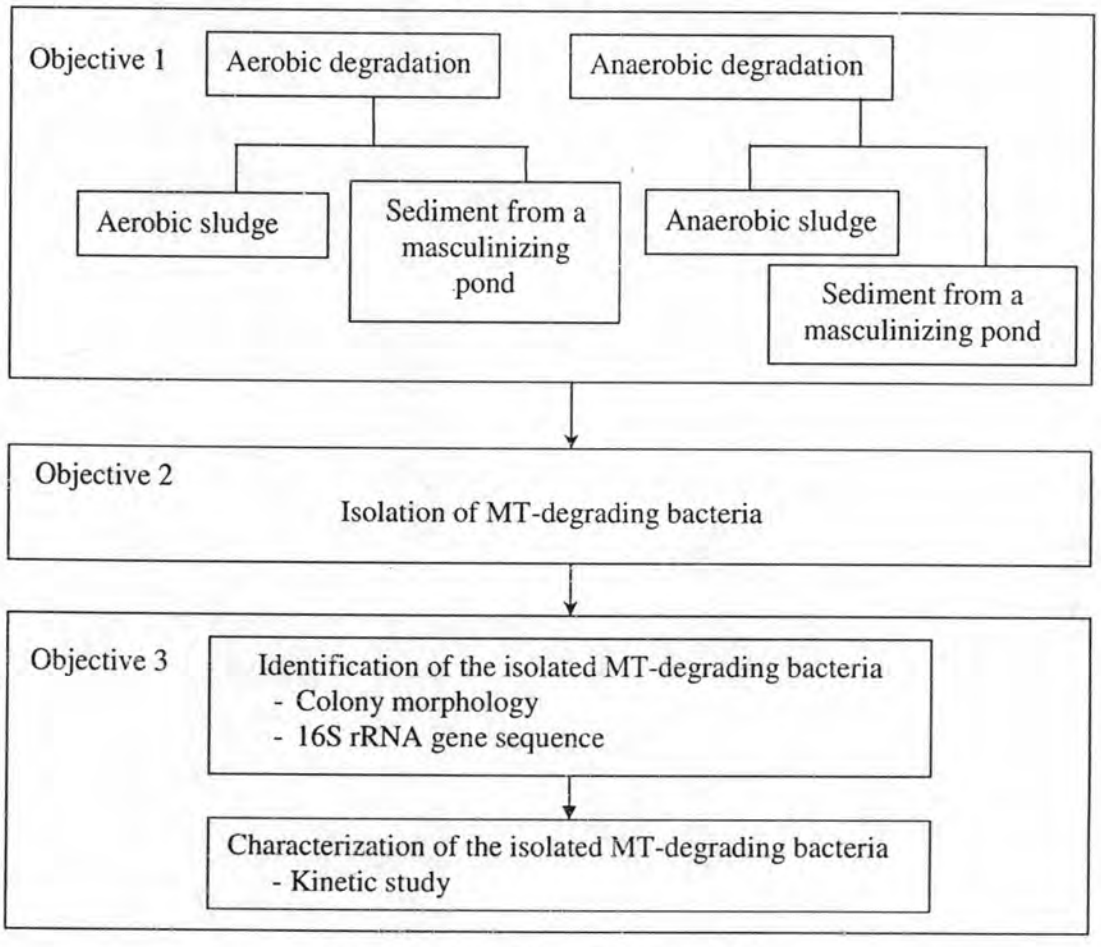

Figure 3.1 Experimental framework

3.2 Materials

3.2.1 Chemicals

MT (> 99% pure, HPLC grade) was purchased from Fluka (Buchs, Switzerland). Acetonitrite and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Individual stock solutions of MT (500 and 5000 mg/l) were prepared in methanol and store at -20°C.

3.2.2 Seed Sludge

3.2.2.1 Aerobic Sludge

Aerobic sludge used in the aerobic biodegradation test was collected from an aeration tank of Toong Kru municipal wastewater treatment plant in Bangkok, Thailand. Mixed-liquor suspended solids (MLSS) concentration of the sludge on the sampling day was 3,160 mg/l.

