CHAPTER 3
RESEARCH METHODOLOGY

3.1 Methodology Outline

The research has focused on screening, isolation and characterization of fungi capable to degrade benzo(a)pyrene. Since benzo(a)pyrene was produced from incomplete combustion of the fossil fuel, burning smoke and/or the incomplete combustion of vehicle exhaust. We hypothesize that the fungi isolated from various parts of plant(s) exposed to the traffic smoke would have the potential to degrade benzo(a) pyrene. Therefore, leaves and barks of *Pterocarpus macrocarpus* Kurz plants located along the Phayathai road, one of the most heavy traffic roads in Bangkok were collected. Then, the isolation of fungi from their leaves and bark, and the screening for their potential BaP degradation were performed. The selected fungi were identified and determined for their biodegradation kinetics. The biotransformation conditions were studied. Finally the biotransformation intermediates were identified and the biotransformation pathway of BaP of the isolate was proposed. The overall experimental outline was summarized in the Figure 3.1.

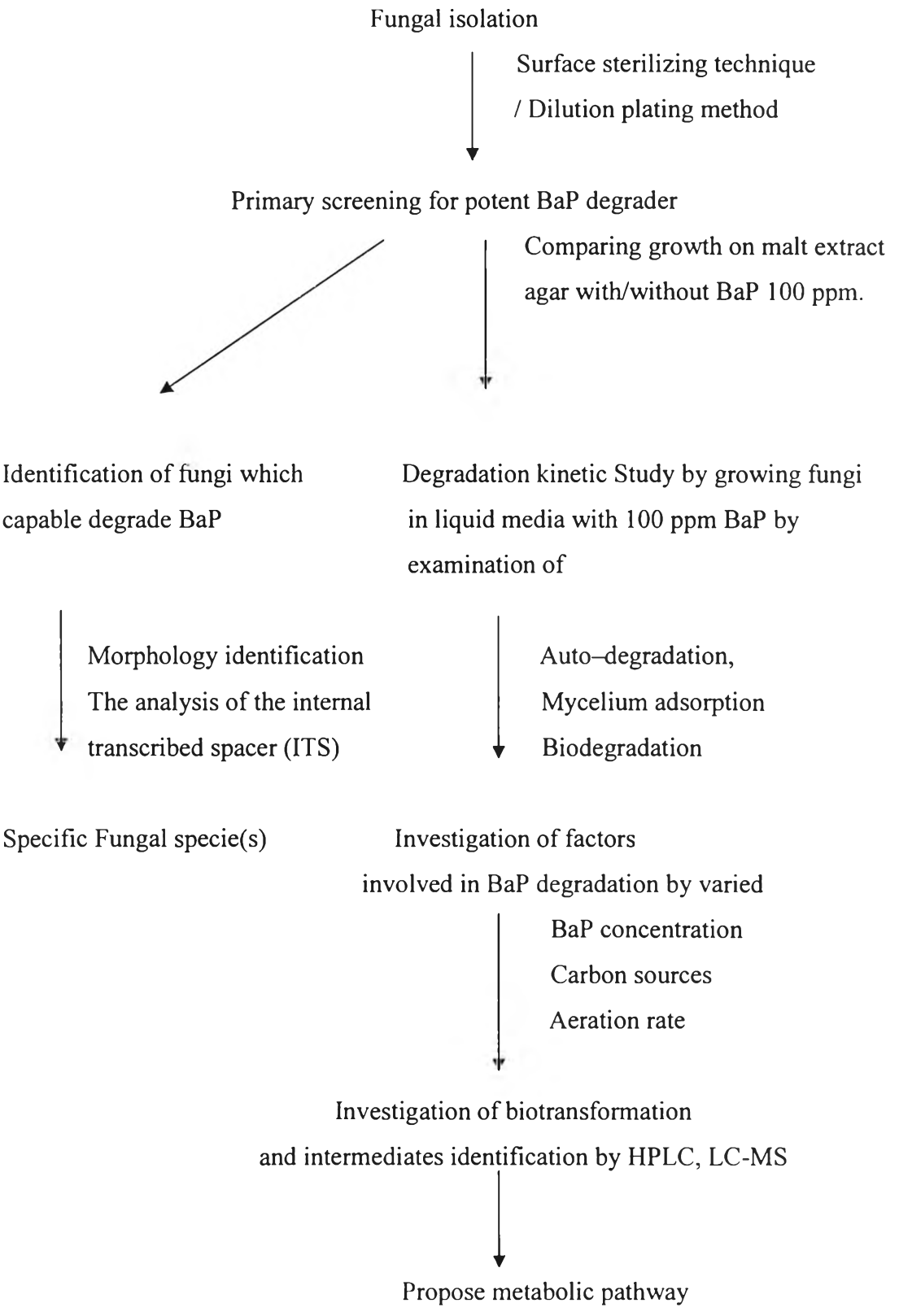

Figure 3.1 Scheme of the flow chart experimental procedures

3.2 Chemicals and Materials

3.2.1 Chemicals

Chemicals used in this experiment were generally supplied from local distributors in Thailand.

3.2.1.1 Tested compound

Benzo(a)pyrene standard (98% purity) was purchased from Fluka chemical.

3.2.1.2 Solvents

For HPLC grade solvents, such as acetonitrile, dichloromethane were purchased from Merck. While the analytical grade solvents as ethanol, methanol, and acetone were purchased from Carlo Erba.

