CHAPTER 3

METHODOLOGY

3.1 Apparatus and Chemical Reagents

3.1.1 Gas chromatography flame ionization detector (GC-FID) model GC-430 from Bruker

3.1.2 Capillary column of GC-FID is VF-1ms, 15m×0.25 mm, 0.25 µm i.d.

3.1.3 The experimental conditions for GC-FID: 100 °C injection temperature, a 130 °C column temperature, and a 150 °C detector temperature

3.1.4 High Performance Liquid Chromatography (HPLC)

3.1.5 Column of HPLC is an Inertsil - ODS3 (5 µm, 250×4.6mm).

3.1.6 GC-8000GC-MS system equipped (30 m × 0.32 mm and 0.25 Mm film thickness)

3.1.7 Chamber size 15.6 L

3.1.8 Ethylbenzene volume preparation of ethylbenzene

Ethylbenzene was injected to generate ethylbenzene concentration of 5 ppm in chamber. Analysis degradation of ethylbenzene in *Z. zamiifolia* and efficiency of microorganisms was injected 300 and 520 ppm of ethylbenzene, respectively. Ethylbenzene analytical grade from merck was used to calculate mole concentration by Eq. (3.1), Mc is mole concentration. P (mmHg) and T (ºC) are pressure and temperature, respectively (Treesubsuntorn C., and Thiravetyan P, 2012).

$$\mathrm{Mc} = 24.47 \times \frac{760}{P} \times \frac{\mathrm{T}+273.15}{298.15} \tag{3.1}$$

5 ppm was specified. MW (molecular weight of ethylbenzene) and V (the volume of the chamber (15.6L)) were substituted to calculate W (ethylbenzene weight (g)) by Eq (3.2).

$$5\ ppm = 10^6 \times \frac{W}{Mw} \times \frac{Mc}{V} \tag{3.2}$$

p (ethylbenzene density (g ml^{-1})) and weight of benzene were used to predict Vb (ethylbenzene volume (ml)) by Eq (3.3). Ethylbenzene volume at 5ppm in this chamber is ~3.8 µg

$$p = \frac{W}{Vb} \tag{3.3}$$

3.1.9 CH_3CN, HPLC grade

3.1.10 bis (trimethylsilyl)-rifluoroacetamide (BSTFA) with 1% Trimethyl chlorosilane (TMCS)

3.1.11 Chloroform

3.1.12 5% of $NaClO_2$ solution

3.1.13 Nutrient broth (NB)

3.1.14 Deionization and distilled water

3.2 Appropriate Plant Screening For Ethylbenzene Removal

Three species of plants, *Z. zamiifolia*, *Sansevieria trifasciata* hort. ex Prain cv. Hahnii Green and *Sansevieria kirkii* var. pulchra Coppertone were purchased from ornamental plant shops in Thailand and used in this study. Leaf area of experimental plant was measured by graph paper and controlled by judicious pruning (130 – 160 cm^2). Plant roots were covered by aluminum foil containing humid coconut coir pith (200g) and placed in a closed 15.6 L chamber that has two pipes for inject (5 ppm of ethylbenzene) and without ethylbenzene after that harvest ethylbenzene. Within 24 hour, air in chamber was analysis by GC. Peak area from chromatogram was use to calculated to ethylbenzene concentration and analyzed to ethylbenzene removal.

3.3 Ethylbenzene Uptake by Each Part of *Z. zamiifolia*

Six sample of 130 cm^2 of *Z. zamiifolia* leaf was sterile by 5% of Clorox ($NaClO_2$) solution for 5 min and washed three times of sterile deionize (DI) water. Three *Z. zamiifolia* was remove leaf to single leaf and use aluminum foil overlay leaf. All plant roots were covered by aluminum foil containing humid coconut coir pith (200g) and placed in a closed 15.6 L chamber. 5 ppm ethylbenzene was injected in six chamber and chamber without plant for control. Ethylbenzene in chamber was analysis by GC within 12 and 24 hours.

3.4 Chemical Analysis in *Z. zamiifolia* by HPLC and GC-MS During Ethylbenzene Removal

Z. zamiifolia was sterile removed soil and coconut meal at roots with tuber by 5% of Clorox ($NaClO_2$) solution for 5 min and washed three times of sterile deionize (DI) water. Place in sterile chamber and wait 1 hour for remove humid by silica. After that 300 ppm of ethylbenzene was injected in treatment chamber and control chamber without plant except control chamber present plant. Air in chamber was analysis by GC within 1, 4 and 5 days. Plant was clean by water and roots with tuber, stem and leaf were separated and ground until fine. After that plant parts were extracted by chloroform (1:1 g fresh weight / ml) for analyzed ethylbenzene, 1-phenylethanol and acetophenone. Supernatant was filtered through membrane syringe filters (13mm, 0.2 µm) to remove particulate matters. For HPLC analysis, supernatant was diluted tenfold and 1ml of supernatant was put in vial. 1.5 ml of supernatant was put in vial for GC-MS analysis.

Plant solution was analyzed by using an Inertsil - ODS3 (5 µm, 250×4.6mm). The mobile phase consisted of CH_3CN, HPLC grade and DI for HPLC (ratio 65/35) with a flow rate of 0.8 mL/min. Separation was done at room temperature and detection was carried out at 240nm for acetophenone and 220nm for ethylbenzene, 1-phenylethanol.

