CHAPTER III

METHODOLOGY

3.1 Methodology Outline

This research study focuses on isolation and identification of microorganisms in biofilter for the treatment of xylene vapor. The microorganisms that islolated from biofilter would have potential to degrade xylene. The performance of biofiltration system inoculated with specific species of microorganisms was evaluated. Biofilter media was consisted of coconut husk and manure at ratio 70: 30 (v/v) with initial moisture content 50 % of dry weight.

3.2 Culture Media

1. Potato Dextrose Agar (PDA)

Potato	200 g
(PDA was use as infusion from potato by boiling 200 g scrubbed and sliced potato in distilled water)	
Dextrose (Difco, 215530)	20 g
Agar (Difco, 0140-1)	15 g
Distilled water	1,000 ml

2. Malt Extract Agar (MEA)

Malt Extract (Merk,1.05391.0500)	20 g
Peptone (BD,211677)	5 g
Dextrose (Difco, 215530)	20 g
Agar (Difco, 0140-1)	15 g
Distilled water	1,000 ml
Autoclaved at 121°C, 15 minutes	

3. Sabouraud Dextrose Agar (SDA)

Glucose (Fisher Chemical,G/0450/60)	20 g
Peptone (BD, 211677)	5 g
Agar (Difco, 0140-1)	15 g
Distilled water	1,000 ml
Autoclaved at 121°C, 15 minutes	
pH 6.8-7.0	

4. Nutrient Agar

Beef Extract (Difco, 211520)	3 g
Peptone (BD, 211677)	5 g
Agar (Difco, 0140-1)	15 g
Distilled water	1,000 ml
Autoclaved at 121°C, 15 minutes	

5. Mineral Medium Agar (MMA) (Verdin *et al.* , 2004)

KCl (Riedel-de Haen, 31248)	0.25 g
$NaH_2PO_4.2H_2O$ (Fisher Chemicals, S/3760/53)	6.5 g
$Na_2HPO_4.7H_2O$ (Riedel-de Haen, 30413)	15.7 g
$MgSO_4.7H_2O$ (Fluka, 63142)	0.5 g
NH_4NO_3 (Univar, A 49-500 g)	1 g
$ZnSO_4.7H_2O$ (BDH, 102994 B)	0.1 g
$MnCl_2.4H_2O$ ((Riedel-de Haen, 31236)	0.01 g
$FeSO_4.7H_2O$ (Riedel-de Haen, 31236)	0.1 g
$CuSO_4.5H_2O$ (Carlo Erba, 476245))	0.05 g
$CoCl_2.6H_2O$ (Riedel-de Haen, 31123)	0.01 g
MoO_3 (Merk)	0.02 g
Agar (Difco, 0140-1)	15 g
Distilled water	1,000 ml
Autoclaved at 121°C, 15 minutes	

3.3 Chemicals and Instruments

1. Xylene : Analytical Grade from Fisher Scientific code x/0250/17
2. Lactophenol Blue Solution:Standard Fluka from Fluka code 418275/121401
3. pH indicator from Merk
4. Pump ; Tank : Tiger and Motor : Toshiba
5. Tedlar Bag : Supelco 24633
6. CCT tube: SKC Tested and Certified Sorbent Sample Tubes Catalog no. 226-01 sorbent : Coconut Charrcoal, 20/40 mesh, 50/100 mg
5. Flow Meter: Dwyer
6. Manometer: Dwyer
7. Desiccator : Temptag from Thai –Asahi Glass
8. Shaker: Ratek : Plate Form Mixer
9. Larminar : Super Clean 120 VC
10. Autoclave: Sanyo Serial no. 31.202
11. Hot air oven : Conerm Model 240 M Serial no. 96150
12. Microscope: Leica A/C 2000
13. Balance: Model Sertorius 50907755
14. Gas Chromatography (FID Detector): HP 6890
15. Gas Chromatography TCD Detector): HP 6890
16. GC/MS/MS : GC; Varian: Star 3400 cx, MS; Varian Saturn2000
    Column: Varian; Fact or Four Capillary Column, VF-5ms, 30 MX, 0.25 MM, ID OF=0.25

3.4 Microorganisms Preparation

3.4.1 Packing Materials

Packing materials or biofilter media in this experiment were consisted of coconut husk (1.5 × 1.0 cm), manure compost and waste water sludge. The waste water sludge collected from waste water treatment plant in Bangkok and from the factory in Rayong province, Thailand, were used as the initial culture.

