CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Shimadzu® High Performance Liquid Chromatography (HPLC) equipped with 4.6 x 150 mm Senshu Pak Pegasil ODS column (C_{18}), pump (model LC-10AD*VP*), autosampler (model SIL-10AD*VP*), and UV-Visible detector (model SPD-10AD*VP*)

ISSCO® laminar air flow (model BVT-124) from International Scientific Supply Co.Ltd., USA.

Gyrotory Shaker (model G10) from New Brunswick Scientific Co.Ltd., USA.

Genesys20® spectrophotometer from Thermo Spectronic Co.Ltd., USA

Incubator from Memmert Co.Ltd., Germany.

Rotary vacuum evaporator (model N-N) equipped with refrigerated pump, digital waterbath (all from Tokyo Rikakikai Co.Ltd., Japan), and circulating aspirator (Shibata type WJ-20, Japan).

Ultrasonicator bath (model RK100) from Bandelin Electronic Co.Ltd., Germany

Mini Sub-Cell GT® Agarose Gel Eletrophoresis System and UV transilluminator (model Universal Hood) from Bio-rad Co.Ltd., USA.

DNA Thermal Cycle® (model 2400) from Perkin Elmer Co.Ltd., USA.

Stainless steel grab sampler from Environmental Research and Training Center, Department of Environmental Quality Promotion, Ministry of Natural Resources and Environment.

3.1.2 Chemicals

PAHs standards: acenaphthylene, fluorene, fluoranthene, dibenzofuran, and pyrene were purchased from Kanto Chemical Co.Ltd., Japan.

PAHs standards: acenaphthene and phenanthrene, ribonuclease A (Rnase A), and proteinase K were purchased from Sigma Chemical Co.Ltd., USA.

Methanol (CH_3OH), ethyl acetate ($CH_3COOC_2H_5$), and dichloromethane (CH_2Cl_2) were obtained from Merck Co.Ltd., Germany.

1Kb DNA standard and *Taq* DNA polymerase were supplied from Promega Co.Ltd., USA.

All chemicals were of analytical grade or higher.

3.1.3 Culture Media

Carbon free mineral medium (CFMM) (Omori *et al.*, 1992), and Luria Bertani (LB) liquid and solid media as shown in Table 3.1 were used as culture media.

Table 3.1 Components of CFMM and LB media

Media	Compositions	Concentrations (g/l)
CFMM[a]	A)KH_2PO_4	0.8
	NH_4NO_3	3.0
	$Na_2HPO_4 \cdot 12H_2O$	5.5
	B)$CaCl_2 \cdot 2H_2O$	0.05
	$MgSO_4 \cdot 7H_2O$	0.05
	$FeCl_3 \cdot 6H_2O$	0.1
LB[b]	NaCl	5.0
	Tryptone	10.0
	Yeast extract	5.0

[a] Part A pH was adjusted to 7.5, autoclaved at 121 ° C, 15 pound/inch^2 for 15 mins. Part B was sterilized by hydrophilic filter.

[b] For the solid medium, 15 g/l of agar powder was added into the medium then adjusted to pH of 7.0, autoclaved at 121 ° C, 15 pound/inch^2 for 15 mins.

3.2 Sediment analysis

3.2.1 Sediment sample collection

Petroleum hydrocarbon contaminated surface sediments were collected in the level of approximately 3-4 meters under the water by stainless steel grab sampler as shown in Figure 3.1. Samples were kept in dark bottle at 4°C until used. The sampling sites in this study are listed in Table 3.2.

Figure 3.1 Stainless steel grab used as sediment collector.

Table 3.2 List of the sampling sites

Sites	Designate	Descriptions
1	S_1	Ratchathewi expressed boat pier, Saen-Saeb Canal
2	S_2	Pratunum expressed boat pier, Saen-Saeb Canal
3	S_3	Panfa-leelard expressed boat pier, Saen-Saeb Canal
4	S_4	Wat Sri-boon-reung expressed boat pier, Saen-Saeb Canal
5	S_5	Phrachulachomklao Royal Navy Dockyard, Chao-Phraya River
6	S_6	See-phraya pier, Chao-Phraya River
7	S_7	Sa-thon pier, Chao-Phraya River
8	S_8	Padungkrungkasem Canal

