CHAPTER II

LITERATURE REVIEW

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbon compounds with multiple benzene rings. PAHs molecules are made up of two or more benzene rings to form linear, angular or cluster structure. PAHs are ubiquitous environmental contaminants that are formed by the incomplete combustion of organic materials, such as wood , charbroiled meat or fossil fuels (Volkering et al., 1992; Freeman and Cattell 1990; Tan et al. 1992). They are typical components of asphalts, crude oil, coal tar, creosote, petroleum and petroleum-derived products. PAHs are generally occured as a complex mixtures, not as single compounds. They enter the environment mostly as releases to air from volcanoes, forest fires, (Cerniglia, 1992), residential wood burning (Perwak et al., 1982) and exhaust from automobiles and trucks. They are found throughout the environment in the air, surface water, groundwater and soil. They can be found in the air, either attached to dust particles or as solids in soil or sediment. However, the compounds are also found in the gaseous phase (Yang et al. 1991). They can enter surface water through discharges from industrial plants and waste water treatment plants, and they can be released to soils at hazardous waste sites if they escape from storage containers.

The following 16 PAHs; naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, benz[*a*]anthracene, chrysene, pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*ah*] anthracene, benzo[*ghi*]perylene and indeno[1,2,3*cd*]pyrene, are classified by the U.S. Environmental Protection Agency (EPA) as priority toxic pollutants (U.S. EPA, 1980) because of their carcinogenicity, mutagenicity and toxicity. These 16 PAHs were chosen to be included in this list because they are suspected to be more harmful than some of the others, they exhibit harmful effects that are representative of the PAHs and there is a greater chance that human could be exposed to these PAHs than to the others and of all the PAHs analyzed. In Thailand, PAHs are also listed as hazardous substance type 7 (mutagen) in Hazardous Substance Act 1992 (Hazardous Substance Act, 1992).

The fate and transport of PAHs in the environment depends on their properties such as solubility and vapor pressure. Normally, PAHs have low solubility in water. They are present in air as vapors or stick to the surfaces of dust or solid particles. Certain PAHs evaporate into the air from surface waters, but most stick to solid particles and settle to the bottom of rivers or lakes. In soils, PAHs are most likely to adsorb tightly to soil particles. Some PAHs evaporate from surface soils to atmosphere. Certain PAHs in soils contaminate groundwater. PAHs can be degraded in the environment by reacting with sunlight and other chemicals in the air. Degradation of PAHs in soil and water generally takes weeks to months and is caused primarily by the actions of microorganisms (Irwin, 1997).

Acenaphthylene

Acenaphthylene, a low molecular weight, two-rings polycyclic aromatic hydrocarbon, consists of two benzene rings and a cyclopentene ring in cluster structural configuration as shown in Fig. 2.1.

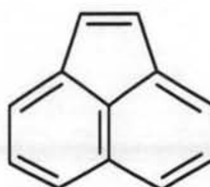

Fig. 2.1 Molecular structure of acenaphthylene

Acenaphthylene is a pollutant of concern due to its potential for causing adverse effects to humans as well as aquatic organisms (Lederer, 1985). This compound has been classified by the U.S Environmental Protection Agency as a priority toxic pollutant because of its toxicity and mutagenicity (Lavoie and Rice, 1988). The drinking water criterion for human health of acenaphthylene is less than 1 ppb and the sediment Effects Range Low (ERL), relating to estuarine ecological health, is 44 ppb. These indicate strong concern for human health and biological effects (Roy, 1997). Moreover, the solubility of this compound is greater than those of some heavier PAHs which increases its potential mobility and risk in certain habitats.