3.2.2.2 Anaerobic Sludge

Anaerobic sludge used in the anaerobic biodegradation test was collected from an Upflow Anaerobic Saludge Blanket (UASB) sysytem from Thai Beverage Public Company Limited, Bangkok Thailand. Mixed-liquor suspended solids (MLSS) concentrations determined on the day of sampling was 75,170 mg/l.

3.2.2.3. Sediment

Sediment used in the aerobic and anaerobic biodegradation tests were collected from a masculinization pond of Nile tilapia fry, in Pathumthani, Thailand.

3.2.3 Media

3.2.3.1 Medium for Aerobic Biodegradation

Medium used in the aerobic biodegradation test with aerobic sludge and sediment was inorganic salt medium (IS medium) containing 100 mg of NH_4Cl, 1 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of EDTA-Fe, 0.05 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of K_2HPO_4, 4 g of HEPES, 0.6 mg of $MnCl_2 \cdot 4H_2O$, 0.5 mg of H_3BO_3, 0.1 mg of $ZnCl_2$, 0.1 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.6 mg of $CoCl_2 \cdot 6H_2O$, 0.12 mg of $NiCl_2 \cdot 6H_2O$, 0.12 mg of

$CuSO_4 \cdot 5H_2O$ dissolved in 1 liter of Milli-Q water and adjusted to pH 7-7.5 with 1 N NaOH solution (Chao et al., 2004).

3.2.3.2 Medium for Anaerobic Biodegradation

Medium used for anaerobic biodegradation test with anaerobic sludge and sediment was a basal medium containing 0.5 g of NH_4Cl, 0.4 g of K_2HPO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$ dissloved in 1 of Milli-Q water with, 1 ml of resazurin solution (stock solution 1 g/l), 10 ml of trace metal solution comprising of 4.5 g of nitrilotriacetic acid (disodium), 0.4 g of $FeCl_2 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot H_2O$, 0.17 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnCl_2$, 0.19 g of H_3BO_3, 0.02 g of $NiCl_2 \cdot 6H_2O$, 0.01 g of $Na_2MoO_4 \cdot 2H_2O$. All trace metals were with 1 liter of Milli-Q water and adjusted to pH 7 with 10 N KOH. The medium was adjusted pH to 6.8 with 1 M HCl.

3.2.3.3 Medium for Enrichment and Isolation

Medium used for enrichment is the same as that used for aerobic biodegradation test. For isolation of bacteria, medium plate was prepared by adding 1.5% agar as the enrichment medium.

3.3 Analytical Method

3.3.1 Extraction and Analysis of MT

One and a half ml of inorganic medium added with 3 ml of methanol was filtered through 0.45 µm filter. MT concentrations in solution were analyzed using a High-Performance Liquid Chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA) with an ODS Hypersil, 250 mm x 5 mm x 4.6 µm column at a flow rate of 0.5 ml/min and column temperature of 40.0 ± 0.5°C. A water–acetonitrile gradient (80:20 (v/v) at time t = 0 min, 4:96 (v/v) at t = 19 min, and 80:20 (v/v) at t = 20 min) was used as the mobile phase. A 10 min post run time was used to equilibrate the column. MT was detected at 245 nm (bandwidth 4 nm) for quantification and 255 nm (bandwidth 4 nm) as qualifier using a diode array detector (DAD). Injection sample volume used was 50 µl (Marwah and Lardy, 2005).

3.3.2 Analysis of 16s rRNA gene sequences

3.3.2.1 DNA Extraction

Twenty µl of 10 % chelex was added to an eppendorf tube containing a single colony and mixed mildly (Drake et al., 1996). The tube was heated in a water bath at 60^{O}C for 60 min and then increased to 95^{O}C for 15 min.