For GC-MS analysis, the sample were added in 200µl bis (trimethylsilyl)-rifluoroacetamide (BSTFA) with 1% Trimethyl chlorosilane (TMCS) and derivatized at 60 ºC for 2 hrs. 0.5 mL of plant solution was injected with a split mold 1:100 and then analyzed by GC-MS. The temperature programming was 80 ºC as the initial temperature, then 7 ºC/min to 150 ºC and 10 ºC/min to 250 ºC holding for 20 min. the

electron impact techniques (70eV) were used. Mass detector was scanned from 30-50 m/z.

3.5 Microbial Screening for Enhancing Ethylbenzene Removal

3.5.1 Epiphytic Bacteria

Z. zamiffolia leaf were harvested and put in bottom that contains nutrient broth (NB) media and incubate in shaker at 37^{o}C, 150 rpm at temperature room, 1day. After that streak on nutrient agar (NA) media and incubate at temperature room, 3 days and purified colony. Pure single colony was analyzed for the species by PCR Technique and used for treatment.

3.5.2 Soil Microorganisms

10 mg of soil from ethylbenzene contaminate put in flask that contain 90ml of 10% NaCl and put in shaker at 37^{o}C, 150 rpm. Dilute solution at ten-fold and streak on NA media with ethylbenzene (0.5 g/L) were added to the medium as the sole carbon source. Incubate at room temperature, 3 days and purified colony. Pure single colony was analyzed for the species and used for treatment.

3.5.3 Other Microorganisms from Review Paper

P. putida TISTR1522 purchased from MIRCEN, Thailand, and *P. aeroginosa* isolated from pesticide-contaminated soil by A. S. Vangnai from Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

All bacteria were spray on *Z. zamiifolia* leaf to study the efficiency of ethylbenzene removal in air by gas chromatography.

3.6 Efficiency of Microorganisms for Enhancing Ethylbenzene Removal

Sterile *Z. zamiifolia* by soaking leaves and stem into 5% of Clorox ($NaClO_2$) solution for 5 min and washed three times of sterile deionize (DI) water was used as control conditions. Microorganisms were incubated in NB media and shaken at 150 rpm and 37^{o}C. Optical density (OD) was measured by spectrophotometer. For treatment conditions, sterile plant was spray 1 ml of inoculum microorganism on the leaves. Microorganisms sprayed on the leaf before and after treatment were harvested in 10% NaCl. After that, microorganisms were poured in a plate with nutrient agar and incubated at 28˚C for 2 days.

After plants preparation, each plant is placed in each chamber. Four glass fumigators chamber (three replicates and one control) are closed and seal by paraffin tape at room temperature (~32^{o}C) and pressure (~760 mmHg). Ethylbenzene were injected to generate the concentration of 5 ppm inside the chamber and stirred for ethylbenzene mixing. In this treatment, plants are fumigated and sampling time is set 12, 24, 36, 48

and 60 hours. 5 ppm ethylbenzene were injected to the chamber and stirred for ethylbenzene mixing, 3 cycles.

Six chamber of sterile plant was spray *P.aroginosa* in first cycle closed and seal by paraffin tape. Condition same before experiment when plant with microorganisms remove ethylbenzene completely, open chamber, plant in three chamber was spray with NB and three chamber was spray with *P.aroginosa*, closed chamber and seal by paraffin tape. Inject 5ppm of ethylbenzene in each chamber. For third cycle do like second cycle. In this treatment has 3 control are sterile plant, sterile plant with NB and sterile plant with *P.aroginosa* which inject 5 ppm of ethylbenzene any cycle.

P.aroginosa and *B. cereus* ZQN5 were culturing 12 hours at 30ºC, 150 rpm in NB medium. OD (0.8) was measured by spectrophotometer and added in 5 ml of NB medium in bottom. Clover bottom by parafilms after that inject 520ppm of ethylbenzene into bottom. Incubation was done in 150 rpm of shaker at 30ºC and measure air bone by GC include harvest cell microorganisms to plant count for prepare grow rate at 12 and 24 hours.

3.7 Fumigation Experiment and GC Analysis

Gas chromatography flame ionization detector (GC-FID) model GC-430 from Bruker. Capillary column: VF-1ms, 15m×0.25 mm, 0.25 μm i.d. The experimental conditions for GC-FID: 200 °C injection temperature and 77.0 ml/min injection flow rate, a 130 °C column temperature, a 50 ˚C oven temperature and a 200 °C detector temperature

0.3μl of air in chamber treatment was sampled by syringe and injected in GC-FID. Three replications per treatment were measured. The amount of ethylbenzene uptake by plant and microorganisms can be calculated by the difference between $ppm_{control}$ (the remaining ethylbenzene concentration in control) and $ppm_{treatment}$ (the remaining ethylbenzene concentration in treatment systems) (equation 3.4).

$$\Delta ppm = ppm_{control} - ppm_{treatment} \quad (3.4)$$

The weight (W) of ethylbenzene uptake by plant (g) was calculated by equation 3.5. V is the volume of the system. M_c is mole concentration of ethylbenzene. MW is molecular weight of ethylbenzene.

$$W = \Delta ppm \times V/M_c \times M_w/10^6 \quad (3.5)$$

Ethylbenzene removal per leaf area (nm mol/m^2) was calculated by equation 3.6. A is leave area.

$$\text{Ethylbenzene removal per leaf area} = W \times 10^6/A \quad (3.6)$$

3.8 Analytical and statistic method

Data were analyzed by one way analysis of variance (ANOVA) using statistical program for social sciences (SPSS) version 16.0. Data are listed as average SD for three replications. One Way ANOVA and Duncan's multiple range tests with 95% confident level was used to classify the group of data. Values in the same column with the superscripted same letter are not significantly different (a = 0.05).