CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals, enzymes and kits

Media and reagents

All of bacteriological media were purchased from Difco (USA). Preparation of media and reagents used are presented in Appendix A and B, respectively.

Chemicals

All chemicals and solvents used in this study such as phenanthrene, pyrene, fluoranthene, authentic 1-hydroxy-2-naphthoic acid, *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosoamide, 2,3-dihydroxybiphenyl, silica gel and HPLC grade solvents are products of Kanto Chemical Co., Inc (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan) and Wako (Tokyo, Japan). Other chemicals were of analytical or molecular biology grade and of the highest purity available.

Enzymes and kits

Enzymes used in this experiment such as restriction endonucleases, calf intestinal alkaline phosphatase (CIAP), lysozyme and proteinase K were purchased from Boehringer Mannheim (GmbH, Germany), Sigma Chemical Co. (USA) and Takara Shuzo Co., Ltd. (Kyoto, Japan). *Tag* polymerase was obtained from Perkin Elmer Cetus (USA). Ligation kit and Gene extraction kit were purchased from Takara Shuzo Co., Ltd.(Kyoto, Japan) and Gibco-BRL (UK), respectively. dNTP mixture and Marker 6 DNA marker was obtained from Appied Biosystems Inc. (USA) and Wako (Tokyo, Japan), respectively.

3.2 Bacterial strains and plasmids

Bacterial strains and plasmids used are shown in Table 3.1

Table 3.1 Bacterial strains and plasmids

Bacteria	Genotype/Phenotype	Reference/Source
Sphingomonas sp. P2	utilize phenanthrene, naphthalene, dibenzofuran, fluorene, anthracene and has possibility to co-metabolize pyrene and fluoranthene in the presence of phenanthrene	(Supaka *et al.*, 1999)
Escherichia coli JM 109	*recA*1 *supE*44 *endA*1 *hsdR*17 *GyrA*96 *relA*1 *thi* Δ (*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lac*^{q} *lacZ*ΔM15	Yanisch-Perron *et al.*, 1985

Plasmid (Appendix E)	Description	Reference/Source
pUC18	Vector; Ap^r; multiple cloning site in *lacZ* α subunit	Yanisch-Perron *et al.*, 1985
pUC19	Vector; Ap^r; multiple cloning site in *lacZ* α subunit	Yanisch-Perron *et al.*, 1985
pUC119	Vector; Ap^r; multiple cloning site in *lacZ* α subunit	Yanisch-Perron *et al.*, 1985
pDI1	Ind$^+$ (*pahA*$^+$) Ap^r; recombinant of pUC119 with 9.5-kb *SalI-SalI* fragment subcloned from 25-kb *SalI-SalI* fragment carrying *pah* gene cluster from OUS82 chromosomal DNA	Kiyohara *et al.*, 1994

3.3 Culture conditions

3.3.1 Culture conditions for bacterial strains

In the study of co-metabolism, *Sphingomonas* sp. P2 was mainly used and cultured in liquid carbon free minimum medium (CFMM) supplemented with 0.1% w/v of phenanthrene (CFMM/phe, Appendix A). Luria-Bertani (LB) or 2×yeast tryptone (2×YT) media (Appendix A) were also employed in supporting the growth of *Sphingomonas* sp. P2 and *Escherichia coli* JM109 that used in genetic experiment. Isopropylthio-β-D-galactoside (IPTG; 0.1mM, Appendix B) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 20 μg/ml, Appendix B) were included in solid LB media to identify recombinant plasmids containing insert DNA in *lac*Z. Ampicillin (100 μg/ml, Appendix B) was supplemented when necessary. *Sphingomonas* sp. P2 was cultured at 30°C, agitation was maintained at 300 rpm for liquid culture whereas *E. coli* JM 109 was cultured at 37 °C, with agitation speed of 200 rpm.

For preservation, *Sphingomonas* sp. P2 was cultured in 5 ml of CFMM/phe for 48 hr, then 500 μl of the culture was mixed with equal volume of 80% sterilized glycerol in microtubes. The glycerol culture was kept at -70 °C. In case of *E. coli* JM109, the cells were cultured in 5 ml of LB liquid medium for 18-24 hr subsequently, mixed with equal volume of 80% sterilized glycerol in microtubes. The glycerol culture was also kept at -70 °C.

