CHAPTER 5

DISCUSSION AND CONCLUSION

Phenanthrene, pyrene and fluoranthene are U.S. Environmental Protection Agency priority pollutants because of their toxicity (Patnaik, 1992). Although they are not genotoxic, they have structures that shared with several carcinogenic PAHs such as benzo(a)pyrene indeno(1,2,3-cd)pyrene (Shutherland *et al.*, 1995). Furthermore, phenanthrene has been found to be toxic to aquatic organisms (Kusk, 1981 and Pipe and Moore, 1986) while pyrene and fluoranthene are considered to be highly recalcitrant and may persist in the environment indefinitely (Cerniglia, 1992). These compounds are commonly found as major PAH contaminants in environment originating from natural and anthropogenic pyrolysis of organic matters; forest fires, automobile exhaust, coat refining processes and the oil industry (Wilson and Jones, 1993). There is also a potential for the bioaccumulation of PAHs in to food chains (Means *et al.*, 1980). Their biodegradation by specific microorganisms may be both a cost-effective and promising means of bioremediation of PAHs in the environment (Cerniglia, 1992). Since many sites are contaminated with a wide variety of PAHs including recalcitrant compounds (Wilson and Jones, 1993), co-metabolism may play an important role in the degradation of such compounds (Mueller *et al.*, 1989). With the aim of remediation and removal of these compounds from the environment, it is essential to understand the degradative pathways of these compounds. Complete metabolic pathways are needed since end toxic metabolites may be produced by incomplete pathways (Timmis and Pieper, 1999).

As described in chapter 1 and 3, *Sphingomonas* sp. P2 has possibility of co-metabolizing pyrene and fluoranthene by using phenanthrene as a growth substrate (Supaka *et al.*, 1999). Consequently, this study has been attempted in order to identify metabolites of pyrene and fluoranthene degradation via co-metabolism with phenanthrene by *Sphingomonas* sp. P2.

In order to investigate co-metabolic metabolites from pyrene and phenanthrene, cultivation of *Sphingomonas* sp. P2 in 15 l-CFMM/phe/pyr medium was carried out. After 4 days incubation period, five compounds were isolated and purified

by silica gel open column, thin-layer and high performance liquid chromatography and identified as coumarin, 1,5-dihydroxy-naphthoic acid, 5,6-benzocoumarin, 7,8-benzocoumarin and 1-hydroxy-2-naphthoic acid, respectively on the basis of gas chromatography-mass spectral, ^{1}H and ^{13}C nuclear magnetic resonance spectral analyses.

On the basis of their chemical structures, these compounds were characterized as metabolites formed from phenanthrene degradation which proposed by Davies and Evans (1964), Evans *et al.* (1965) and Kiyohara *et al.* (1976). Based on the metabolites isolated, the phenanthrene degradation pathway of *Sphingomonas* sp. P2 grown in the present of pyrene could then be proposed as shown in Figure 5.