The ratio of packing materials of coconut husk: manure compost: sludge was 75 : 20 : 5 (v/v). This ratio was reported to be the optimum ratio of packing media for biofiltration process (PCD, 2001). Water was added in mixed filter media untill the moisture content reach 50% of dry weight approximately. Then all biofilter media was packed in acrylic column.

3.4.2 Column

The biofilter column was made of acrylic (5.0 cm inside diameter and 200 cm long) and provided with four sampling ports located at 20, 60, 100, and 140 cm from the bottom for gas sampling. The bed of column packed with gravel (1 cm) to the height of 20 cm and packed with filter material up to the height of 120 cm on top of gravel layer.

3.4.3 Biofilter Compartment

Flow meter, air pump and mixing chamber were connected with columns to control biofilter condition. The air and water vapor were pumped to mix with xylene vapour in mixing chamber. Then xylene vapor in moisture saturated air was pumped into 4 columns with constant flow rate.

3.4.4 Operation System for Acclimatization

The instrument is consisted of column, pump, flow meters, water and xylene containers and mixing chamber(Figure3-1). To maintain moisture content in column, moisture saturated air and xylene vapor were pumped into the mixing chambers at the same flow rate of 2 L/ min. Then, xylene vapour from mixing chamber was feed to the column at flow rate of 1L/min to acclimatize xylene degrading microorganisms for 1 and 2 months.

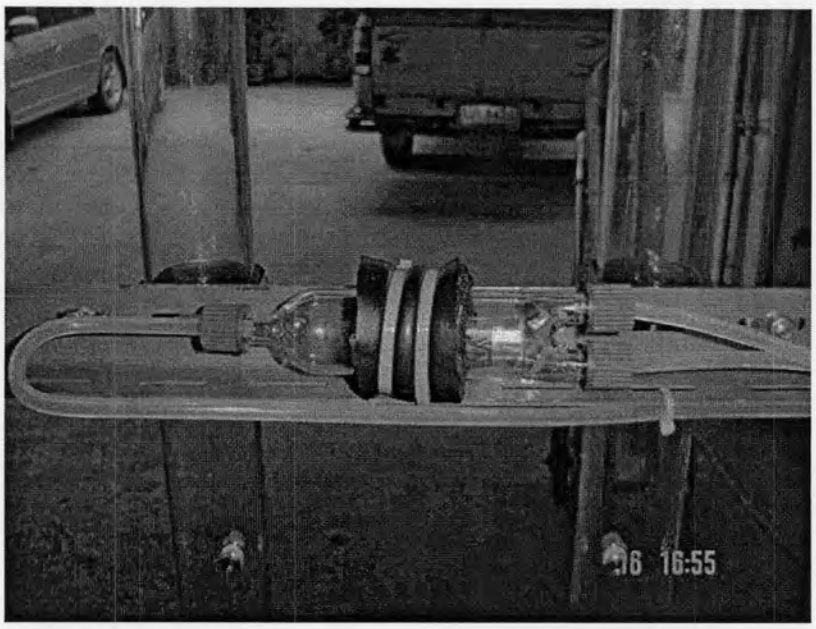

Figure 3-1 Mixing Chamber

3.4.5 Microorganisms Acclimatized System

Xylene vapor was pumped through the biofilter media in the column continuously 8 hrs per day for 1 and 2 months to acclimatize xylene degrading microorganisms. During this time the dominant species of xylene degrading microorganisms were well grown up. The removal efficiency (nearly 100 %) was monitored to ensure that the xylene degrading microorganisms were the dominant species.

3.5 Isolation and Identification

Xylene-degrading microorganisms were isolated from biofilter media after 1or 2 months of acclimatization. The biofilter media was sampling for 10 grams and put into enriched liquid media such as malt extract medium, nutrient, heart brain infusion or peptone broth to enhance the growth of microorganisms .The biofilter materials in liquid media was shaken at 200 rpm for 10 hrs. After that the microorganisms in the liquid media was transfer to the other media to enhance the growth of microorganisms. All microorganism strains from filter media were developed on agar

Nutrient Agar, Malt Extract Agar or Potato Dextrose Agar (PDA). Then all single colonies were isolated at room temperature and stored at 4°C as a stock culture. The stock cultures were cultivated in selective media that were added with different xylene concentrations, and on carbon-free medium at different xylene concentrations.