3.3.2 Large scale cultivation

Sphingomonas sp. P2 stock culture was streaked on LB agar plate and incubated for 2 days. Three isolated colonies were inoculated into individual of 4 tubes of 5 ml CFMM supplemented with 0.33% (w/v) succinate (CFMM/Suc, Appendix A) then incubated at 30°C, on shaker at the agitation speed of 300 rpm for 24 hr. The cells were collected by centrifugation at 3,000 rpm for 2 min and washed twice with CFMM, then inoculated into two media systems. Medium A was 10 ml of CFMM supplemented with phenanthrene and pyrene each 0.1% (w/v) (CFMM/phe/pyr, Appendix A). Medium B

was 10 ml of CFMM supplemented with phenanthrene and fluoranthene each 0.1% (w/v) (CFMM/phe/flu, Appendix A) and incubated for another 2 days with the same conditions as above to obtain PAH-pregrown cells. The cells were transferred to 500 ml flask containing 100 ml of the same media. All cultures were incubated at 30°C, on a rotary shaker at the agitation speed of 300 rpm. These cultures were served as starter cultures (2%v/v) for large-scale cultivation.

For large-scale cultivations (15 litre) each 600 ml of starter culture from media systems A and B was inoculated to two separated 30 l fermenters (model MSJ-U3, B. E. Marubishi Co., Ltd., Tokyo, Japan) containing 15 l of media systems A and B, respectively. Cultivation temperature was 30 °C with aeration rate of 15 l/min as well as 300 rpm agitation speed. The pH of the culture media was maintained at 7 with 0.1 M NaOH. Cultivation period was 4 days.

3.4 Methods and chemical analyses

3.4.1 Solvent extraction

Bacterial cells were removed from 5 litre of culture fluid by centrifugation using continuous centrifuge (Tomy Seiko Co. Ltd., Japan), the supernatant obtained was adjusted to pH 2-3 with concentrated HCl followed by three times extraction with equal volume of ethyl acetate. The ethyl acetate phase was further extracted for three times with equal volume of alkaline water (10 mM NaOH). The ethyl acetate phase was collected as 'Neutral extract' while the aqueous phase was brought to pH 2-3 by the addition of concentrated HCl and extracted three more times with equal volume of ethyl acetate and designated 'Acidic extract'.