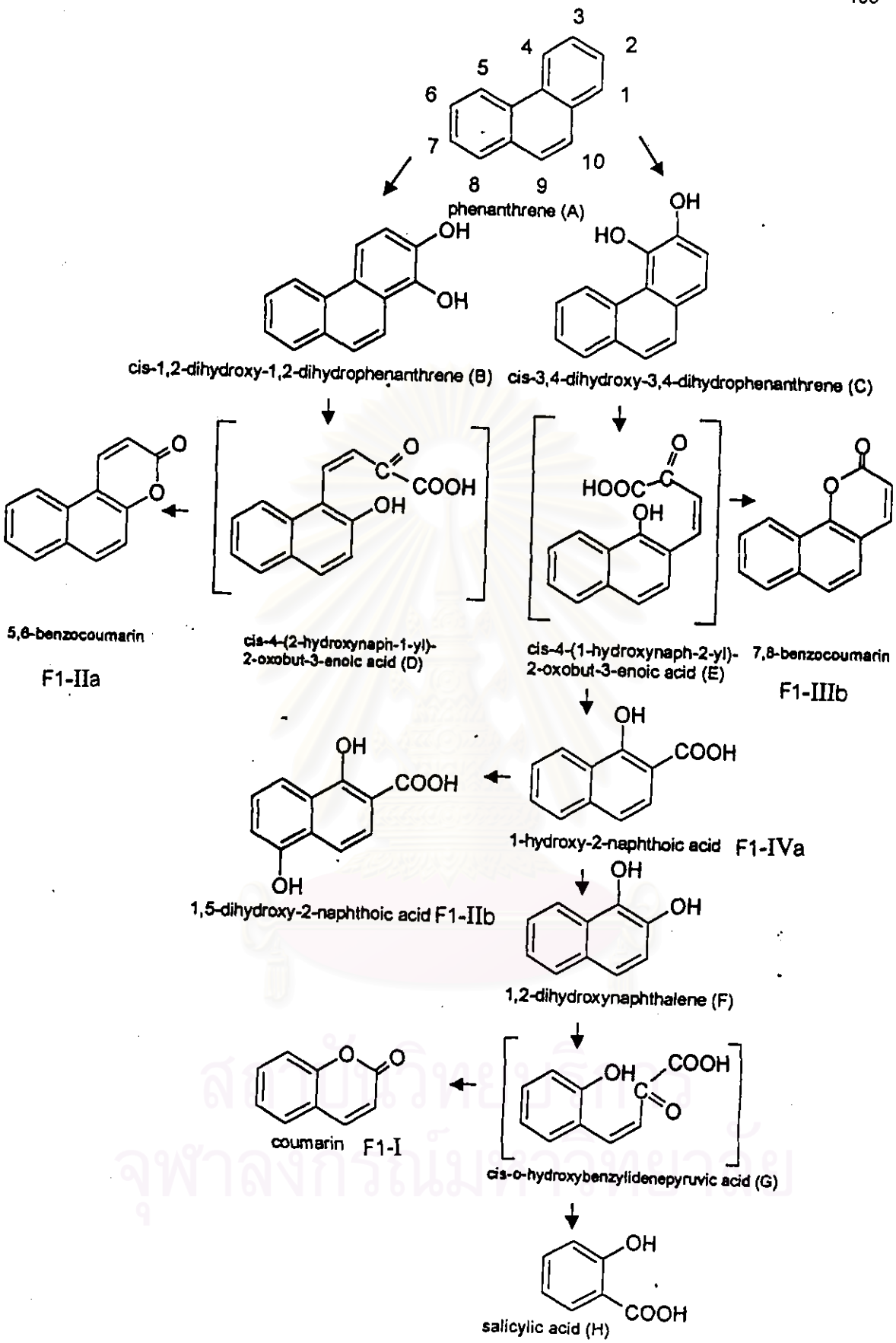

Figure 5 Proposed pathway for phenanthrene degradation by *Sphingomonas* sp. strain P2 grown in the presence of pyrene

