CHAPTER 3

METHODOLOGY

3.1 Apparatus

3.1.1 2 and 4 decimal analytical balance
3.1.2 Hot air oven
3.1.3 Glass desiccator
3.1.4 Gas chromatography with flame ionization detector, GC-FID (Bruker, 400GC-series)
3.1.5 Hot plate
3.1.6 Volumetric flask
3.1.7 Test tube
3.1.8 Aluminium foil and plate
3.1.9 Paraffin tape
3.1.10 Gas tight syringe
3.3.11 Light microscope
3.1.12 Chlorophyll fluorometer, kings lynn, England
3.1.13 Watman filters paper No. 2
3.1.14 GC-MS, DB5 capillary column
3.1.15 FT-IR
3.1.16 Vacuum pump model VE115N with 2.0 CFM free air displacement
3.1.17 Retsch ultra centrifugal mill

3.2 Material and chemical reagent

3.2.1 99.8% Benzene, panreac, E.U.
3.2.2 Hexane
3.2.3 Ethyl alcohol
3.2.4 BSTFA with 1% TMCS

3.3 Plant preparation

Ornamental plants such as *Chamaedorea seifrizii, Scindapsus aureus, Sansevieria trifasciata, Philodendron domesticum, Ixoraebarbata craib, Monster acuminate, Epipremnum aureum,* and *Dracaena sanderiana* were purchased from plant shops in Thailand. Similar leaf area appearance in each species was chosen. The roots had been covered by wet tissue paper in every species, and then the roots had been wrapped again by aluminum foil. These plants after preparation had been used to study benzene absorption in static system.

3.4 Plant leaves material preparation

Twenty-one plants such as *Homalomena rubescens, Citrus hystrix, Musa paradisiaca, Mangifera indica, Catura metet, Lagerstroemia inermis, Cananga odorata, Cassia siamea, Bougain villea, Litchi chinensis, Coccinia grandis, Dieffenbachia picta, Attacus atlas, Polyalthia longifolia, Acrostichum aureum, Ficus religiosa, Alstonia scholaris, Anthurium andraeanum, Plerocarpus Indicus, Lagerstroemia macrocarpa,* and

Dracaena sanderiana were purchased from plant shops in Thailand. Leaf of each plant was cut and dried under 60 ^{o}C for 2 days. Dry leaf of plants had been powdered by Retsch ultra centrifugal mill with 14,000 cycles min^{-1} and dried again under 60^{o}C for 2 days in oven. Leave materials had been collected in dry bottle to control the humidity. These materials had been used in 3 kinds of experiment such as static adsorption, dynamic adsorption and biofilter. In static system, 0.2 g of each plant leave material was put in fumigator for passive benzene uptake. In dymamic system, selected plant leave materials were immobilized on glass bead by cassava powder glue. Plant leave materials-immobilized glass bead were oven under 60 ^{o}C for 2 days. In biofilter, *A. aureum* and *A. scholaris* leave materials immobilized glass bead were applied as a media for *P. putida*. The UV was applied for sterilization of *A. aureum* and *A. scholaris* leave materials immobilized glass bead.

3.5 Crude wax extraction and crude wax quantity analysis

One g of plant leave and leave materials had been immersed in 50 mL of hexane at 4 ^{o}C over night for extraction of hydrophobic wax. Extracted solution was filtrated by Whatman number 2-filtration paper with 8 µm of pore size. After filtration method, the wax extracted solution was put in ceramic cup, and nitrogen was applied to flow though the sample for hexane evaporation on ceramic cup. Weight of crude wax was calculated by the different of ceramic cup weight before and after wax immobilization. In this experiment, 4 decimals balance had been used.

3.6 Microorganism preparation

Freeze dry *Pseudomonas putida* TISTR1522 was purchased from Bangkok Microbiological Resources Centre (MIRCEN), is the main service collection in Thailand. 25 mL NB nutrient was used to grown this stain, and room temperature was applied for *P. putida* TISTR1522 incubration overnight by 150 cycles min^{-1}. This microorganism had been inoculated in biofilter.