3.3.2.2 Polymerase Chain Reaction

DNA was amplified by Polymerase Chain Reaction (PCR) with universal primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3'). The total volume of PCR reaction was 25 µl and the PCR mixture was prepared using tag from QIAGEN, Germantown, Maryland, USA following the manufacturer's instruction. PCR amplification was performed in PTC-DNA engine cycler (Biorad, USA) under the conditions of 3 min at 94 ^{0}C followed by 30 cycles of 1 min at 94 ^{0}C, 1 min at 57 ^{0}C, and 1 min at 72 ^{0}C, followed by 10 min final extension at 72 ^{0}C. The product from PCR was purified by QIAquick® PCR purified kit (QIAGEN, Germantown, Maryland, USA).

3.3.2.3. Sequencing

PCR samples were sent for sequencing to Macrogen Inc. laboratory, Seoul, Korea. Sequences were analyzed by BLAST (Basic Local Alignment Search Tool), (National Center for Biotechnology Information, Bethesda, Maryland, USA) which provides a method for rapid searching of nucleotide and protein databases.

3.4 Biodegradation of MT by Microorganisms from Wastewater Treatment Systems and Sediment under Aerobic and Anaerobic Conditions

3.4.1 Aerobic Biodegradation of MT Using Aerobic Sludge

To investigate the degradation of MT by aerobic sludge, three parallel batch tests comprising of a degradation test, a recovery test, and a control test were carried out in autoclaved 16 ml amber vials with PTFE screw caps. MT dissolved in methanol was added to amber vials to obtain a final concentration of 0.3, 5.0, 7.0, and 10.0 mg/l. Methanol was dried by blowing nitrogen gas mildly 5.0 ml of IS medium was then added to redissolve the MT. In the biodegradation test, 0.47 ml of aerobic sludge was added into

the IS medium to obtain a final MLSS concentration of 150 mg/l (Shi et al., 2004). The final volume in the amber vials were 5 ml. Recovery test and control test were prepared in the same manner except that for the recovery test, autoclaved aerobic sludge was used to determine the recovery of MT to the presence of biomass. No sludge was added to the control test vials. Triplicates were prepared for each concentration for the degradation tests whereas recovery and control tests were done in duplicate. The vials were incubated in the dark at 25 °C and rotated at a speed of 200 rpm. Whole vials were sacrificed periodically depending on the initial MT concentrations.

3.4.2 Aerobic Biodegradation of MT using sediment

Aerobic biodegradation tests using sediment were carried out in the same manner as the experiments for aerobic sludge except that sediment were used instead of aerobic sludge. Sediment was added to achieve a concentration of 10% (v/v) measured by Tuberculin syringe. In this test 0.5 ml of slurry sediments were added.

3.4.3 Anaerobic Biodegradation of MT Using Anaerobic Sludge

To investigate the biodegradation of MT using anaerobic sludge, three parallel batch tests comprising of a degradation test, a recovery test, and a control test were carried out in 10 ml amber serum bottles. Five MT concentrations of 0.1, 1.0, 3.0, 5.0 and 10.0 mg/l were tested. MT stock solution were added into serum bottles to reach the required final concentration. The methanol was dried by blowing nitrogen gas mildly. In the biodegradation test, 0.34 ml of anaerobic sludge was added into the basal medium to obtain a final MLSS concentration of 5,100 mg/l (Ong et al., 2005). The final volume in the vial was 6 ml. All serum bottles were capped with butyl rubber stoppers and aluminum caps and remaining oxygen in the bottle was purged by nitrogen gas to create anaerobic conditions. All serum bottles were confirmed for the absence of oxygen by using 1 g/l of resazurin as an indicator. The recovery test, and the control test were prepared in the same manner as the anaerobic biodegradation test except that the recovery test used autoclaved anaerobic sludge to determine the recovery of MT from biomass and the control test did not have sludge added. Triplicate serum bottles were prepared for each concentration for the biodegradation test. Duplicate serum bottles were prepared for recovery and control tests. The vials were incubated at 25°C and rotated at a speed of 200

rpm. Serum bottles were sacrificed periodically and samples taken depending on the initial MT concentrations.