The first step of reaction, phenanthrene was dioxygenated at position 1,2 and 3,4 then dehydrogenated and subsequently ring was cleaved. As the ring-cleavage products, *cis*-4-(2-hydroxynaph-1-yl)-2-oxobut-3-enoic acid and *cis*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid (compounds D and E in Figure 5) were unstable themselves, thus they can be readily decarboxylated and oxidized via nonenzymatic conversion to 5,6-benzocoumarin (F1-IIIa) and 7,8-benzocoumarin (F1-IIIb), respectively. While the latter compound has been previously described by Evans *et al.* (1965), the former compound the 5,6-benzocoumarin is by for the first ever reported. This finding confirms the possibility that the initial dioxygenation of phenanthrene can also be occurred at 1,2-positions which generally, it is metabolized by initial dioxygenation at 3,4-positions (Evans *et al.*, 1965; Kiyohara *et al.*, 1976 and 1978). Most of the metabolites in this pathway were already elucidated, but little is known about degradation via initial dioxygenation at 1,2-positions. Previously, Jerina *et al.* (1976) reported the dioxygenation of phenanthrene at 1,2-positions which give a small amount of *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene by *Beijerinckia* sp. B-836 and *Pseudomonas putida* 119. Another compound from this route in *Pseudomonas putida* AC10 carrying *pah* gene cluster of *P. putida* OUS82 is 2-hydroxy-1-napthoic acid which has been described by Kiyohara *et al.* (1994). But in the present work, 2-hydroxy-1-naphthoic acid could not be isolated, even though a 30-litre fermenter was used in the cultivation of *Sphingomonas* sp. P2. In this strain, the substrate specificity of isomerase or aldolase, responsible for further catabolism of ring-cleavage compound of phenanthrene and naphthalene, may allow the degradation via initial dioxygenation at the 3 and 4 positions of phenanthrene but not at the 1 and 2 positions.

The formation of 1-hydroxy-2-naphthoic acid (F1-IVa), the key metabolite of phenanthrene catabolism in many bacteria has been revealed by several researchers, for example, Evans *et al.* (1965) and Kiyohara *et al.* (1976) reported the finding of this compound in phenanthrene metabolism pathway by soil Pseudomonads and *Aeromonas* sp. Furthermore, *Alcaligenes dinitrificans* WW1 (Weissenfels *et al.*, 1991), *Pseusomonas fluorescens* 5R (Menn *et al.*, 1993) and *Pseudomonas putida* NCIB 9816 (Yang *et al.*, 1994) were also metabolized phenanthrene via 1-hydroxy-2-naphthoic acid.

The formation of this metabolite was from hydration of *cis*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid then aldol clevage and finally dehydrogenation.

In the case of coumarin (F1-I), this compound formed from nonenzymatic conversion of *cis*-o-hydroxybenzylidenepyruvic acid (H) (Davies and Evans, 1964).

In this study, a large amount of 1,5-dihydroxy-2-naphthoic acid (metabolite F1-IIb) was found and identified as a major metabolite. As some initial dioxygenase attacks a wide variety of aromatic compounds (Kiyohara *et al*, 1994), phenanthrene dioxygenase or other dioxygenase in *Spingomonas* sp. P2 may catalyze *cis*-dihydroxylation at the 5 and 6 positions of 1-hydroxy-2-naphthoic acid. This compound might be transformed to give 1,5-dihydroxy-2-naphthoic acid due to the fact that dihydrodiol forms of aromatic compounds easily undergo the specific loss of water to yield monohydroxylated aromatic ring compounds upon extraction with ethyl acetate under acidic conditions (pH 2-3) (Nojiri *et al.*, 1999).

In order to elucidate co-metabolic metabolites of fluoranthene and phenanthrene, cultivation of *Sphingomonas* sp. P2 in 15 l-CFMM/phe/flu was then performed. After 4 days, isolation and purification of metabolites were carried out. The HPLC elution profiles of major metabolites obtained from this experiment were almost the same as those of pyrene and phenanthrene experiments. Different peaks were chosen for further analyses. Unfortunately, it was found that all of them were minor components, thus the quantities of such products were not sufficient to be performed additional analyses. Nevertheless, major metabolites formed from this experiment were similar to those from pyrene and phenanthrene experiments as described above.

These results revealed that no metabolites formed from pyrene or fluoranthene co-metabolism with phenanthrene by *Sphingomonas* sp. P2 in acidic extract could be identified. Although one of these metabolites (1-hydroxy-2-naphthoic acid) has also been found as metabolite in pyrene degradation pathway of *Mycobacterium* sp. strain PYR-1 and ATCC700033 (Heitkamp *et al.*, 1988; Cerniglia, 1992 and Dean-Ross and Cerniglia, 1996), but other high molecular weight intermediates from those pathways were not observed in this study.