3.7 Nutrient for microorganism culture

Lysogeny Broth (LB) was used as enriched medium, and minimum medium was prepared from 20 mg $CoCl_2$•$6H_2O$, 30 mg H_3BO_3, 10 mg $ZnSO_4$•$7H_2O$, 1 mg $CuCl_2$•$2H_2O$, 2 mg $NiCl_2$•$6H_2O$, 3 mg $NaMoO_4$•$2H_2O$, 10 mg $FeSO_4$•$7H_2O$, 2.6 mg $MnSO_4$•H_2O, and 1000mL ultrapure water (Kragelund and Nybroe, 1994). Before nutrient injection, 121 ^{o}C and 20 minute with pressure of 15-pound inch^{-2} was applied for autoclave-sterilized nutrient.

3.8 Fumigatory chamber preparation

Glass desiccators, which contain 15.6 L of volume, were used to fumigate plants and it materials with benzene. The static system was closed and sealed by paraffin tape. Three aluminum plates were also put in the chamber to help benzene homogenous. The special lid of the chamber that had been specifically designed contained 2 rubber septums for injection and sampling. The desiccators had been applied as a benzene exposure system in every static experiment. Living plants and plant leave materials were exposure with benzene in this chamber.

3.9 Benzene removal in a static system by living plants

Eight ornamental plants, which contain leaf area more than 130 cm^2, were placed in designed desiccators under normal indoor conditions (3 repetition chambers in each treatment). Desiccators were closed and sealed by paraffin tape at temperature (~32 ^oC) and pressure (~760 mmHg). These temperature and pressure were used to calculate mole concentration (M_c) by Eq 3.1.

$$Mc = 24.47 \times \frac{760}{P} \times \frac{T + 273.15}{298.15} \quad \text{(Eq 3.1)}$$

$$20\ ppm = 10^6 \times \frac{W}{M_w} \times \frac{M_c}{V} \quad \text{(Eq 3.2)}$$

$$\rho = \frac{W}{V_b} \quad \text{(Eq 3.3)}$$

M_c is mole concentration. P (mmHg) and T (^oC) are pressure and temperature, respectively. 20 ppm was specified. M_W (molecular weight of benzene) and V (the volume of the chamber (L)) were substituted to calculate W (benzene weight (g)) by Eq 3.2. ρ (benzene density (g/ml)) and weight of benzene were used to predict V_b (benzene volume (mL)) by Eq 3.3. 99.8% benzene, which was purchased from Panreac (made in E.U.), should be injected at 1±0.1µL for 18±2 ppm of benzene concentration. From the system optimization, four hours were required for benzene equilibrium in this system, so the samples in every experiment were collected after 4 h. Remaining benzene had been collected in 4, 6, 8, 24, 48, and 72 h and analyze by gas chromatography with flame ionization detector, GC-FID (Bruker, 400 GC-series)

3.10 Benzene removal by living plants under dark and light conditions

Same size of *D. sanderiana* had been grown and exposure with gaseous benzene under light and dark conditions. In light condition, *D. sanderiana* was grown under 24 h normal lamplight. In dark condition, black paper was used to cover the benzene exposure chamber, creating 24 h dark conditions. Liquid benzene was injected in to the chamber follow on the protocol of benzene removal in static system by plant. Benzene uptake in both conditions had been analyzed and compared in a long-time exposure (4 cycles) to make sure that this technology could be sustainable benzene removal method. Seven days were applied in each cycle, and 20 ppm of benzene was started in each cycle. 0.3 mL of gaseous benzene in the chamber was collected by gas tight syringe and measured by gas chromatography with flame ionization detector, GC-FID (Bruker, 400GC-series). Three replication and 3 samples in each treatment were required. Remaining benzene concentration had been observed in day 1, 2, 3, 5, 7 of each cycle.