3.5.1 Isolation and Identification of Bacteria

Liquid media in flakes from the shaker were spread on PCA by plate technique and on Nutrient Agar or Blood Agar by streak plate technique. The microbial colony on Steak plate was transferred to one portion of the agar plate which is then streaked across with an inoculation loop to another portion of the plate in order to separate individual spores and make the single colonies available for isolation. Isolated pure colonies from plate were then checked for bacterial morphology by staining. Identification was done by biochemical test or polymerase chain reaction (PCR) technique and confirmed with DNA sequencing method. The flow diagram of bacteria isolation and identification is shown in Figure 3-2.

3.5.2 Isolation and Identification of Fungus

Liquid media in tubes from the shaker were inoculated to the agar plate in order to isolate the pure colony .The media are potato dextrose agar, malt agar and sabouraud dextose agar supplemented with antibiotic such as gentamicin, streptomicin or penicilin. In general, molds and yeasts were isolated within 3 to 7 days after inoculation. Then slide culture technique was applied to study morphology of fungus. The identification was done by phenotype technique and DNA sequencing. Fungal classification and identification is based entirely on the structures, fruiting body shape, spore features, mating tests or DNA analysis provides a different way of looking at fungi. The flow diagram of fungus isolation and identification is shown in Figure 3-3.