3.2.1.3 Minimal media

The chemicals used to prepare minimal media, such as NH_4NO_3 was purchased from J.T. Baker, USA. The common chemicals such as $Na_2HPO_4.7H_2O$, $NaH_2PO_4.2H_2O$ were purchased from BDH England. KCl was obtained from UNILAB, Australia. The mineral salt media were prepared from $MgSO_4.7H_2O$, $FeSO_4 .7H_2O$, $MnCl_2.4H_2O$, $ZnSO_4. 7H_2O$, $CuSO_4 .5H_2O$, $CoCl_2.6H_2O$, and $Na_2MoO_4 .2H_2O$ (obtained from UNIVAR Australia) as described in APPENDIX C.

3.2.1.4 Microbiological media

Malt extract, glucose, peptone, agar, streptomycin were purchased from Merck chemical.

3.2.1.5 Chemicals for molecular analysis

The molecular chemicals such as polyvinlpropanol (PVP), ascorbic Acid, and CTAB were purchased from Serva. EDTA and Tris were obtained from Scharlau. The followed chemicals, such as, 2-mercaptoethanol, isopropanol, NaCl, polyethylene glycol, isoamyl alcohol/ chloroform were purchased from Merck chemical. Agarose gel was from ISC Bio express. Ethidium bromine, DNA marker, and Taq polymerase were purchased from Fermentas. The ITF1F and ITF4 primers and dNTP were obtained from Bio basic.

3.2.2 Source of fungi

Fungi were isolated from leaves and barks of *Pterocarpus macrocarpus* Kurz located along Phayathai road of Bangkok. Leaves and barks of *Pterocarpus*

macrocarpus Kurz. (Figure 3.2) were collected from an adult tree that showed no signs of disease, fragments were placed in an icebox and processed within 4 hours after the collection.

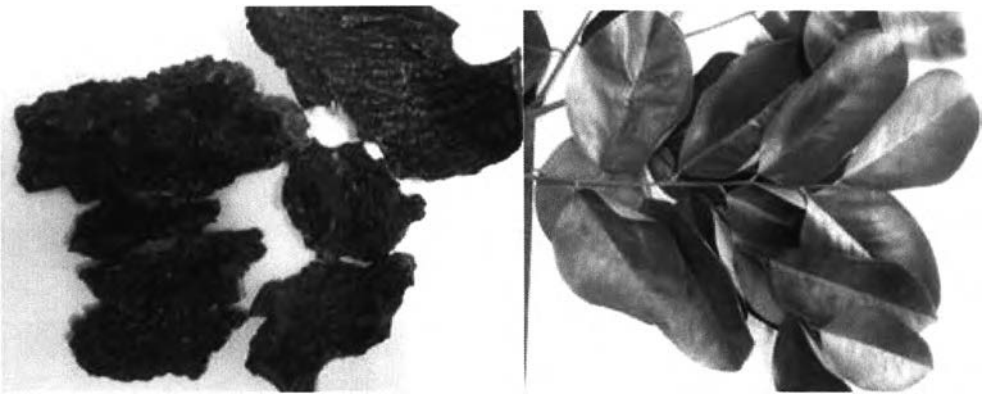

Figure 3.2 Leaves and barks of *Pterocarpus macrocarpus* Kurz.