3.11 Benzene removal by wax of plant

A 130 cm^2 sized of leaves of *D. sanderiana* were cut and collected, and crude wax was extracted by hexane. The leaves were immersed in 200 mL hexane overnight at 4^oC of temperature. Crude wax solution was transferred in to a 130 cm^2 aluminum plate that was the similar size of leaves of *D. sanderiana*, and hexane was evaporated by the flowing of nitrogen. The aluminum plate with crude wax was used to study the benzene uptake by wax. Crude wax immobilized on aluminum plate was fumigated with 20 ppm

of benzene in a chamber that follow on the method in benzene removal by plant, and 0.3 ml of gas was collected in each chamber at temperature (~32 ^{o}C) and pressure (~760mmHg). There are 3 replications and 3 samples in each treatment. The samples were collected at 24, 48, 72, 120, and 168 h. The result had been used to calculate the benzene adsorption by crude wax and investigate benzene uptake pathways.

3.12 Stomata observation

Replica techniques, nail varnish could be applied to copy the pattern of stomata on the leaves. Nail varnish was painted on the stomata side on leaf surface, and dry nail varnish after pattern copying was carefully keep out. A light microscope with 100 x was applied to observe the structure of stomata and count number of stomata that occurred on dry nail varnish. The number of stomata had been used to find the relation with benzene removal efficiency, and structure of stomata had been observed from growing *D. sanderiana* under light and dark conditions.

3.13 Benzene removal in a static system by plant leaf materials

Plant leaf materials, each containing about 0.2 g, were placed in modified desiccators with injection and sampling pot under indoor conditions (3 repetition chambers in each treatment). Desiccators were closed and sealed by parafilm at temperature (~32 ^{o}C) and pressure (~760 mmHg). In this experiment, the equation from benzene removal by plant under static system had been applied. 20 ppm of benzene was used to be initial benzene concentration, and the remaining benzene concentration had been measured in day 1, 2, 3 because from preliminary study, benzene uptake by cuticle had been saturated normally in 3 days. The experiment had been done in 21 materials from plant leaves. The result was analyzed to select the high benzene adsorption capacity materials.

3.14 Benzene adsorption by a dynamic system

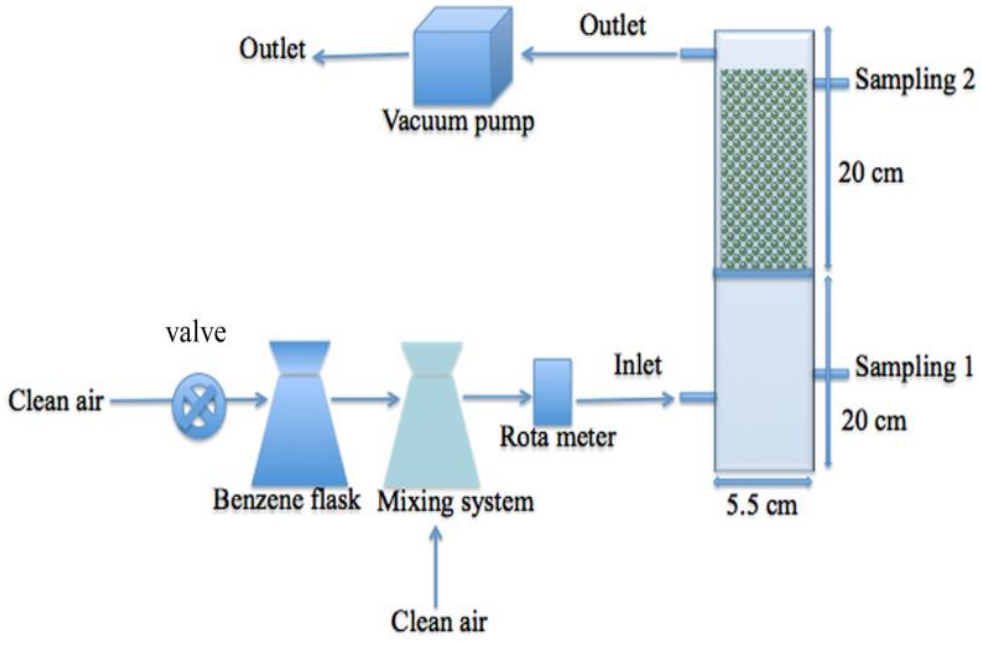

Figure 3.1 Benzene adsorption system.