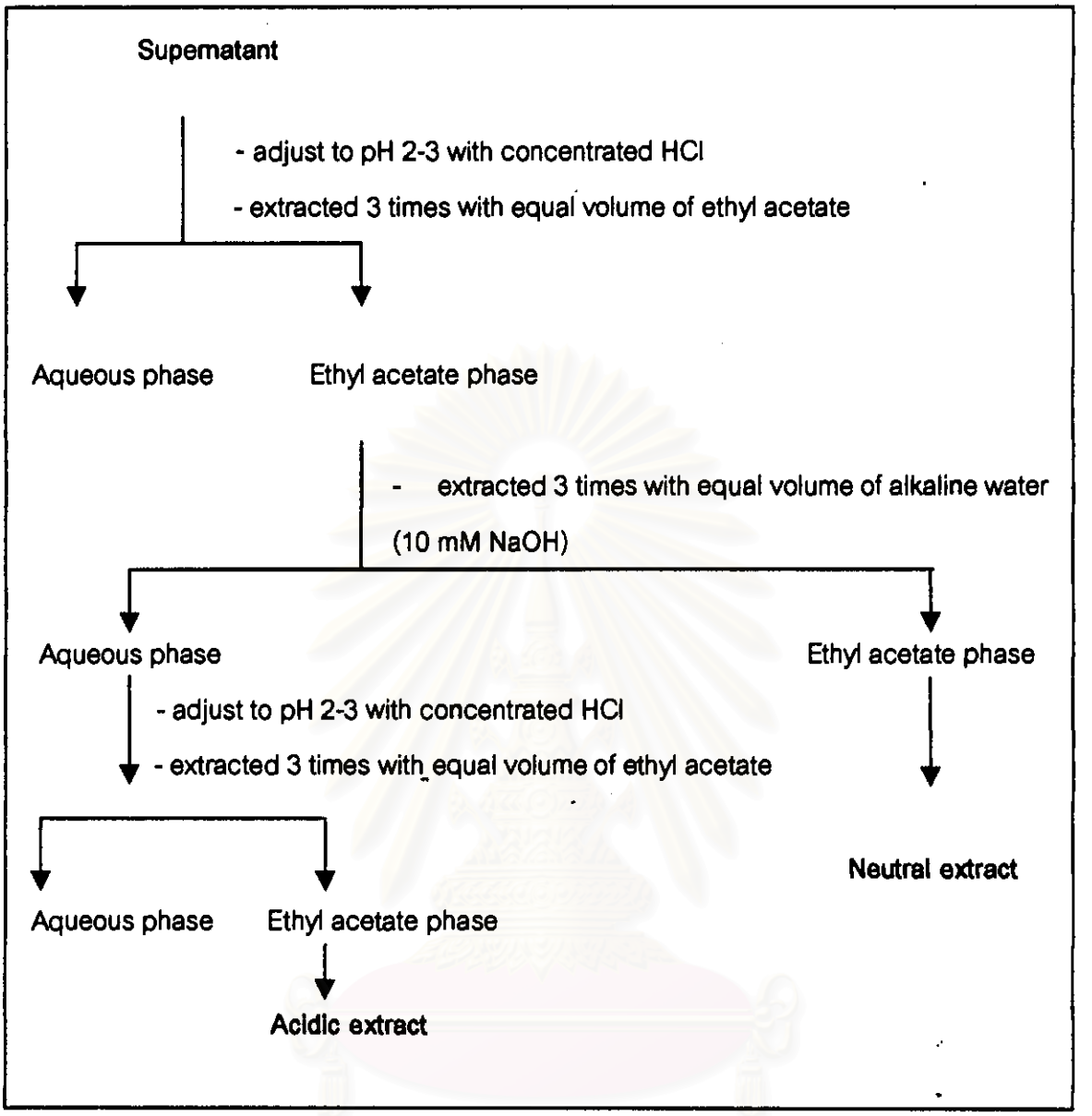

Figure 3.1 Metabolites extraction procedure (after Grifoll *et al.*, 1995).

The acidic extracts were dehydrated over anhydrous disodium sulphate (Na_2SO_4) and evaporated to 10 ml by using rotary evaporator (Iwaki Co. Ltd., Japan), then transferred to 13X100 mm tubes and evaporated to dryness under reduced pressure at 20^0C (EYELA, Japan). The residues were dissolved in suitable volume of methanol and used for further purification.

3.4.2 Silica gel open column chromatography

The crude acidic extracts were partially purified by silica gel open column chromatography. The column was prepared by placing a cotton at the bottom of column firstly, then filling the column with 150 ml *n*-hexane and slowly adding a slurry of silica gel (wakogel C-300, 45-75 μm) suspended in *n*-hexane.

The crude extract dissolved in methanol was mixed with zeolite at ratio of 1:5 by weight. After drying in desiccator, the mixture was added to the top of silica gel packed column. For purifying crude extract of CFMM/phe/pyr, ratio of the crude extract to the adsorbent was 1:25 by weight whereas for crude extract of CFMM/phe/flu, ratio of the crude extract to the adsorbent was 1:10 by weight.

The columns were eluted with increasing amount of ethyl acetate in *n*-hexane (stepwise 0-100 % by volume) (Table 3.2). The volumes of eluting solvents used for each fraction were 150 ml and 100 ml for crude extract of CFMM/phe/pyr and CFMM/phe/flu respectively.

The collected fractions were dehydrated over anhydrous Na_2SO_4 and subsequently evaporated to 10 ml by using rotary evaporator then evaporated to dryness under reduced pressure at 20°C. The residues were dissolved in suitable volume of methanol and used for further purification.

Table 3.2 Concentration ratios of the solvent system used for stepwise elution of the samples.