3.2.2 Sediment extraction

The sediment samples were subjected to temperature measurement and PAHs quantification. Temperature was measured immediately after collection by thermometer. In the present study 7 PAHs were focused as following; acenaphthylene, acenaphthene, dibenzofuran, fluorene, phenanthrene, fluoranthene and pyrene. The sediments were dried in the dark at room temperature and sieved by sieve No.8. Five grams of air-dried and sieved sediment sample was used in each case, the residual water were removed by the addition of anhydrous Na_2SO_4. Triplicate extractions were performed with 3 ml of dichloromethane. The sediments were mixed by vortex at maximum speed for 2 mins and sonicated for 15 mins; the remaining supernatant was pooled into new tube. The combined extracts were concentrated under reduced pressure at 30°C and evaporated to dryness by the rotary vacuum evaporator. The residue was brough up in 1 ml of methanol and filtered through 0.22 μm syringe PTFE filter (Juhasz *et al.*, 1997).

3.2.3 PAH concentration analysis

Twenty microliters of the extracts were injected into HPLC to determine the PAH concentrations. HPLC was operated under the following condition: column temperature 40°C, flow rate 1.0 ml/min, mobile phase 80% methanol solution and detection by UV detector at 275 nm. The PAH concentration can be calculated by comparing peak area with the PAH calibration curve. The calibration curve was conducted by the authentic individual PAH ethanol solution.

3.3 Isolation of the PAHs degrading bacteria

3.3.1 Enrichment culture

The enrichment cultures were conducted using modified method as described in Saiphet (2002). Ten grams (wet weight) of contaminated sediment were added in 50 ml of CFMM and incubated at room temperature with agitation at 200 rpm for 24 hrs. The suspensions were left at room temperature for 2 hrs in order to settle the large sediment particles. Five milliliters of supernatant were transferred into 45 ml fresh sterilized CFMM supplemented with individual PAHs solution either fluoranthene, fluorene or pyrene (Appendix A) at the final concentration of 100 mg/l and incubated at room temperature with the agitation of 200 rpm (Bauer and Capone, 1988). The cultures were daily visual examined for turbidity, colour and declination of PAH crystals which represented the growth of bacteria. After bacteria grown, 5 ml of enrichment culture was transferred to the fresh CFMM containing respective PAH at the same concentration and incubated in the same condition. The serial transfer was performed for five times in order to enrich the PAHs degrading bacteria.

3.3.2 Isolation of the PAHs degrading bacteria

PAHs degrading bacteria were isolated by the spray plate technique (Kiyohara *et al.*, 1982). After five successive transfers, bacterial suspension from 3.3.1 was diluted and spreaded with sterilized glass rod onto CFMM agar surface. The individual 2 % PAH solution in diethyl ether (Appendix A) was sprayed on the plate as a sole carbon and energy source for bacterial growth and incubated at 30°C up to 3 weeks. This treatment resulted in visible and uniform surface coat of PAH compound on the agar plate. Colonies surrounded with a clear zone were inoculated in liquid CFMM containing the same PAH at the same final concentration in order to confirm their PAH degrading ability. Bacterial culture was checked for their purity by spreading on the CFMM agar in the present of respective PAH in vapor phase. The single and well isolated colonies were purified by repetitive streaking or spreading on CFMM agar containing respective PAH in vapor phase. The isolated pure strains were grown in liquid CFMM supplemented with respective PAH to ensure that pure strain certainly degraded respective PAH at concentration of 100 mg/l. After obtained the PAH isolated bacteria, they were cultured in same medium and concentration of respective PAH to maintain their PAH degrading activity.

For long-term preservation, the pure culture was grown to log phase in liquid CFMM supplemented with respective PAH at the final concentration of 0.1 mg/ml. The culture broth was mixed with sterilized glycerol at the ratio of 50:50, and 30:70 in the sterilized cryotube and kept at –20°C or –70°C, respectively. Bacteria can be maintained by this method for maximum of one year.

3.4 Identification and characterization of the PAH degrading bacteria

3.4.1 Morphological and biochemical characterizations

Morphological and biochemical characterizations were conducted according to standard procedure (Krieg and Hott, 1994), including Gram stain reaction, cell and colony morphology, oxidase, nitrate reduction, gelatinase, urease, motility, citrate utilization, H_2S production, oxidation-fermentation and carbohydrate fermentation test.