Vacuum pump model VE115N with 2.0 CFM free air displacement was used to flow clean air. Clean air was flow though controlled valve and was pumped though benzene flask that contain 50 mL benzene. Contaminated air was diluted with clean air in mixing flask, and Rotameter was applied for benzene contaminated airflow rate regulation. Initial waste gas of benzene (~55 ppm) was flowed into glass column, which have 40 cm height and 5.5 cm diameter. Above column bottom 20 cm, glass pore disc was used to create layer of adsorbent. Contaminated gas was flowed though absorbent layer, and out from the system. Before waste gas was flow out of the system, activated carbon was used to treat completely waste gas. Sampling pot 1 and 2 on the column was used for sample collection (Fig 3.1). The continuous study was followed by condition in Table 3.1.

Table 3.1 Continuous system conditions in each selected plant leaf materials.

	D.sanderiana	***D.picta***	***F.religioza***	***L.macrocarpa***	***A.scholaris***	***A.aureum***
Weight of leaf (g)	15	15	15	15	15	15
Flow rate (L min^{-1})	0.03-0.05					
Filter volume (mL)	93.75	103.5	108.75	120	120	144
Filter Depth (cm)	15.79	18.95	15.79	12.63	12.63	15.16
EBRT (min)	3.125	3.45	3.625	4	4	4.8
Initial benzene concentration (ppm)	55					

Sampling pot 1 was collected to measure benzene concentration of inlet, and sampling pot 2 was used to analyze outlet waste gas. Plant leaf materials-immobilized glass bead was used to be the treatment. Starch glue-immobilized glass bead and only glass bead were used as control.

3.15 Benzene adsorption mechanism

Hexane was applied to desorb benzene from absorbent after capacity saturation of adsorbent was occurred. 1 g of every plant leaf materials was immerged in 50 mL hexane. Benzene desorption from plant leaf materials had been analyzed by GC-FID (Bruker, 400GC-series) with 250 ^{o}C Injector, 200 ^{o}C Detector and 100 ^{o}C Oven and split mode 1:10, and percentage benzene desorption were calculated. In surface functional group observation both before and after benzene adsorption, the samples of every plant leaf materials were analyzed by Fourier transform infrared spectrometer (FT-IR), and Perkin-Elmer spectrum one. The KBr disc technique was used to carry spectra of absorbents. In BET porosity analysis, Surface porosity of each material was measured by surface area and porosity analyzer model Autosorb-1 (Quantachrome).

3.16 Biofilter with packing bead from plant leaf material

3.16.1 Suitable loading rate evaluation

Highest benzene removal efficiency from adsorption experiment such as *Acrostichum aureum* and *Alstonia scholaris* leaf materials immobilized glass bead were selected in bio filter study. 25 mL of 23 × 10^{7} cfu g^{-1} was inoculated in UV-sterilized column that contain sterilized applied media, and 0.45 micron filtration paper by Whatman was used to protect microorganism loss from the system.