3.3 Experimental procedures

3.3.1 Fungal isolation

3.3.1.1 Isolation of fungi from leaves

For epiphytic fungi, leaves were cut into circle-shape pieces with an approximate 5-mm diameter. Pieces of leaves were soaked in sterile distilled water and mixed with tween 80 (the ratio of leaves: water: tween 80 was1:10:0.1 w/w). Then the series dilution of solution was prepared from 10^{-3} to 10^{-5}, and then 0.1 ml of each dilution were spreaded on the surface of a solidified Malt Extract Agar (MEA) with adding 0.5% streptomycin in a petri plates. Each dilution was prepared in triplicate. The plates were then incubated for 2 days at room temperature (35-37° C), until the colonies appeared.

For endophytic fungi were used modified surface sterilization technique of Petrini, (1991) was performed. Healthy mature leaves were cut into circle shape pieces and dipped successively several steps into 70% ethanol for 1 min followed by 5% sodium hypochlorite (3-5 min) and, again, in 70% ethanol (1 min) and finally rinsed twice in sterile distilled water. The fragments were placed on the MEA solid agar and incubated in the dark at room temperature. After 7 days of

incubation, the fungal mycelium which came out from the leave fragments was collected.

3.3.1.2 Isolation of fungi from barks

Barks were blended with sterilized water in the sterilization blender with 1:100 (w/w). The series dilution of 10^{-3}, 10^{-4}, and 10^{-5} of the blended bark in sterile distilled water were prepared and spreaded on MEA agar as described in 3.3.1.1.

The fungi which obtained from those isolation steps were collected and sub-cultured onto new medium until obtained pure cultures and were used for the further experiments.

3.3.2 Primary screening for fungi degraded benzo(a)pyrene

Fungi isolates were grown on malt extract agar with and without adding 100-ppm BaP. Mycelial extension growth was measured as the colony diameter (Faleiro et al., 1996) at 14 days. The potential BaP-degraders were selected depending on their greater growth and growth rate compared to that of the control as shown below (Figure 3.3). Then, the greater fungal colony growth (which assumed that they were able to degrade BaP) was selected for further study.

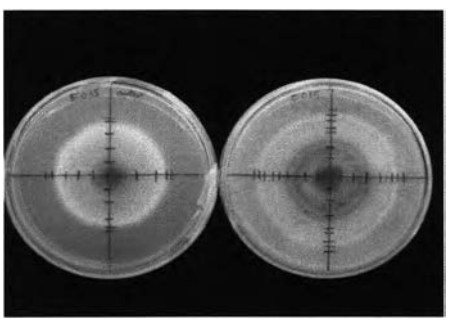

Figure 3.3 Comparison of fungal growth by measuring colony diameter on the media supplemented with BaP (right) and in the absence of BaP (left)

3.3.3 Molecular fungal identification

The isolated fungal strains which capable to grow well on BaP supplemented media was identified morphologically and genetically using 18s rDNA sequence comparison (White et al., 1990; Larena et al., 1999). All experiments were carried out in duplicate.

3.3.3.1 Morphological analysis

3.3.3.1.1 Morphology observation by slide culture

The morphology of the three selected fungi was examined on glass slide after cultured for three days. The mycelium and spore were stained with the phenylalanine solution and observed under light microscope.

3.3.3.1.2 Ultra structure observation under Scanning Electron Microscope (SEM)

Ultra structure of fungi were examined under scanning electron microscope model Genesis 4000 XMS 60 running under the ESEM mode of analysis equipped with the Gaseous Secondary Electron (GSE) detector.

The photographed with scanning electron microscope for the fungal morphology of the promising fungi was under the magnification of 500X, 800X, or 1200X or higher magnification in necessary depending on the specimens.

3.3.3.2 Molecular technique for fungal identification

For the genetically analysis, the procedure were comprised of DNA extraction, PCR amplification DNA sequencing and sequence analysis. The details of these two processes were described below. The protocol of the genetically analysis can be simplified as shown in Figure 3.4.

The genomic DNA was prepared from mycelium cultured in MEB and extracted with cetyl-trimetyl-ammonium bromide (CTAB) as described in Zhou et al., 1999. PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 50 µl which comprised approx. 100 ng genomic DNA, 1X Taq buffer, 0.25mM each dNTP, 1µM of each primer, 1.5mM MgCl, 2.5 units Taq DNA Polymerase (Fermentas, California, USA), and the primer ITS1f (Gardes and Bruns

1993) and ITS4 (White et. al. 1990). The amplification was performed in a thermal cycles (TP 3000; Takara Shuzo,Tokyo, Japan). with 94 °C for 5 min , followed by 38 cycles of 94° C for 1 min, 51° C for 1 min and 72 °C for 1 min, with a final extension of 72° C for 5 min. PCR product was purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and cloned using PCR-Script ™ Amp. Cloning Kit. Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced externally by Macrogen (Seoul, Korea) using the same primers as for amplification. The ITS sequence of the three promising fungi were submitted to GenBank.