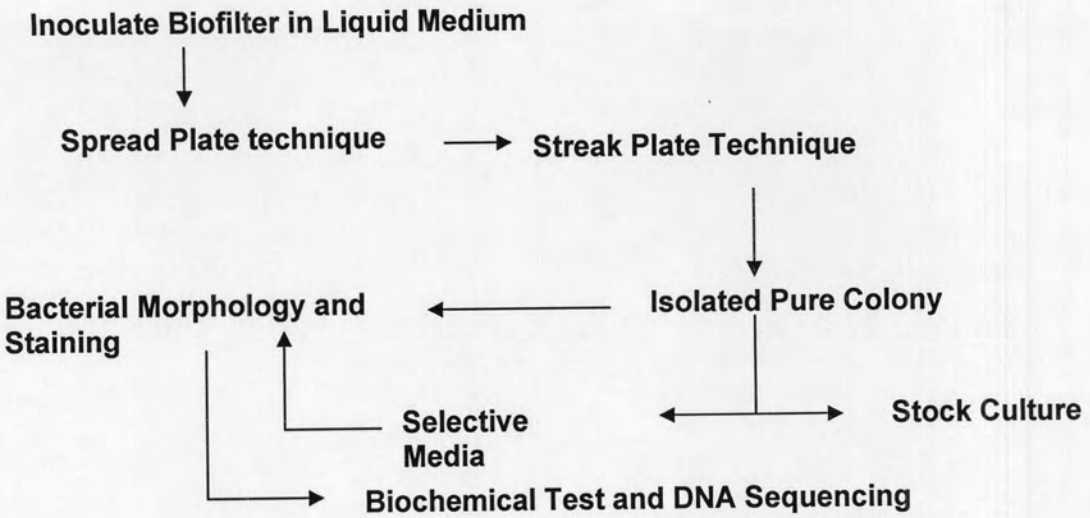

Figure 3-2 Bacterial Isolation and Identification

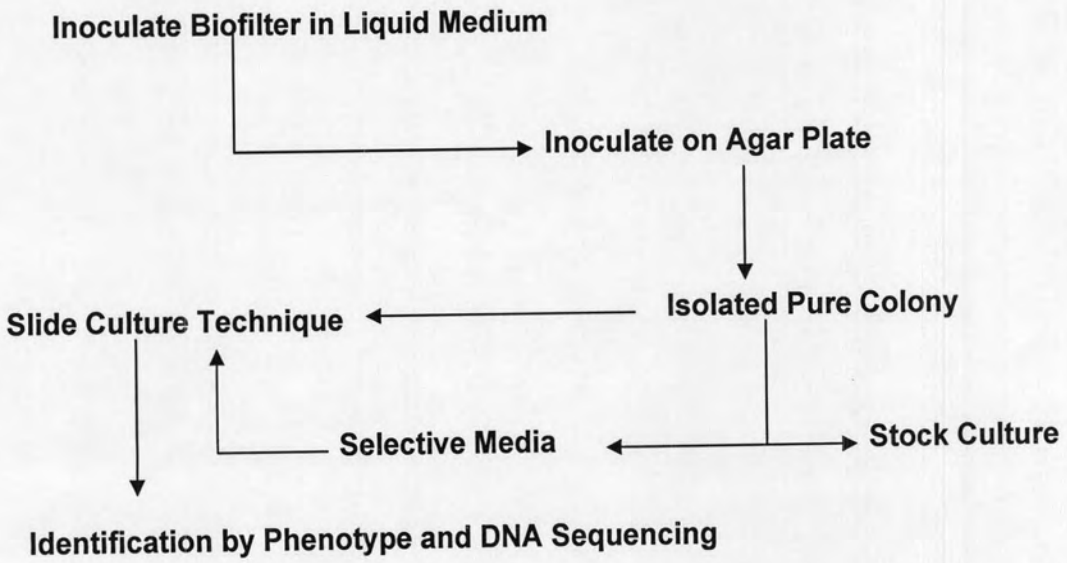

Figure 3-3 Fungal Isolation and Identification

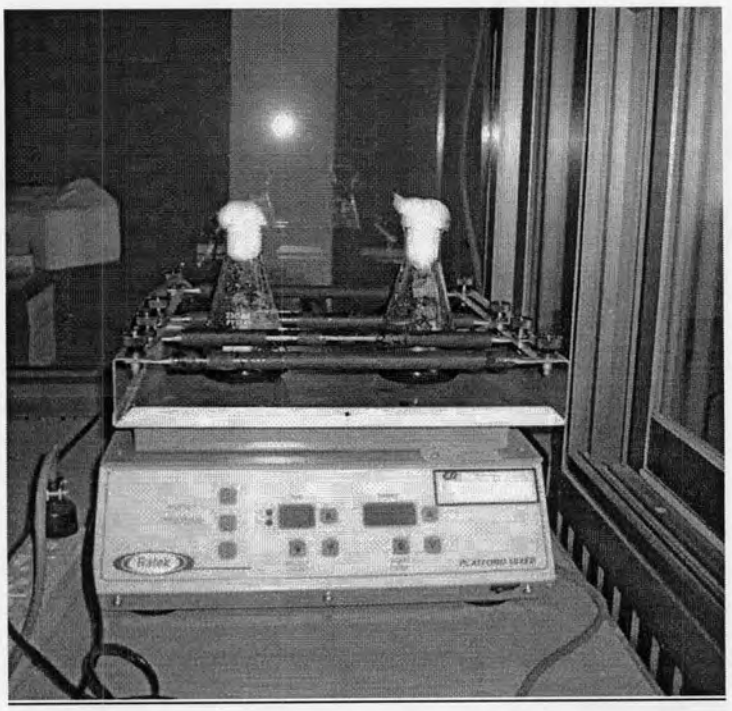

Figure 3-4 Inoculation of Biofilter Media in Liquid Medium

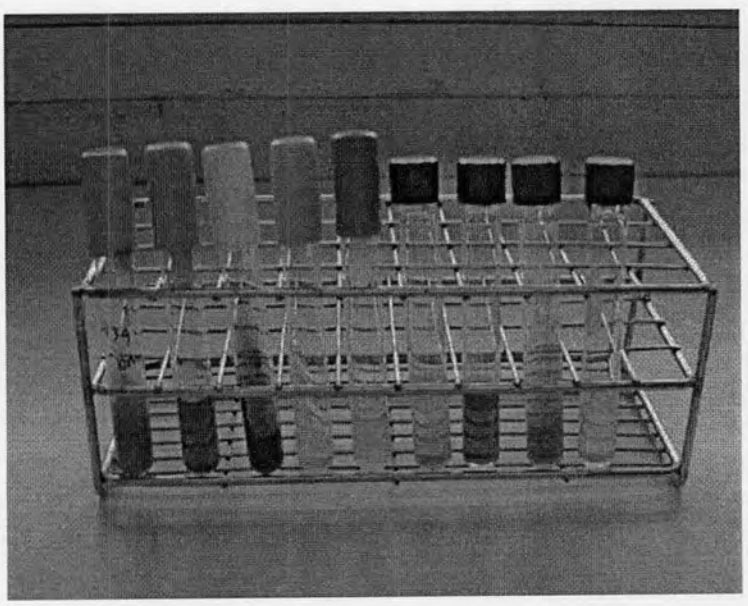

Figure 3-5 Biochemical Test

3.5.3 Slide Cultures Technique for Fungi

The steps of slide culture are as follows. The slide was put in culture plate on the small curve glass tube. Potato dextrose agar was cut as a small piece of 1x