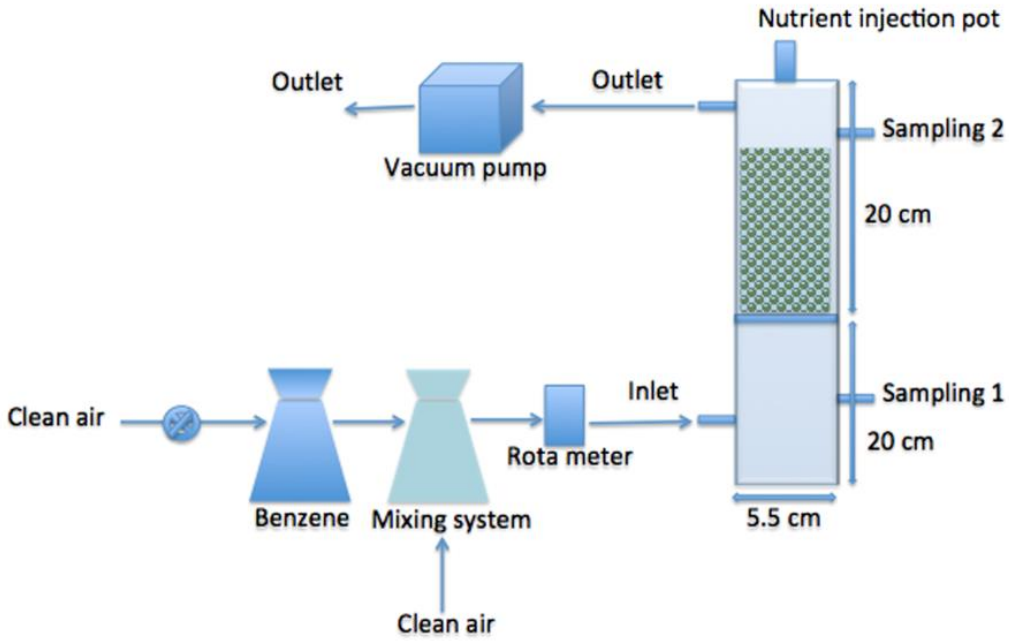

Figure 3.2 Fed batch biofilter system in this experiment.

Clear air was flowed by vacuum pump model VE115N with 2.0 CFM free air displacement. Clean air was flow though controlled valve and was pumped through 50 mL benzene flask. Contaminated air was diluted with clean air in mixing flask, and flow meter was applied for benzene contaminated airflow rate regulator. The experiment was separated to be 4 phases, about 5 days per each phase. Flow rate in phase 1, 2, 3, 4 was controlled as 0.03, 0.1, 0.2, and 0.3 L min^{-1}, respectively. Initial waste gas of benzene (55 ppm) was flowed into glass column, which have 40 cm height and 5.5 cm diameter. Above column bottom 20 cm, glass pore disc was used to create layer of adsorbent. Contaminated gas was flowed though bio filter part, and out from the system. Finally, activated carbon was applied to treat completely waste gas. Sampling pot 1 and 2 on the column was used for sample collection (Fig 3.2). 5 mL nutrient such as enrich medium was feed into the system every day. Waste gas concentration was analyzed and calculated benzene removal efficiency. The bio filter was followed by condition in Table 3.2. Glass bead with *P. putida* and starch glue immobilized glass bead with *P. putida* were used a control. *A. aureum* and *A. scholaris* leaf materials immobilized glass bead with *P. putida* was a treatment.

Table 3.2 Fed batch biofilter conditions in each selected plant leaf materials.

		A.scholaris	***A.aureum***
Weight of leaf (g)		15	15
Flow rate (L/min)	**Phase 1**	0.03-0.05	
	Phase 2	0.1	
	Phase 3	0.2	
	Phase 4	0.3	
Filter volume (mL)		120	144
Initial benzene concentration (ppm)		55	

Percentage removal efficiency of benzene in the system was calculated by Eq 3.4.

$$\%\ Removal\ efficiency = \frac{(C_i - C_o)\times 100}{C_i} \quad \text{(Eq 3.4)}$$

C_i and C_0 are the benzene concentration in inlet of treatment and outlet of treatment, respectively. Elimination capacity (g m^{-3} h^{-1}) and benzene loading rate (g m^{-3} h^{-1}) were calculated follow Eq 3.5 and 3.6, and the relationship was analyzed. Q and V_b in the equation were airflow rate and volume of bed, respectively.

$$Loading\ rate = \frac{QC_i}{V_b} \quad \text{(Eq 3.5)}$$

$$Elimination\ capacity = \frac{Q(C_i - C_o)}{V_b} \quad \text{(Eq 3.6)}$$

The relation of loading rate and elimination capacity was analyzed, and suitable loading rate that the system could still removal completely benzene had been reported.

3.16.2 Evaluation of suitable nutrients

The similar experiment set up with loading rate evaluation was done. Suitable loading rate from loading rate evaluation experiment was applied in this study, and the experiment was separated as 2 phases such as phase 1 and 2 that contain 0.03 and 0.1 L min^{-1}, respectively. 5 mL nutrient such as enrich medium, minimum medium and sterilized water were feed into the system every day. The biofilter was followed by condition in Table 3.3.