3.4.4 Anaerobic Biodegradation of MT Using Sediment

Anaerobic biodegradation tests using sediment were carried out in the same manner as the experiments using anaerobic sludge except that sediment were used instead of anaerobic sludge. Sediment was added to achieve a concentration of 10% (v/v) measured by Tuberculin syringe. In this test 0.5 ml of slurry sediments were added.

3.5 Isolation of MT-degrading bacteria from aerobic sludge

3.5.1. Enrichment of MT-degrading bacteria

Enrichment experiment was carried out with 3 initial MT concentrations of 10, 100 and 500 mg/l. Stock solution of MT was added a 250 ml Erlenmeyer flask to obtain the desired concentrations. Methanol in solution was dried with mild nitrogen gas flow. Five ml of inoculum (activated sludge) was added to the flask with 95 ml of IS medium to obtain a final volume of 100 ml. The flasks were incubated at 25 °C and rotated at a speed of 200 rpm. MT concentrations were monitored and analyzed until 70 % of the initial MT was degraded. Five ml of inoculum were then transferred into 95 ml of fresh medium in new flasks. The subcultures were enriched five times.

3.5.2 Isolation of MT-degrading bacteria

Enriched inoculums were diluted with Milli-Q® water by 10^{-4}, 10^{-5} 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} fold and spread on a 1.5 % agar plate coated with 100 mg/l of MT. Plates were incubated at 25°C until colonies appeared.

3.6 Identification and characterization of the isolated MT-degrading bacteria

3.6.1 Prescreening Using Colony Morphology

Colonies were prescreened by examining the colony morphology. Colonies were identified for the following traits: color, form, elevation, margin, diameter, surface, opacity, and texture. The colonies morphologies were used to initially group the enriched inoculums.

3.6.2 Identification Using 16S rRNA Gene Sequence

After extraction of DNA in the same manner of DNA extraction explained in Section 3.3.2.1 and PCR amplification using the universal primer, the sample was identified using 16S rRNA gene sequencing to analyze the genus and species of the microorganisms isolated from the aerobic sludge. Analysis based on sequence of 16s rRNA gene was performed for all samples.

3.6.3 Degradation Kinetics of MT-degrading Bacteria

To investigate the degradation kinetics of MT-degrading bacteria, two parallel batch tests comprising of a degradation test and a control test were carried out in 16 ml amber vials with PTFE screw caps. All tests were conducted in duplicate. To determine the biodegradation rate constant, four initial MT concentrations of 3, 5, 15, 100 mg/l were prepared. In the biodegradation test, inoculums of MT-degrading bacteria was added into the 5 ml of IS medium to obtain a final concentration of 2.4 x 10^7 CFU/ml by using plate count technique (Zhang et al., 2007). The total volume in the vial was 5 ml. Control test were prepared in the same manner except that no inoculums were added. All vials were incubated at 25 ^{O}C and rotated at a speed of 200 rpm. Samples were collected every 6 hr and analyzed for MT.

3.6.4 Biodegradation Activity Test of MT-Degrading Bacteria

To investigate the aerobic biodegradation of MT by MT-degrading bacteria, two parallel batch tests comprising of a degradation test and control test were carried out in 16 ml amber vial with PTFE screw cap. All tests were conducted in duplicate. In this experiment, 10 mg/l and 500 mg/l of initial MT concentration was test for MT-degrading bacteria isolated at 10 mg/l and 500 mg/l (their culture concentration), respectively. In the biodegradation test, inoculums of MT-degrading bacteria was added into 5 ml of IS medium to obtain a final concentration of 2.4 x 10^8 CFU/ml by plate count technique (Zhang et al., 2007). The final volume used in the vial was 5 ml. Control test were prepared in the same manner except that no inoculums were added. All vials were incubated at 25 ^{O}C and rotated at a speed of 200 rpm.