1 cm, and then placed on the slide in culture plate. A flamed inoculating needle was used to obtain a small fragment of the fungus from the primary culture medium. Moisture was provided by sterilize water in culture plate about 5 ml then cover slip was put on top of the potato piece. The incubation was done at room temperature of 28-32 °C for 7 days. The organism would grow onto the underside of each cover slip without interruption. The exact morphologic relationships between conidia and conidiophores were maintained. Both under slide and cover slips were taken out and stained with lacto phenol cotton blue with clean slide and cover slip to get 2 slides for studying of morphology of fungus. Microscope was used to investigate all morphology of fungi.

3.5.4 Gram Staining for Bacteria

A drop of the suspended culture was transferred to be examined on a slide with an inoculation loop or pick colony from Petri dish or a slant culture tube. The culture was spread with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a dime. The culture was air-dried and fixed over a gentle flame, moving the slide in a circular fashion to avoid localized overheating. Adding crystal violet stain over the fixed culture did gram staining. Let stand for 10 to 60 seconds. For thinly prepared slides, it is usually acceptable to pour the stain on and off immediately. Pour off the stain and gently rinse the excess stain with a stream of water from a faucet or a plastic water bottle. Add the iodine solution on the smear, enough to cover the fixed culture. Let stand for 10 to 60 seconds. Pour off the iodine solution and rinse the slide with running water. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. Counterstained with basic fuchsin solution for 40 to 60 seconds and wash off the solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may be shaken to remove most of the water and air-dried (Xu, 1997).

3.5.5 Selected Dominant Species and Degradation Limit Test

All pure colonies from bacteria and fungus isolation were selected for dominant species by culture on Mineral salt agar that use xylene as carbon source at

0.3 ml, 0.5 ml, 1 ml and 2 ml per plate. The observation on microbrial growths was performed in 7-14 days. Then, 3 dominants species were selected for testing on biofiltration performance.

3.6 Biofiltration Experiments

3.6.1 Experiments Set up

The experiment consisted of 4 columns, 3 types of microorganisms were inoculated in 3 columns and one was served as a control column.

3.6.2 Column

Four acrylic columns with 5 cm ID and 200 cm long were used in the experiment. The column was provided with four sampling ports located at 20, 60, 100, and 140 cm from the bottom. However, the gas sampling was done from ports located at 20 and 140 cm only.

3.6.3 Biofilter media

Biofilter media was prepared by mixing coconut husk (size 1.5 × 1.0 cm) and manure compost in the ratio of 75 to 25 (v/v) with moisture content 50 %. The biofilter media was sterilized at 15 psi for 15 min.

3.6.4 Inoculum Preparation

Isolated microorganisms species were maintained on nutrient agar or PDA at 30°C then store at 4°C. The inoculum was prepared in flask containing liquid medium solution (malt extract broth) 50 ml shaking at 200 rpm at 30 C for about 10 hrs. The single culture was inoculated onto the biofilter media inside the column. The schematic of biofiltration is shown in Figure 3-6.

3.6.5 Moisture Content

In the experiment, the initial moisture content in media at the each column was maintained at 50 % of dry weight. In column 2, 3 and 4 that were

inoculated with microbial M1, M2 and M3, moisture content was adjusted with sterilized mineral medium and malt extraction broth from culture step. For control column, sterilized distilled water was used to adjust moisture content. The sterilized technique was necessary for this study to avoid contamination from another strain in each column.

3.6.6 Pollutant Concentration Measurement

A lab-scale biofiltration system was used to treat xylene vapor. Xylene concentrations in the inlet and outlet streams were monitored with a Gas Chromatography (FID detector) HP 6890. Gas Chromatography (thermal conductivity detector, TCD), HP 6890was used to measure carbon dioxide production.