Fraction	% *n*-Hexane (v/v)	% Ethyl acetate (v/v)
1	100	0
2	90	10
3	80	20
4	70	30
5	60	40
6	50	50
7	40	60
8	30	70
9	20	80
10	10	90
11	0	100
12	100% Methanol	

3.4.3 Thin layer chromatography (TLC)

Analytical TLC

Analytical TLC was used to verify fractions collected from both siliga gel open column chromatography and HPLC whether they contain metabolites or not. Five microlitres of samples and authentic compounds; phenanthrene, pyrene and fluoranthene individually dissolved in methanol (1 mg/ml) were spotted at 1.5 cm from the lower edge on a silica gel precoated TLC plates which had length, width and gel thickness of 8 cm, 7 cm and 0.25 mm, respectively (Merck, Darmstadt, Germany). Distance between each spot was kept at 1 cm. TLC plate was developed in TLC chamber equilibrated with 10 ml of a mixture of toluene, dioxane and acetic acid at the

ratio of 90:25:4 (by volume), then air dried and visualized under ultraviolet light at 365 nm (UV lamp UVGL-15, UVP. USA).

Preparative TLC

Preparative TLC was used to purify metabolites which could not be purified after using HPLC. Two hundred microlitres of samples dissolved in methanol (1 mg/ml) were applied on a silica gel precoated TLC plates which had length, width and gel thickness of 20 cm, 20 cm and 1 mm, respectively (Merck, Darmstadt, Germany) as a line at 2 cm from the lower edge. After drying, TLC plate was developed in TLC chamber equilibrated with a *n*-hexane: ethyl acetate: acetic acid (50:50:0.5, v/v/v) solvent system, then air dried and visualized under ultraviolet light. The desired band was scraped off and eluted with water-saturated ethyl acetate. The resultant eluate was dehydrated over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 20^0C. Residue was dissolved in methanol and subjected to reversedphase HPLC with isocratic solvent system.

3.4.4 High performance liquid chromatography (HPLC)

The fractions containing metabolites after verification by TLC were further purified by the following HPLC preparative system.

Reversed phase HPLC system

For reversed phase HPLC system, samples were dissolved in methanol (1 mg/10 µl) whereas authentic compounds; phenanthrene, fluoranthene and pyrene were dissolved in methanol (1mg/2ml). 200 µl of sample was subjected to HPLC (Waters 600E chromatograph, Waters, MA, USA) equipped with an ODS-4253-D column (10 mm by 250 mm) (Senshu Scientific Co. Ltd., Tokyo, Japan). Mobile phase used were 50 to 100 % by volume of methanol in water with 1 % acetic acid (linear gradient within 30 min) (Appendix C) with flow rate of 3 ml/min. Elution was monitored at 254 nm by using

photodiode array detector (Waters 996, Waters, MA, USA) and samples were collected every minute. Fractions collected were evaporated to 500 µl under reduced pressure at 20°C, then run on TLC to characterize the products. Other three isocratic solvent systems; a methanol-water-acetic acid at 45:55:1 v/v/v, 50:50:1 v/v/v, 80:20:1 v/v/v were also employed using the condition mentioned above for further purification.

Normal phase HPLC system

Sample dissolved in 40% v/v of 2-propanol in *n*-hexane (1 mg/10µl) was subjected to HPLC equipped with Silica-4251-N column (10 mm by 250 mm) (Senshu Scientific Co. Ltd., Tokyo, Japan). Mobile phase used was a *n*-hexane: 2-propanol: acetic acid (94:5:1, v/v/v) isocratic solvent system with flow rate of 3 ml/min. Elution was detected at 254 nm by using photodiode array detector and collected every minute. Solvent of desired fractions collected was evaporated under reduced pressure at 20°C.

Conditions used for operating HPLC preparative system were summarized in Table 3.3

Table 3.3 HPLC operating conditions

Column	Reversed phase HPLC system; ODS-4253-D column (10 mm by 250 mm) (Senshu Scientific Co. Ltd., Tokyo, Japan) Normal phase HPLC system; Silica-4251-N column (10 mm by 250 mm) (Senshu Scientific Co. Ltd., Tokyo, Japan)
Column temperature	40°C

Table 3.3 (continued)

Injection volume	200 µl
Mobile phase system	Reversed phase HPLC system; 1) linear gradient solvent system; - 50 to 100 % by volume of methanol in water with 1 % acetic acid (linear gradient within 30 min) 2) isocratic solvent systems; - a methanol: water:acetic acid =45:55:1 by volume - a methanol: water:acetic acid =50:50:1 by volume - a methanol: water:acetic acid =80:20:1 by volume Normal-phase HPLC system; 1) isocratic solvent systems; - a *n*-hexane: 2-propanol: acetic acid = 94:5:1 by volume
Flow rate	3 ml/min
Detector	Photodiode array detector (Waters 996, Waters, MA, USA) Operating at 254 nm