3.4.2. Identification of the isolated strain by 16S rDNA sequence analysis

3.4.2.1 Genomic DNA extraction (Ausubel *et al.*, 1999)

The selected isolate was grown in 5 ml of LB (liquid medium) for 18-20 hrs. The cell was harvested by microcentrifuge at 5,000 rpm at room temperature for 2 mins. Pellet was resuspened with 576 µl of TE buffer (Appendix B). Thrity microliters of 10% SDS and 3 µl of 20 mg/ml proteinase K were added to the suspension (Appendix B). The cell suspension was mixed well and incubated at 37°C for 1 hr. To the reaction, 100 µl of 5 M NaCl was added, and mixed thoroughly. Eighty microliters of CTAB/NaCl solution was added, mixed, and incubated for 10 mins at 65°C. The reaction was extracted by equal volume of chloroform/isoamyl alcohol followed by centrifugation at 13,000 rpm for 5 mins at 4°C. Then, the aqueous phase was transferred into a fresh tube and extracted again with equal volume of phenol/chloroform/isoamyl alcohol followed by centrifugation at 13,000 rpm for 5 mins. The aqueous phase was transferred into a fresh tube. Isopropanol 0.6 volume was added and mixed gently until DNA precipitate was seen, and then centrifuged at 13,000 rpm for 15 mins at 4°C. DNA was washed twice with 1 ml of 70% ice-cold ethanol and centrifuged at maximum speed for 15 mins at 4°C. The supernatant was discarded. DNA pellet was dried and then

resuspended in 50 µl of steriled-distilled water. Two microliters of 10 mg/l RNase solution was added for elimination of RNA.

3.4.2.2 Polymerase Chain Reaction (PCR)

PCR amplification method was used to amplify 16S rDNA with oligonucleotide primer. The experiment was performed under the cooled condition. The final reagent concentrations of the reaction components were as following; 25 mM $MgCl_2$ was 3 µl, 10 mM of dNTP was 1 µl, 200 ng/µl of each forward primer 10f (5'-AGTTTGATCATGGCTC-3') (Auburn University Environmental Institute, 2002) and reverse primer 1540r (5'-AAGGAGGTGATCCAGCC-3') (Lambert *et al.*, 1998) 2.5 µl, 2.5U of *Taq* DNA polymerase 0.5 µl, *Taq* DNA polymerase buffer 5 µl, steriled distilled deionized water 34.5 µl and DNA template 1 µl. Total volume of 50 µl was thoroughly mixed. PCR condition was carried out as following:

Hot start	95 °C	5 mins	
Denaturation	94 °C	1 min	35 cycles
Annealing	46 °C	1 min	
Extension	72 °C	2 mins	
Final extension	72 °C	10 mins	
End	4 °C	-	

3.4.2.3 Agarose gel electrophoresis

Agarose gel was completely melted in 1X TAE buffer (Appendix B) using microwave. After cooling down, the agarose gel was poured into gel casting apparatus with comb. The agarose was left at room temperature to solidify and thereafter placed into electrophoresis chamber containing 1X TAE buffer. DNA samples and 1 Kb DNA marker were mixed with loading dye before loading with the gel slot. The electricity was applied at 100 volts to the agarose gel. After the tracking dye migrated to the end of agarose gel, the power supply was disconnected. Agarose gel was stained with ethidium bromide solution for 5 mins in the dark and at room temperature and destained

with distilled water. The gel was illustrated with the UV transilluminator. The expected molecular size of 16S rDNA was approximately 1,500 bp.

3.4.2.4 16S rDNA sequence analysis

The PCR products were subjected to analyse the nucleotide sequences, which was performed by Bioservice Unit (National Centre for genetic Engineering and Biotechnology, National Science and Technology Development Agency). The primers used for nucleotide sequencing were following:

Forward primer 10f 5'- AGTTTGATCATGGCTC-3' (Auburn University Environmental Institute, 2002)

Reverse primer 350f 5'- TACGGGAGGCAGCAG-3' (Weber *et al.*, 2001)

Forward primer 1240r 5'- CCATTGTAGCACGTGT-3' (Laurie *et al.*, 2002)

Forward primer 1540r 5'- AAGGAGGTGATCCAGCC-3' (Lambert *et al.*, 1998)

The 16S rDNA nucleotide sequences were compared with the presented sequences available in GenBank using BlastN program (Altschul *et al.*, 1997).

3.5 PAHs degrading ability of the isolated strains

Ability of isolated bacteria to utilize PAH as substrate for growth can be monitored by the depletion of PAH in the culture and the bacterial cell count.