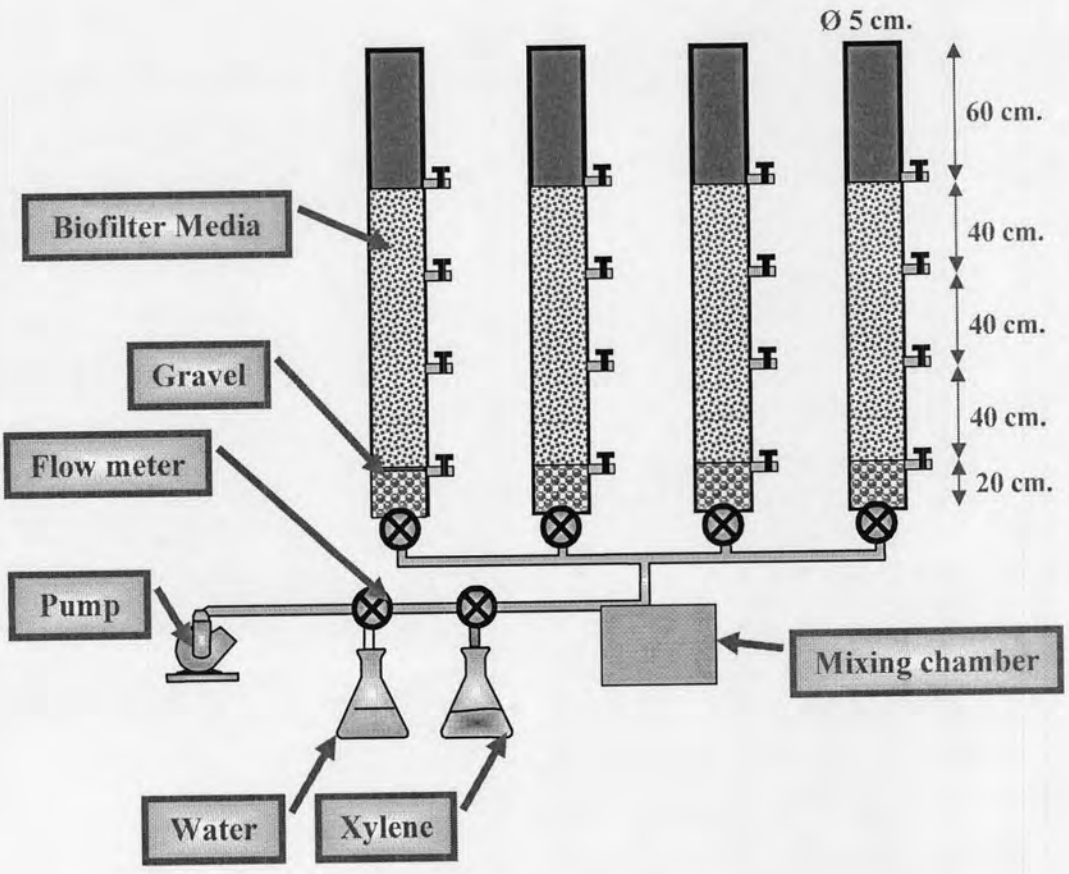

Figure 3-6 Schematic of Four-Columns Biofiltration System

3.6.7 Operating and Performance Parameters

Operating and performance parameters include loading, elimination capacity, and detention time. Mass loading rate takes into account the gas flow rate and contaminant concentration and was defined as the mass of pollutant introduced in a unit volume of biofilter material per unit time. Detention time or empty bed retention time (EBRT) was the time the contaminant spends in the empty volume of column containing the active media. Elimination capacity (EC), a performance indicator, was related to mass loading rate in that it is defined as the fraction of the mass-loading rate biodegraded in the biofilter. Performance of biofilter in treatment of xylene was evaluated in 4-6 months by varying airflow rate and xylene inlet concentrations. Temperature, pressure drop and pH were monitored. Microbial activities were determined by measuring xylene degradation and CO2 production rates in biofilters.