3.4.5 Gas chromatography-Mass spectrometry (GC-MS)

Samples obtained after purification by HPLC were prepared by dissolving in ethyl acetate (10 µg/100 µl) and methylated with diazomethane (Appendix B) by dropping 1-2 drop of diazomethane into sample tubes, incubation at room temperature for 10 min, evaporation to dryness under reduced pressure at 20°C and redissolving in ethyl acetate (10 µg/50 µl). One microlitre of methylated metabolite was subsequently analyzed by JMS-Automass 150 GC-MS system (JEOL, Ltd., Tokyo, Japan) using DB-5

column having inside diameter, length and film thickness of 2.5 mm, 15m, 0.25 μm, respectively (J&W Scientific Inc., Köln, Germany). The column temperature was held at 80°C for 2 min and then programmed to 280 °C at rate of 16 °C/min, and held at 280°C for 3 min (Appendix D). Injection temperature used was 230°C. The head pressure of helium carrier gas was 65 kPa.

3.4.6 Nuclear magnetic resonance (NMR)

^{1}H NMR and ^{13}C-NMR spectra of each metabolite dissolved in 10 % v/v of deuterated methanol (CD_3OD) in deuterated chloroform ($CDCl_3$) (1mg/600μl) were recorded on JNM-A500 spectrometer (JEOL, Tokyo, Japan) operated at 500 and 125 MHz respectively. Two-dimensional NMR experiments including, heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (two- and three-bond) (HMBC), nuclear overhauser enhancement spectroscopy (NOESY), and ^{1}H-^{1}H shift- correlated spectroscopy (^{1}H-^{1}H COSY) were performed under operating conditions as mentioned above. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated solvent ($CDCl_3$,δ = 7.24 ppm)

3.5 Molecular genetics methods

3.5.1 Preparation of total DNA and plasmid DNA

3.5.1.1 Total DNA extraction

Total DNA extraction from *Sphingomonas* sp. P2 was performed as described by Ausubel *et al.* (1990) with slightly modification. Bacterial strain was inoculated into a 5 ml LB medium and incubated at 37°C on rotary shaker with 300 rpm for 18-24 hr. Cell suspension was poured into Eppendorf tubes and centrifuged at 5,000 rpm in a microcentrifuge (Beckman, USA) for 2 min. Cells were resuspended in 517 μl Tris-EDTA buffer pH 8 (TE buffer, Appendix B) by vortex, added with 50 μl of lysozyme solution (60 mg/ml in TE buffer) and incubated at 37 °C for 60 min. Thirty microlitres of 10% sodium

dodecyl sulphate (SDS) and 10 µl of proteinase K solution (10 mg/ml in sterilize distilled water) were added then mixed thoroughly and incubated at 37 °C for 60 min. One hundred twenty microlitres of 5 M NaCl (Appendix B) was given to the mixture and gently mixed by inverting 4-5 times, then 200 µl of hexadecyl trimethyl ammoniumbromide/ sodium chloride solution (CTAB/ NaCl solution, Appendix B) which was prewarmed at 65 °C was added. After mixed well this was incubated at 65 °C for 30 min. DNA was purified twice by an approximately equal volume of phenol:chloroform (25:25, Appendix B). DNA in aqueous phase was precipitated with 0.6 volume of isopropanol. The solution was mixed well by inverting until a stringy white DNA precipitate appears. The precipitate was collected by centrifugation at 13,000 rpm for 1 min at room temperature and washed with 70% ethanol and centrifuged for 5 min. After removal of the supernatant, pellet was dried in desiccator and redissolved in 100 µl of TE buffer containing 0.2 µl of DNA free RNase solution (10 mg/ml in 0.01M sodium acetate pH 5.2), followed by incubation at 37 °C for 60 min. DNA obtained could be stored at 4 °C until used.