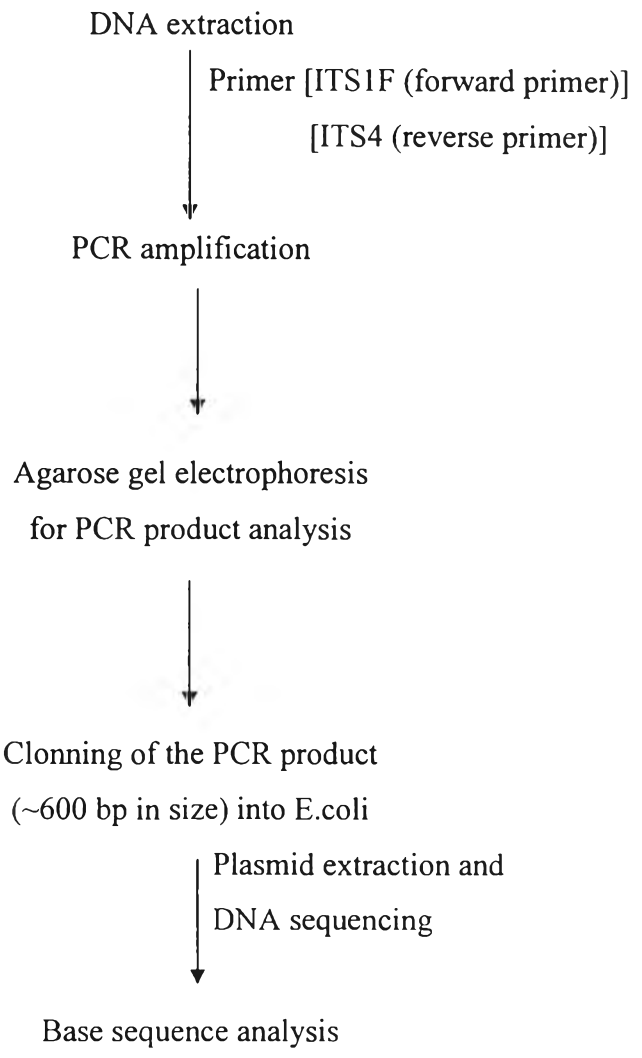

Figure3.4 The diagram of protocol of the molecular genetic process for fungal identification

The analysis of the Internal Transcribed Spacer (ITS) region of the nuclear rRNA gene, which has been used to identify and clarify the species of fungi were applied to our three promising fungi. The ITS 1 and ITS 2 region as well as the primers applied to identify the specific regions were shown in the Figure 3.5 and Figure 3.6