Co-metabolism of pyrene and fluoranthene via phenanthrene as a growth substrate by *Sphigomonas* sp. P2 was possible due to the change of culture broth colour from colourless to green (in the medium containing pyrene and phenanthrene) and from colourless to red (in the medium containing fluoranthene and phenanthrene), while in the medium containing only phenanthrene gave yellow colour. The green and the red compounds were expected to be the metabolites formed during the degradation of pyrene and fluoranthene by the co-metabolic reaction with phenanthrene.

However, these metabolites could not be purified as green and red colour were disappeared during extraction. These phenomena might suggest the possibility that the compounds possessing green or red colour were unstable either to the process used for the extraction. Or else, it is possible that the metabolites might be dissolved in neutral extract as these metabolites from pyrene or fluoranthene degradation. Heitkamp *et al.* (1988b) demonstrated that *cis*- and *trans*-4,5-pyrene dihydrodiol, pyrenol and 4-hydroxyperinaphthenone, metabolites formed by incubation of *Mycobacterium* sp. in minimal medium containing pyrene and cofactor were isolated from neutral extract resemble that of 9-hydroxyfluorene, 9-fluorenone and 7-methoxy-8-hydroxy-fluoranthene which were metabolites from degradation of fluoranthene by *Mycobacterium* sp. (Kelley *et al.*, 1993). Generally, compounds isolated from neutral extract are hydroxy or ketone compounds whereas compounds isolated from acidic extract are carboxy derivatives. Interestingly, three ketone compounds were also isolated from acidic extract in the present study, these ketone compounds were formed from nonenzymatic conversion of carboxy compounds during chemical isolation (Davies and Evans, 1964 and Evans *et al.*, 1965). Nevertheless, only the acidic extract being chosen for metabolite identification is simply that carboxy compounds obtained from acidic extract can served as a model for understanding of the ring-cleavage of the substrates.

Possibility also remains that co-metabolic products did not accumulate in significant amount within that time.

To our knowledge, 5,6-benzocoumarin and 1,5-dihydroxy-2-naphthoic acid identified in this study are novel metabolites in the degradation of phenanthrene by bacteria. This finding strongly supports that in addition to the 3,4-positions, the initial dioxygenation of phenanthrene can also be occurred at 1,2-positions. Hence, the informations obtained from the experimental results suggested that dioxygenase of *Sphingomonas* sp. P2 had broad substrate specificity making the study of dioxygenase genes in this strain interesting.

The second objective of this study was to preliminary study of dioxygenase genes in *Sphingomonas* sp. P2. It has been demonstrated that dioxygenase which catalyses the insertion of molecular oxygen into aromatic substrate is the first enzyme of PAH degradation pathway (Butler and Mason, 1997). In order to verify whether dioxygenase of *Sphingomonas* sp. P2 similar to that of other bacteria which were previously studied, additional experiment was carried out by using PCR with two of primer pairs which targeting genes that encode the iron-sulfur protein large subunit (ISPα). From the primers used, one can specifically target the *nah*-like gene (Lloyd-Jones *et al.*,1999) or *phn*-type phenanthrene catabolic genes (Lloyd-Jones *et al.*,1999). The amino acid sequence homology of these two genes is only 56% (Laurie and Lloyd-Jones, 1999b). PCR amplification with total DNA of *Sphingomonas* sp. P2 as template gave no PCR product.

Thus other two of degenerate primer pairs which target different position of consensus sequences of genes encoding the iron-sulfur protein large subunit (ISPα); pPAHforward/ pPAHreverse primers (Hedlund *et al.*, 1999) and Rieskeforward/ Rieskereverse primers (Hamann *et al.*, 1999) were also used to detect dioxygenase gene. However, PCR product still could not be obtained.

The absence of PCR product in *Sphingomonas* sp. P2 could be due to the dioxygenase from gene in this strain has less nucleotide sequence-similarity or even non-homology with those of *nah*-like group and *phn*-type.