3.5.1.2 Plasmid extraction

Plasmids were extracted by rapid alkaline extraction method modified from Sambrook *et al.*, (1989). Colony of *E. coli* JM109 containing plasmid was inoculated in a 5 ml LB medium supplemented with 100 µg/ml of ampicillin and incubated at 37°C on rotary shaker 200 rpm for 18-24 hr. After centrifugation in microcentrifuge at 5,000 rpm for 2 min, the cell was resuspended in 100 µl Tris-EDTA/glucose buffer pH 8 (TEG buffer, Appendix B), mixed by vortex and stored at room temperature for 5 min. The tubes were then added with 200 µl of freshly prepare lysis solution sodium hydroxy-sodium dodecyl sulphate solution (NaOH-SDS, Appendix B), mixed by inverting and incubated on ice for 5 min. One hundred fifty microlitres of 5 M potassium acetate solution (KOAc, Appendix B) was add, suspended well and incubated on ice for 10 min. The mixture was extracted with 450 µl (25:25) phenol:chloroform solution and centrifuged at 13,000 rpm for 5 min. Aqueous phase was transferred to new Eppendorf tube. Plasmid was precipitated with 450 µl isopropanol and collected by centrifugation

at 13,000 rpm for 5 min. The supernatant was removed by autopipette, followed by addition of 400 µl of 70% ethanol. Plasmid was repelleted by centrifugation at 13,000 rpm for 5 min. Pellet was resuspended in100 µl of TE buffer mixed with 25 µl of 12.5 M of lithium chloride (LiCl) and incubated on ice for more than 15 min. After centrifugation at 15,000 rpm for 15 min at 4°C, supernatant was transferred into fresh Eppendorf tube and added with 300 µl of TE buffer. Plasmid was precipitated by adding with 1 ml of chilled absolute ethanol, followed by centrifugation at 13,000 rpm for 30 min at 4 °C, then washed with 400 µl of chilled 70% ethanol and repelleted by centrifugation at 13,000 rpm for 30 min at 4 °C. Supernatant was removed and pellet was dried. Plasmid was resuspended in 50 µl of TE buffer containing 0.1 µl of 10 mg/ml DNase free RNase solution. The solution was incubated at 37 °C for 60 min. Plasmid obtained could be stored at -20 °C until used.

3.5.2 Recombinant DNA techniques and DNA analyses

3.5.2.1 Restriction endonuclease digestion

The total DNA of *Sphingomonas* sp. P2 and plasmids; pUC18, pUC19, pUC119 were completely digested with one of restriction endonucleases; *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sph*I or *Sal*I using appropriate buffer provided with enzyme under condition recommended by manufacturer. The digested total DNA was characterized in 1% (w/v) agarose gel. 1-10 kb of DNA-fragments were extracted from agarose gel by using Gene extraction kit. The digested plasmid was purified by phenol:chloroform extraction and ethanol precipitation then dephosphorelated with calf intestinal alkaline phosphatase (CIAP) according to manufacturer's instruction, followed by purification again.

3.5.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to characterize sizes of DNA fragments after digestion by restriction endonuclease. One percent (w/v) agarose gel in 1×Tris-acetate/EDTA buffer (TAE buffer, Appendix B) was prepared by heating until

agarose was completely melted. The melted agarose gel was cooled down to approximately 50°C and pored in chamber with comb. DNA sample was mixed with 0.1 volume of gel-loading buffer containing bromophenolblue (Appendix B) and loaded into slot of agarose gel. Submerged gel electrophoresis was carried out at 50 or 100 V for 30-60 min in minigel electrophoresis chamber with 1×TAE as running buffer (Mupid, minigel electrophoresis set, Tokyo Co. Ltd., Japan). Gel was stained with ethidium bromide (0.5 μg/ml in distilled water) for 15 min and destained with distilled water for 5 min. DNA was visualized under UV-light transilluminator (TOYOBO Co. Ltd., Japan). Marker 6 as presented in Figure 3.4 was used as a standard DNA marker.