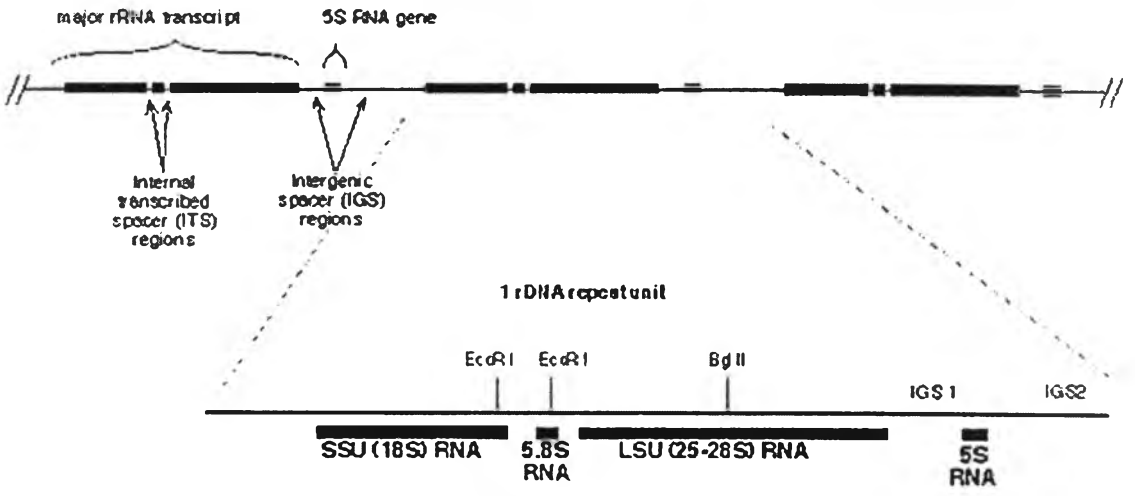

Fig. 3.5 Schematic representation of the rDNA region of fungi. The SSU represent the Small Sub Unit, and LSU represent the Large Sub Unit. The EcoR, Bgl represent the restriction sites conserved in the rDNA of fungi.

Source: Vilgalys et al., 1994

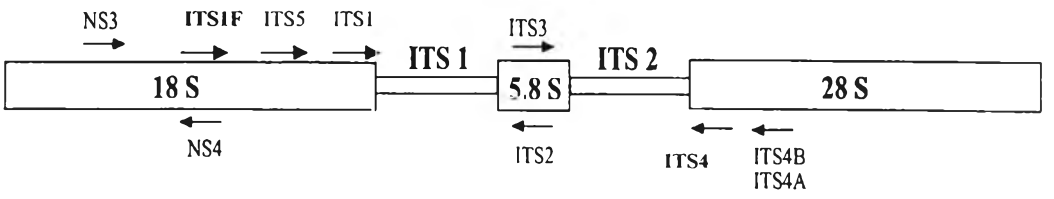

Fig. 3.6 The primers used in this study (ITS1F and ITS 4; bolded) for identification of the ITS region of the fungi of interest.

Source: Vilgalys et al., 1994

3.3.4 Determination of the BaP biodegradation kinetics in liquid media

The selected fungal strain(s) were tested for their degradation kinetics of BaP when grown in liquid media. Abiotic controls were performed in order to compensate for adsorption loss and photo-oxidation, if any. HPLC were used to determine the reduction of BaP as described in 3.4.2. The flow chart of the experiment was simply illustrated in Figure3.7

3.3.4.1 Culture medium preparation

To grow each fungal isolate, 5 plugs of 6 mm. mycelia on the solid agar were conducted into 250-ml. Erlenmeyer flask containing 50 ml of liquid culture having the compositions as followed to Verdin et al. (2004) shown in APPENDIX C. Glucose (5 mM) was provided as a carbon source. In order to determine the BaP degradation ability, benzo(a)pyrene dissolved in acetone was also added to the final concentration of 100 ppm (0.4 mM). After that, flasks were then sterilized at 120 $^{\circ}$C for 20 min.

3.3.4.2 Experimental conditions for BaP biodegradation kinetic study

The cultures were grown in the presence of 100 ppm BaP at room temperature (~30-32°C) with orbital shaking at 120 rpm for 30 days. Abiotic controls were conducted to ensure that the disappearance of BaP was caused by the biodegradation. As well as the determination of adsorption loss and photo-oxidation were observed. Biotic control was used-the three times autoclaved-killed culture. The mycelia sample was collected every 5 days interval, washed and filtered. The mycelia were then lyophilized and the dry weight was examined. BaP was extracted from both the filtrate and the lyophilized mycelium. BaP in the filtrate as well as BaP adsorbed on the surface of the mycelia were separately extracted three times with equal volume of dichloromethane (DCM). BaP contained in the cell mycelium was also extracted using DCM after lyophilization and well grinding. Amount of BaP remained in the filtrate and mycelium extracts were then analyzed by HPLC. In all experiments, cultures were grown in triplicate and the results are expressed as means with standard deviations.