Table 3.3 Fed batch biofilter conditions in each selected plant leaf materials.

		A. scholaris	***A. aureum***
Weight of leaf (g)		15	15
Flow rate (L min^{-1})	**Phase 1**	0.03-0.05	
	Phase 2	0.1	
Filter volume (mL)		120	144
Initial benzene concentration (ppm)		55	

Percentage removal efficiency of benzene in the system was calculated by Eq 3.7, respectively.

$$\%\ Removal\ efficiency = \frac{(C_i - C_o)\times 100}{C_i} \quad \text{(Eq 3.7)}$$

C_i and C_0 are the benzene concentration in inlet of treatment and outlet of treatment, respectively. Elimination capacity (g m^{-3} h^{-1}) and benzene loading rate (g m^{-3} h^{-1}) were calculated follow Eq 3.8 and 3.9, and the relationship was analyzed. Q and V_b in the equation were airflow rate and volume of bed, respectively.

$$Loading\ rate = \frac{QC_i}{V_b} \quad \text{(Eq 3.8)}$$

$$Elimination\ capacity = \frac{Q(C_i - C_o)}{V_b} \quad \text{(Eq 3.9)}$$

Highest benzene removal condition had been reported following the efficiency of benzene removal and value of loading rate.

3.17 Wax composition analysis

Wax of selected plant leaf materials from *D. picta, A. aureum, C. siamea, A. scholaris, D. sanderiana, L. Macrocarpa, P. longifolia, M. paradisiaca,* were extracted by hexane chilling of plant material. Ten mg pure cuticular wax was immerged in 10 mL hexane for cuticular wax dilution. BSTFA and 1% TMCS were used for cuticular wax solution derivatization (Beck and Lynn, 1997). 0.5µL of sample was applied as injection volume. GC-MS was used to analyze the composition of wax by 30 m DB5 capillary column temperature programmed at 80 ^{o}C to 250 ^{o}C and split mode 1:100. Electron impact mode (70eV) and 30 to 550 atomic mass units scanning were condition to mass analysis.

Composition of wax was classified in 2 groups such as fatty acid (8-20 carbon atoms) and alkane. In each group, percentage of each wax composition was calculated, and the relation of percentage of each wax composition and benzene removal by 21 leaves material had been investigated. Correlation coefficient of each kind of wax composition and benzene removal was also reported.

3.18 Gas analysis

Every day, samples of 0.3 mL waste gas were collected from chamber in chamber. GC (gas chromatography, 400GC-series bruker) was used to analyze benzene concentration. Benzene was measured by 105-meter length and 0.53 mm ID column. Diphenyl/Dimethyl polysiloxane phase were filled as a stationary phase. N_2 was used as a carrier gas. FID cylindrical electrode detector with detection limits of 3×10^{-12} g s^{-1} and dynamic range of 10^7 were used. The benzene uptake by biomaterial from plant was calculated follow the equations:

$$\Delta ppm = ppm_{control} - ppm_{treatment} \quad \text{(Eq 3.10)}$$

$$W = \Delta ppm \times \frac{V}{M_c} \times \frac{M_w}{10^6} \quad \text{(Eq 3.11)}$$

$ppm_{control}$ and $ppm_{treatment}$ are the remaining benzene concentration in control and treatment systems, respectively. The differences between $ppm_{control}$ and $ppm_{treatment}$ were used as benzene uptake by biomaterial from plants (Δppm), and benzene uptake by plants was calculated to the weight (W) of benzene uptake by biomaterial from plants (g) following Eqs 3.10-3.11. V (L) is the volume of the system. M_c is mole concentration of benzene. M_W is molecular weight of benzene. Weight of benzene uptake per weight of plant leaf material was reported.

3.19 Statistical analysis

Descriptive statistic that was used usually in this experiment was mean and standard deviation. One Way ANOVA with 95% confident level was applied to compare mean of each treatment, and Duncan's multiple range tests are used to classify significantly the group of all data in this experiment. Correlation coefficient was used to analyse all relationship. Statistic package for social science (SPSS) version 19 was the program to analyse the data in this study.