The operating and performance parameters were performed on the level of the xylene inlet load (IL), the corresponding removal efficiency and elimination capacity (EC). The definitions for these parameters are as the following:

$$IL = \frac{QCin}{V} \qquad \textbf{(Equation 3-1)}$$

$$EC = \frac{Q\,(Cin-Cout)}{V} \qquad \textbf{(Equation 3-2)}$$

$$\%\,RE = \frac{Cin-Cout\ x100}{Cin} \qquad \textbf{(Equation 3-3)}$$

$$EBRT = \frac{V}{Q} \qquad \textbf{(Equation 3-4)}$$

$$PCO2 = \frac{Q(CCO_2\ out-CCO_2\ in)}{V} \qquad \textbf{(Equation 3-5)}$$

Where C is Xylene concentration (g m^{-3}), Q the volumetric gas flow rate (m^3 h^{-1}) and V is the volume (m^3) of the packing bed considered; Subscripts "in" and "out" are referred to the inlet and outlet of the packing bed considered. EBRT is the

empty bed retention time and PCO2 is carbon dioxide production rate . EC is an actual removal capacity in the biofilter. It is usually less than load (IL) and sometimes equal to IL when 100% removal efficiency is achieved.

3.6.8 Mass Balance of Xylene

The organic pollutants are aerobically degraded to water and carbon dioxide and used as the essential carbon source for the microbial growth. The ratio of CO_2 production and EC, i.e. the mass of CO_2 produced per mass of xylene removed should be 3.3, in case of complete oxidation of xylene to water and carbon dioxide. The stoichiometric reaction of complete oxidation is as follow:

$$C_8H_{10}+10.5O_2 \rightarrow 8CO_2+5H_2O \qquad \textbf{(Equation 3-6)}$$

In case of biodegradation of organic pollutants, a fraction of consumed organic carbon was used for the microbial growth according to the following metabolism:

Organic pollutant + Oxygen → Carbon dioxide + Water + Cellular material

(Equation 3-7)

3.7 Monitoring on Microorganisms

Type and amount of microorganisms from each column were monitored at the end of the experiment to compare with the start up microorganisms.

3.8 Analytical Methods

3.8.1 Xylene Vapor Measurements

Gas samples for xylene analysis were periodically collected from inlet and outlet of the column by charcoal tubes, CCT (SKC Catalogue number 226–01). Vapor was passed through the tube at a constant flow rate. Each tube was packed with coconut activated carbon and consisted of a front part of 100 mg and a rear part of 50

mg. The samples were analyzed by a Gas Chromatography with a flame-ionization detector (model HP6890). The analysis conditions were oven, inlet and column temperature. Oven initial temperature was 45 °C and maximum temperature was 180 °C with initial time 3 min. Detector temperature was 250 °C and inlet initial temperature was 200 °C . Capillary column (model number: Agilent 19091X-133 HP-Wax Bonded Polyethylene Glycol) temperature was 250 °C.

3.8.2 Carbon dioxide (CO_2)

Gas samples for CO_2 analysis were collected from gas sampling plot with Tedlar bag (1 Liter Supelco 24633). CO_2 concentrations were measured by Gas chromatography (Thermal Conductivity Detector, TCD) (model HP 6890). Oven maximum temperature was 320 °C and maximum temperature of capillary column (model number: Agilent 19091P-MS4 HP-PLOT Molesieve 5A) was 350 °C. Detector temperature was 250 °C.

3.8.3 Intermediate Species

Gas samples for intermediate species of xylene analysis were collected from gas sampling plot with Tedlar bag. Intermediated species of xylene were measured by gas chromatography mass spectrometry (GC/MS/MS: GC; Varian: Star 3400 cx, MS; Varian Saturn2000; Column: Varian; Fact or Four Capillary Column, VF-5ms, 30 MX, 0.25 MM, ID OF=0.25)

3.8.4 Moisture Content Determination

Mixed packing materials were placed in the hot air oven (Conerm Model 240 M Serial no. 96150) at 105 °C for 24 h and put sample in desiccator (Temptag from Thai –Asahi Glass) 24 hrs to evaporate all water. After drying, the moisture content in the packing material was determined by measuring the weight loss. (Carter (editor), 1993)

$$\% \text{ Moisture content} = \frac{\text{Weight of wet –dry packing media}}{\text{Weight of dry packing media}} \times 100 \quad \textbf{(Equation 3-8)}$$

The moisture content in packing materials was adjusted with sterilized water to reach 50 % moisture content before use.