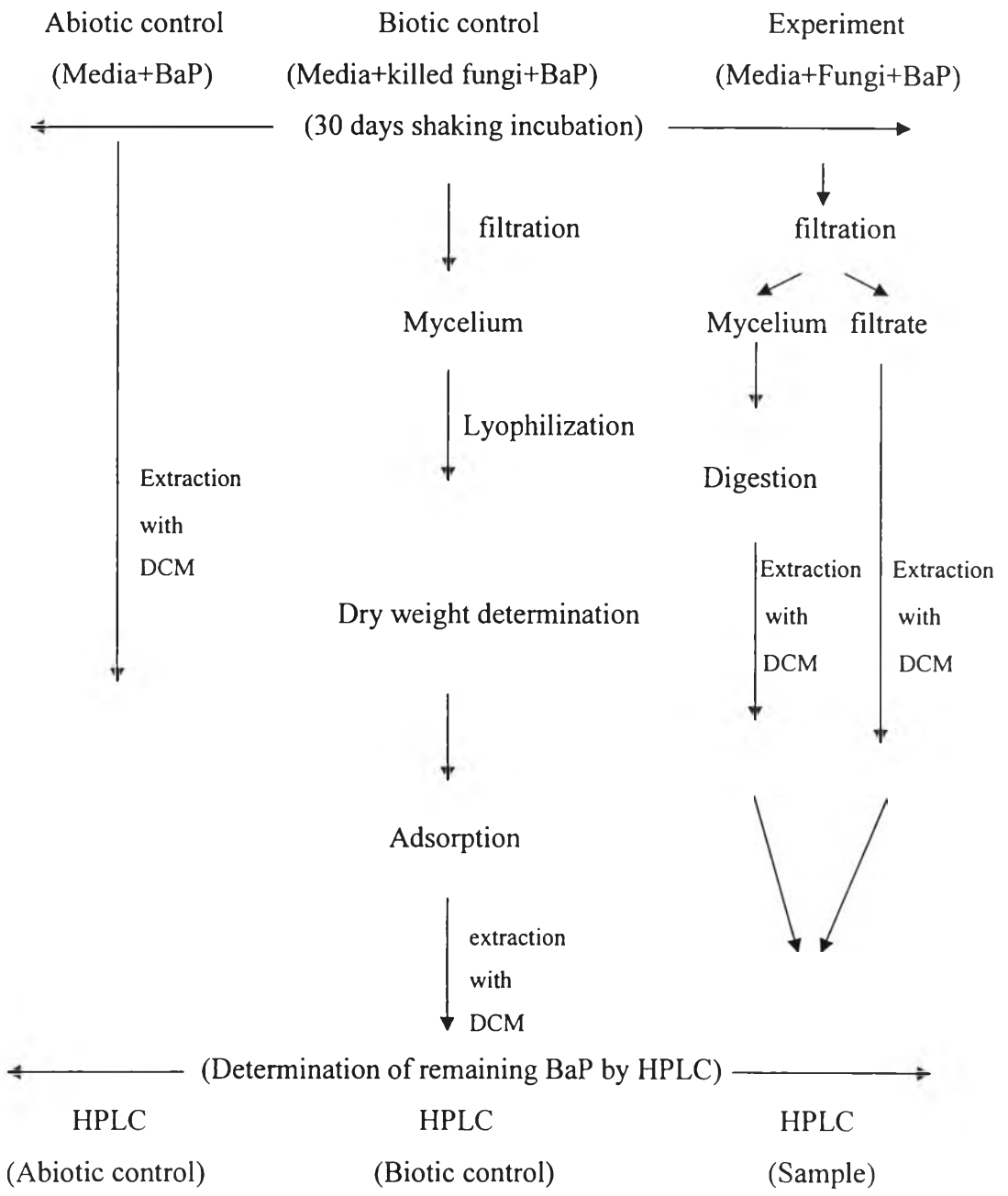

Figure 3.7 Summarized diagram of the degradation kinetic study.

BaP degradation rates were calculated by subtracting the amount of BaP extracted from the abiotic control (without fungi) and from the amount on cell surface in the other samples. The three times autoclaved killed cultures (Juhasz et al., 2002) were used to quantified as adsorbtion losses. The BaP degradation rate was calculated as:

BaP degradation rate (%) = Abiotic control extraction (100%) – (sample extraction + adsorption losses)

Where sample extraction = filtrate extraction + mycelium extraction

3.3.5 Factors affecting BaP biodegradation

The conditions involved in the biodegradation of BaP by the fungal isolated strain(s) were investigated. The factors affecting the biodegradation of BaP include:

3.3.5.1 The aeration rate

Since the BaP degradation reactions were reported to be oxidation (Cerniglia&Gibson, 1979, 1980; Launen et al., 1995), the effect of aeration rate on the BaP degradation by the chosen fungi were examined. The effect of various shaking rates at 60 rpm, 120 rpm and 180 rpm. on 100 ppm BaP biodegradation was examined.

The culture media and other growth conditions were similar to those described in 3.3.4.1. The depletions of BaP in the system were recorded and investigated over 30 days of incubation. The biomass was also measured.