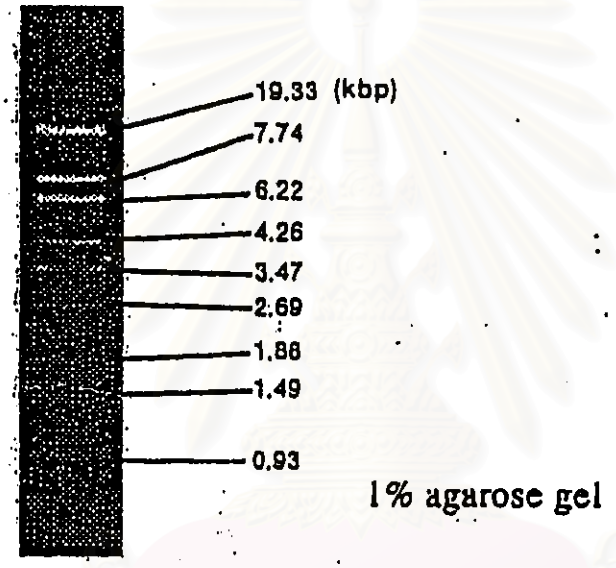

Figure 3.4 Marker 6

3.5.2.3 Recovering DNA fragment from agarose gel

DNA fragments were extracted from agarose gel by using Gene extraction kit Protocol according to the manufacturer's instructions was used. The area of gel containing the DNA fragment was cut using a clean and sharp blade. Gel (≤ 400 mg) was placed into a 2 ml polypropylene tube. Thirty microlitres of gel solubilization buffer (provided) was added for every 10 mg of gel. The solution was incubated at 50 °C for 15 min and mixed every 3 min. After gel slice was dissolved, incubated for further 5 min. The solution was pipetted into a spin cartridge placed into a 2 ml wash tube, followed by centrifugation at 13,000 rpm for 1 min. The flow-through was discarded.

Seven hundred microlitres of wash buffer (provided) was added to the spin cartridge and incubated at room temperature for 5 min then centrifuged at 13,000 rpm for 1 min. The flow-through was discarded. Then centrifuged at 13,000 rpm for 1 min again. The spin cartridge was placed into a new 1.5 ml Eppendorf tube. Fifty microlitres pre-warmed TE buffer was added directly to the center of the spin cartridge, incubated at room temperature for 1 min. Eppendorf tube with spin cartridge was centrifuged at 13,000 rpm for 2 min. The DNA obtained could be stored at -20°C until used.

3.5.2.4 Ethanol precipitation

This method used for concentrating nucleic acid or changing the dissolving buffer. A 0.1 volume of 3 M sodium acetate (Appendix B) was added into DNA solution then mixed gently, followed by addition with 2 volume of chilled absolute ethanol then incubated at -20°C for at least 30 min. The recovered DNA pellet was collected by centrifugation at 15,000 rpm at 4 °C for 30 min and the pellet was washed with chilled 70% ethanol then centrifuged at 15,000 rpm at 4°C for 30 min. Pellet was completely dried and finally dissolved in an appropriate volume of TE buffer.

3.5.2.5 Ligation of DNA fragment into plasmid vector

1-10 kb of DNA-fragments were ligated into appropriately digested and dephosphorelated plasmids using the protocol according to the manufacturer's instructions. DNA insert and plasmid vector were mixed together with the molar ratio was 3:1 and 5:1 and finally mixed with same volume of Ligation-solution (provided). The reaction mixture was incubated at 16°C for 12-16 hr then served for transformation into competent *E. coli*.

3.5.3 Transformation of recombinant plasmid into *Escherichia coli*

3.5.3.1 Preparation of competent *Escherichia. coli*

Preparation of competent *E. coli* was performed following method described by Hanahan (1989). *E. coli* JM109 was streaked directly from a glycerol stock onto the surface of an ψb medium plate (Appendix A) and incubated at 37°C for 16 hr. A colony was inoculated into 5 ml of ψb medium and grown at 37°C in shaker until OD_{550} was 0.3. Cells were transferred into 100 ml of ψb medium and shaked at 37°C until OD_{550} was 0.48. The culture cell tube was kept on ice for 5 min and centrifuged at 3,000 rpm at 4°C for 5 min. The pellet was resuspended in 40 ml of TfbI solution (Appendix B) by vortexing and incubated on ice for 5 min then centrifuged at 3,000 rpm at 4°C for 5 min. The pellet was resuspended in 4 ml of TfbII solution (Appendix B) and kept on ice for 15 min then dispensed into aliquots (50 µl in 1.5 ml tube). The aliquots were snap-freezed in dry ice and stored at -70° until used.