3.5.1 Degradation of PAH as growth-substrate

3.5.1.1 Inoculum preparation

The inoculum was prepared by the modified resting cell method (Saiphet, 2002). Briefly, the isolated strain was grown in solid CFMM with single PAH crystal (either fluorene, fluoranthene or pyrene) on the lids. Colonies were added in 0.85 % NaCl 5 ml and shaken at 200 rpm for 12-15 hrs to ensure that the bacteria utilized the

entire intracellular nutrient. The cell suspension was adjusted for the optical density at 600 nm to 0.1.

3.5.1.2 PAHs utilization

The 100 µl of obtained cells were inoculated in 5 ml of liquid CFMM supplemented with respective PAH as growth substrate at concentration of 0.1 mg/ml. The cultures were incubated at room temperature with agitation 200 rpm. Two set of controls i.e. "substrate control" or CFMM containing individual PAH only and "growth control" consisting of CFMM and bacteria without PAH substrates were also included. The summary of this experiment was shown in Figure 3.2. The triplicate samples were taken at day 0, 1, 3, 5, 7, 10, 14 to examine the remaining PAH concentration and total cell number. The increasing in bacterial cell number and reduction of PAH concentration indicates that the isolate is able to degrade that kind of PAH.

The bacterial cell number was determined by the viable plate count technique by spreading the diluted bacterial culture on LB agar. The remaining PAH was extracted from the culture broth by method described by Grifoll *et al.* (1992). Briefly, after acidification to pH 2.5 with concentrated HCl, the culture broth was extracted three times with 1 volume of ethyl acetate by using vortex mixer. The water was removed from the extracts with anhydrous Na_2SO_4 and evaporated to dryness in the rotary vacuum evaporator. The residue was resuspended with 1 ml of methanol and filtered through 0.2 µm syringe PTFE filter. The extracts were analyzed for the PAH remaining by HPLC, which was operated under the condition as described previously in 3.2.3

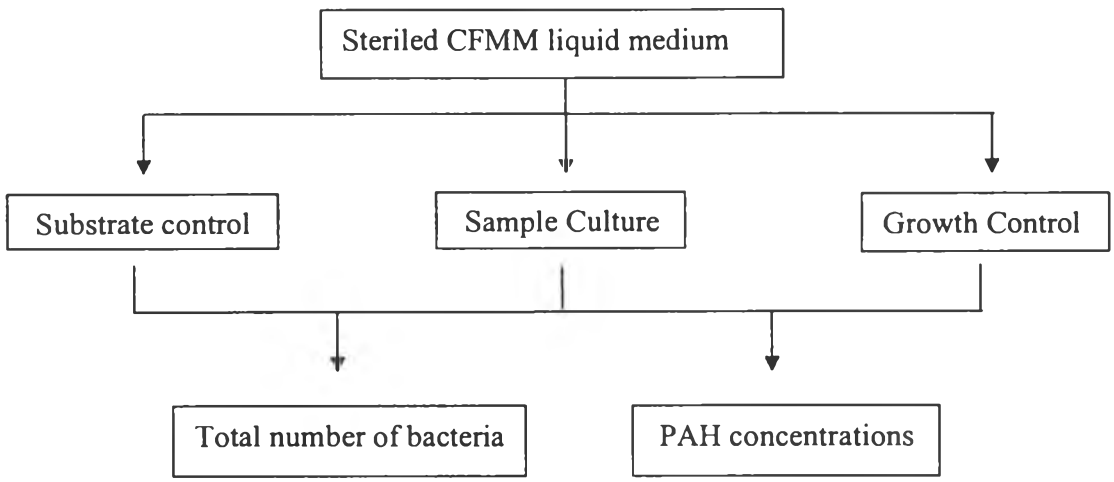

Figure 3.2 Diagram of PAH utilization experiment

3.5.2 Substrate specificity

The substrate specificity of the isolates was determined. The inoculums were prepared as described previously in 3.5.1.1 and then were added into 5 ml sterilized CFMM supplemented with other individual PAHs (acenaphthene, acenaphthylene, dibenzofuran or phenanthrene) at final concentration of 100 mg/ml. The cultures were incubated at 200 rpm in room temperature. Two sets of controls and triplicate samples were taken at day 0, 2, 4, 7 of incubation time to examine the number of bacteria and PAH concentrations as described in 3.5.1.2.