3.3.5.2 The initial BaP concentration

According to the previous reports, the maximum concentration of BaP which fungi can degrade was at 100 ppm (Launen et al., 1995; Verdin et al., 2004). In our study, the higher BaP concentrations were used to test the toleration ability and the degradation efficiency of selected fungi. The concentrations of BaP varied at 200 ppm and 300 ppm. The disappearances of BaP as well as the incremental biomasses were investigated.

3.3.5.3 The carbon source

The type and concentration of carbon source is one of the limiting factors for microbiological growth and biodegradation (Liebeg&Cutright, 1999). Glucose has been widely used as a carbon source for fungal growth. In this study, glucose with various concentration, 0 mM, 5 mM, or 50 mM, was applied as the supplemented carbon source concomitant with 100 ppm BaP. The BaP degradation efficiency and fungal growth at various glucose concentrations were examined.

3.3.5.4 Bioavailability of BaP

The bioavailability of BaP to the fungi is also one of the limiting factors. BaP has a low water solubility of approximately of 0.038 mg/l at 25 °C indicating low bioavailability to the organism. Therefore; in order to enhance BaP bioavailability, ethanol and methanol at the concentrations of 5 mM were added to increase the bioavailability of BaP to fungi and also as the supplementary carbon source for fungi. Then, the effect of ethanol and methanol to the reduction of BaP as well as the fungal growth over 30 days of incubation were determined.

3.3.6 Determination of the biotransformation product(s) to propose the biodegradation pathway of BaP

The biotransformation intermediate(s) obtained during the biodegradation of BaP were determined from the intracellular and extra-cellular samples. Culture free extract sample was prepared from the fungal mycelium. The mycelium was lyophilized, well grinded and sonicated before extracted with an equal volume of DCM. The necessary techniques were applied to identify the intermediate(s), for example HPLC, LC-MS, GC-MS. Finally, the information obtained was gathered to propose the biodegradation pathway of BaP by the newly isolated strain(s).

3.4 Analytical methods

3.4.1 Benzo(a)pyrene extraction from liquid sample

BaP was extracted from liquid sample by using liquid-liquid extraction. The collected samples were transferred to the glass tube. The liquid sample was extracted with equal volume of dichloromethane (2 times). The mixture was mixed vigorously for 5 min. using vortex, then left to allow the solution to separate. The upper phase of

aqueous liquid was discarded. The extracted solvents containing were pooled together and left over night in the chemical hood to evaporate the solvent. The BaP residue was subject to analyze by HPLC, therefore the mobile phase was added to re-dissolve the substance before HPLC analysis. All experiments were carried out in triplicates.

3.4.2 Quantitative analysis of BaP by liquid chromatography

BaP was qualitatively and quantitatively analyed by high performance liquid chromatography (HPLC) using a 97.5% purity standard BaP. HPLC was run on an Agilent instrument model 1100 series equipped with UV detector (254nm.) using a Hewlette Packard hyposil C18 reverse phase (250mm x 4mm. id) column. The mobile phase was 100% acetonitrile and passed through the column at the flow rate of 1.0 ml/min. The retention time of BaP was received at 3.96 (+/-) 0.1 min. as shown in Figure 3.8. The amount or the concentration of BaP achieving from the chromatogram was calculated based on the calibration curve.

Figure 3.8 The HPLC chromatogram of benzo(a)pyrene eluted at 3.9 (+/-)0.1 min.

Peak area of the chromatogram were used to calculate for amount and/or concentration of BaP when compare to the calibration curve shown in Figure E-1 (APPENDIX E)

3.4.3 Analysis of biotransformation product(s) using Liquid Chromatography- Mass Spectrometry (LC-MS)

The extractable samples both in liquid media and fungal mycelium were analyzed and separated using liquid chromatograph (Agilent 1100) equipped with a mass spectrometry (Bruker DALTONICS) with electro-spray ionization/ ion trap mass analyzer

The direct infusion using syringe pump at 260 µl/hr. The samples were diluted 1:10 with 50% acetonitrile in water. The scan acquisition range was from m/z = 50-500 in positive ion mode generated by 0.1% formic acid. The capillary voltage was -4000 V. The dry gas was run at 8 l/min and dry temperature was at 200°C. The nebulization was generated at 10 psi. The results were compared to the molecular mass of the possible intermediate(s) in BaP degradation pathway in order to identify it.