These finding are not totally unexpected since numerous reports have indicated genetic diversity among degradative genes. Hamann *et al.* (1999) could not obtain PCR product from *Gordona* sp., *Mycobacterium* sp., *Pseudomonas* sp. and *Rhodococcus* sp. which were able to assimilate phenanthrene, anthracene, pyrene

and fluoranthene by using ndoB-specific primers derived from *ndoB* gene in *P. putida* NCIB9816. Lloyd-Jones *et al.* (1999) demonstrated that *nahAc* could be amplified from only 45% (20/44) of naphthalene-degrading bacteria and *phnAc* could not be amplified from any naphthalene-degrading bacteria (44 isolates) or phenanthrene (35 isolates) while Laurie and Lloyd-Jones (1999b) showed that no PCR product was amplified from *Burkholderia* sp. RP007 by using primers based on the *pah* genes of *P. putida* OUS82 and the *nah* genes of *P. putida* G7.

Nevertheless, other experiments such as southern hybridization using probes based on dioxygenase genes or shot gun cloning using specific activity of these enzymes for screening of the positive clone should be carried out to provide enough information about genes involved in phenanthrene metabolism in *Sphingomonas* sp. P2.

Shot gun cloning may be a promising method for obtaining certain particular genes (Fowler *et al.*, 1993), thus shot gun cloning was employed by the ligation of 1-10 kb of DNA fragments from *Sphingomonas* sp. P2. digested with one of these restriction enzymes; *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sph*I or *Sal*I, with one of plasmids; pUC18, pUC19 or pUC119 digested with similar enzyme used for fragment generations. The recombinant plasmids were transformed into *E. coli* JM109. Approximately 3,000 white colonies were obtained from the selection media (LB-Ap-IPTG/X-Gal plates).

These white clones were further screened for aromatic oxygenase activity (indigo formation) since genes involved in upper pathways of naphthalene and phenanthrene degrading bacteria could be isolated by screening for indigo (Kurkela *et al.*, 1988; Kiyohara *et al.*, 1994; Goyal and Zylstra, 1996; Laurie and Lloyd-Jones, 1999b). However, no positive clone was found in this study.

Furthermore, *meta*-cleavage dioxygenase activity was also used for screening positive clone, since this method was reported as valuable method to obtain gene involved in *meta*-cleavage operon of PAH degrading bacteria (Laurie and Lloyd-Jones, 1999a). Unfortunately, no positive clone could be detected in this study.

According to Fowler *et al.* (1993), the total number of about 1,800-18,000 clones is needed to have one clone with particular gene from the insert site of 1-10 kb. However, the target gene encoding multicomponent of dioxygenase had the size approximately

3.3 kb (Kiyohara *et al.*, 1994) therefore, the number of about 6,000 is needed in order to obtained one positive clone. The present work obtained only 3,000 white colonies which may be the reason why a positive clone could not yet be obtained.

Further attempts to study genes involved in phenanthrene degradation of this strain can be done by other methods. Lloyd-Jones *et al.* (1999) used southern hybridization with probe base on *nahAc* and *phnAc* genes for detection of gene encoding ISPα of dioxygenase from naphthalene and phenanthrene degrading bacteria, Yen and Gunsalus (1982) studied on organization and regulation of genes for naphthalene metabolism in *Pseudomonas* sp. by using transposon mutagenesis method. Foght and Westlake (1996) also used transposon mutagenesis to reveal that *Pseudomonas fluorescens* LP6a which utilizes naphthalene, phenanthrene and anthracene as sole source of carbon and energy has two gene clusters corresponding to naphthalene degradation upper and lower pathway operons found in *P. putida* G7. Despite these methods, transposon mutagenesis, can also be used to obtain mutants lacking genes encoding enzymes in part of metabolic pathway, such that the study of these mutants would be an alternative method to understand genetic of phenanthrene degradation in *Sphingomonas* sp. P2.