3.5.3.2 Transformation of *E. coli* with plasmid DNA

Standard method (Sambrook *et al.*, 1989) was used to transformed plasmid DNA into competent *E. coli* JM109. Plasmid DNA or ligated products were mixed with 50 µl of the stored competent cells and the tubes were kept on ice for 30 min. Then the mixture was subjected to heat shock at 42°C for exactly 90 sec and immediately chilled into a water-ice bath for 2 min. One ml of 2×YT medium was added into the mixture tubes and incubated at 37°C for 1 hr. A 50- 200 µl of the mixture was spreaded onto LB agar plates containing X-gal, IPTG and ampicillin. The plates were incubated at 37°C for 18-24 hr. White colonies were picked and replicated on LB or 2×YT plates supplemented with ampicillin and served for further screening method.

3.5.3.3 Screening of aromatic oxygenase and *meta*-cleavage dioxygenase activities

Assay for aromatic oxygenase activity (indigo formation)

Aromatic oxygenase activity was assayed in liquid 2×YT medium as described by Ensley *et al.* (1983); white colonies were inoculated into 5 ml 2×YT medium containing 0.1mM of IPTG and 100 μg/ ml of ampicillin and incubated at 37°C, 200 rpm for 16-18 hr. Dark blue of indigo colour indicated the aromatic oxygenase activity. *E. coli* carrying pDI1 plasmid was used as a positive control.

Assay for meta-cleavage dioxygenase activity

Meta-cleavage dioxygenase activity (extradiol dixoygenase activity) was assayed as described by Kimura *et al.* (1996); 20 μl of 50 mM 2,3-dihydroxybiphenyl dissolved in DMSO were dropped on top of each colony grown on LB agar plates supplemented with 0.1mM of IPTG and 100 μg/ ml of ampicillin. Changing of colony's colour to yellow indicated *meta*-cleavage dioxygenase activity. *Sphingomonas* sp. P2 was used as a positive control.

3.5.4 Polymerase chain reaction (PCR)

Primers

Primers used in the study on dioxygenase gene by PCR are shown in Table 3.3

Table 3.3 Primers used in PCR experiment

Primer	Reference/source
nahAc primers: forward primer; 5'TGGCGATGAAGAACTTTTCC	Lloyd-Jones *et al.*, 1999
(Position in *nahAc* gene: nt 63-82)	
reverse primer; 5'AACGTACGCTGAACCGAGTC	
(Position in *nahAc* gene: nt 1036-1055)	
phnAc primers: forward primer; 5'TTCGAGCTGGAATGTGAGC	Lloyd-Jones *et al.*, 1999
(Position in *phnAc* gene: nt 82-100)	
reverse primer; 5'AATAACCGGCGATTCCAAAC	
(Position in *phnAc* gene: nt 1057-1076)	
modified pPAH primers	Hedlund *et al.*, 1999
forward primer; 5'GGYAAYGCNAAAGGNTTC	
GTNTGYWSHTAHTAYCAY	
reverse primer; 5'CCANACTTCNGTNGTRTT	
HGCATCRATSGGRTKCAA	
Rieske primers: forward primer; 5'TGYCGSCAYCGNGGNA	Hamann *et al.*, 1999
(Position: 240-255)	
reverse primer; 5'CCANCCRTGRTARCTRCA	
(Position: 300-317)	

IUB CODES

A = Adenosine, C = Cytidine, G = Guanosine, T = Thymidine, R = A or G (puRine),

Y = C or T (pYrimidine), K = G or T, M = A or C, B = C, G or T, D = A, G or T,

H = A, C or T, V = A, C or G, S = G or C (Strong-3H bonds)

W = A or T (Weak-2H bonds), N = aNy base

PCR conditions

Amplification was performed with 20 pmol for oligonucleotide primers or 50 pmol for degenerate primers of the forward and reverse primers in total volume of 100 µl with 200 mM dNTP, 2.5 mM $MgCl_2$, 2.5U *Tag* DNA polymerase and template DNA at 0.2 µg. Polymerase chain reaction was carried out in a GeneAmp PCR system 2400 (Perkin Elmer Cetus, CT, USA). PCR-program was 5 min at 94°C, followed by 30 cycles (nahAc primers and phnAc primers) or 40 cycles (modified pPAH primers and Rieske primers) of 94°C for 2 min, 55°C (nahAc primers and phnAc primers) or 50°C (modified pPAH primers) or 42°C (Rieske primers) for 1 min, 72 °C for 1 min, maximal ramp rates throughout, with the final 72 °C segment of the cycle extended to 10 min before cooling to 4 °C.