3.8.5 DNA Sequencing Method

DNA sequencing is the process of determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. The sequence of DNA constitutes the heritable genetic information in nuclei, plasmids, mitochondria, and chloroplasts that forms the basis for the developmental programs of all living organisms. Determining the DNA sequence is therefore useful in basic research studying fundamental biological processes, as well as in applied fields such as diagnostic or forensic research. Because DNA is key to all living organisms, knowledge of the DNA sequence may be useful in almost any biological subject area. (Wikipedia , 2007b)

Dominant species of microorganisms in biofilter were confirmed identified by DNA sequencing method. The fungal strains were incubated and allowed to proliferate on Saubouraud dextrose agar plates at 30°C until colonies were recovered. Fungal DNA was extracted using the method of Makimura *et al.* (1994). The internal transcribed spacer (ITS) was amplified using the primer pairs ITS-1 (5' TCC GTA GGT GAA CCT GCG G) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC). Amplification was carried out in packaged PCR tubes (Ready-to-Go PCR Beads; Amersham Bioscience, Piscataway, N.J) in a final volume of 30 µl with 5 µl of DNA extract and 40 pM of primers. Amplification was carried out in a thermal cycler (model 9700; Applied Biosystem, Foster City, Calif.) with an initial 5 min denaturation at 94°C, follow by 35 cycles that consisted of 30 sec at 94 °C, 60 sec at 51°C and 45 sec at 72°C, and a final 10 min extension at 72 °C. The PCR products were electrophoresis on a 1.5 % agarose and were purified with GeneElute ^{TM} PCR Clean-up Kit (Sigma, Saint Louis, Missouri) according to the manufacturer's instructions. The PCR products were sequence using the same primers. All cycle sequencing reactions were performed with an ABI 3130 XL automated sequencer (Hitachi) according to the manufacturer's instructions (PE Applied Biosystems, Foster

City, Calif). Each sequence were aligned and analyzed to ensure a high quality of sequence data. All DNA sequences were submission to Gen Bank.

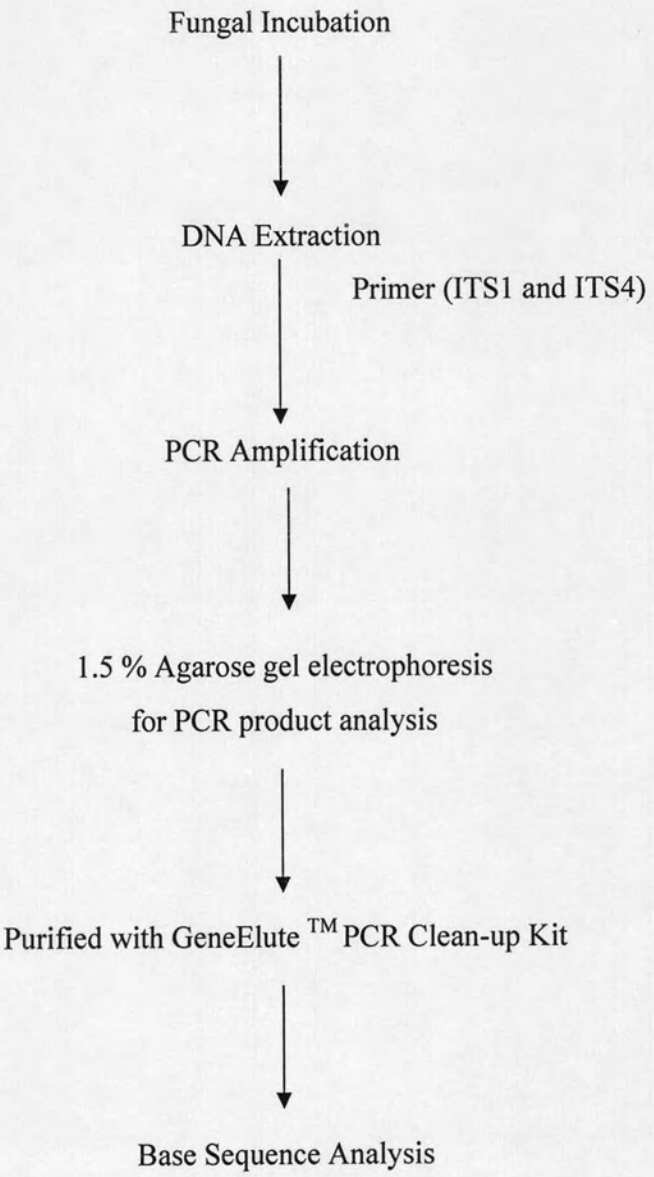

Figure 3-7 The Protocol of DNA Sequencing Method for Fungal Identification