Acenaphthylene is found as constituent of creosote, petroleum, petroleum-derived products and tobacco smoke. Emissions from petroleum refining and coal tar distillation processes are major contributors of acenaphthylene to the environment. Because of the widespread use of materials containing acenaphthylene, its release to the environment also occurs during improper handling, processing, transportation and disposal through municipal waste water treatment facilities and municipal waste incinerators via stack emission and as a component of grate and fly ash. It is also found in soil, sediment and water in petroleum contaminated sites and as one of the major components of PAHs in urban air of Thailand (Wise et al., 1988; Panther et al., 1996). Since acenaphthylene and other PAHs exhibit carcinogenic, mutagenic and toxic properties, they bring about serious public concern about their presence in the environment and subsequent remediation process. There are several approaches to remediate the environment contaminated with acenaphthylene and other PAHs including physical methods such as filtration, drying, solvent extraction, absorption, chemical method (Cockson, 1995), incineration, landfill and biological method. Bioremediation, the method of using microorganisms to transform hazardous contaminants into less toxic compounds, is emerging as a feasible technology for the treatment of PAHs contaminated environments (Mueller et al., 1989). Several advantages of bioremediation make it an interesting method. Using the natural process has the advantage of being safe and environmental friendly. Microorganisms are capable of breaking down practically all hydrocarbons contaminated in the natural environment. Some bacteria can mineralize PAHs completely to water and carbon dioxide for their growth (Wison and Jones, 1993) while some bacteria transform PAHs to less toxic substances. Another advantage of bioremediation over other methods is that the pollutants are actually degraded or transformed instead of simply being moved from one media to another such as landfill. A final and often most important factor is the cost. Although comparisons of cost and effectiveness of bioremediation strategies strongly depend on each problem, the cost savings are estimated to range from 30-90% (Patrinos, 2005). Some bioremediation technologies such as bioventing, landfarming and biostimulation involve the modification of the environment to stimulate the activity of indigenous bacteria capable of bioremediation. Biostimulation includes addition of nutrient, oxygen and pH adjustment of the environment while bioaugmentation introduces other microorganisms that have ability to degrade the pollutants into the contaminated site.

PAHs Catabolic Pathway

The biochemical pathways for the biodegradation of aromatic compounds have been well described (Gibson and Subramanian, 1984). Naphthalene is a 2-benzene ring polycyclic aromatic hydrocarbon and serves as a model for understanding the properties of a large class of environmentally prevalent polycylic aromatic hydrocarbons. Yen and Gunsulas (1982) reported that *Pseudomonas putida* G7, able to utilize naphthalene as a sole source of carbon, could metabolize naphthalene to salicylic acid which could be further oxidized to central intermediates such as pyruvate. Similarly, naphthalene could be mineralized through salicylic acid to water and carbon dioxide when incubated in the culture of *Sphingomonas yanoikuyae* B1 (Kim et al., 1997). The naphthalene catabolic pathway was well documented as summerized in Fig. 2.2. Naphthalene was initially dioxygenated to form *cis*-naphthalene-1,2-diol by the activity of naphthalene dioxygenase enzyme. Subsequently, *cis*-naphthalene-1,2-diol would be oxidized by naphthalenedihydrodiol dehydrogenase to 1,2-dihydroxynaphthalene. Dihydroxynaphthalene dehydrogenase oxidizes 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylic acid (HCCA) which could be further oxidized to *trans*-O-hydroxybenzylidenepyruvic acid (*t*HBPA) by the activity of HCCA isomerase enzyme. *t*HBPA would then be oxidized to salicylaldehyde and salicylic acid by hydratase-aldolase enzyme and salicylaldehyde dehydrogenase enzyme, respectively. Salicylic acid was found to be a central intermediate for the degradation of many PAHs such as the degradation of anthracene by *Pseudomonas aeruginosa* (Sutherland et al., 1995), fluorene by *Arthrobacter* sp. F101 (Casellas et al., 1997), and phenanthrene by *Pseudomonas* sp. s47p1 (Kiyohara et al., 1994). Two main pathways are well known in the oxidation of salicylic acid by bacteria. The first one is the decarboxylation of carboxyl group to form carbon dioxide and catechol by the activity of salicylate hydroxylase (Suzuki et al., 1991; Balashova et al., 2001 and Zhao et al. 2005) and then catechol would be further converted to tricarboxyliac acid cycle intermediates (Meer et al., 1992) as shown in Fig. 2.3.

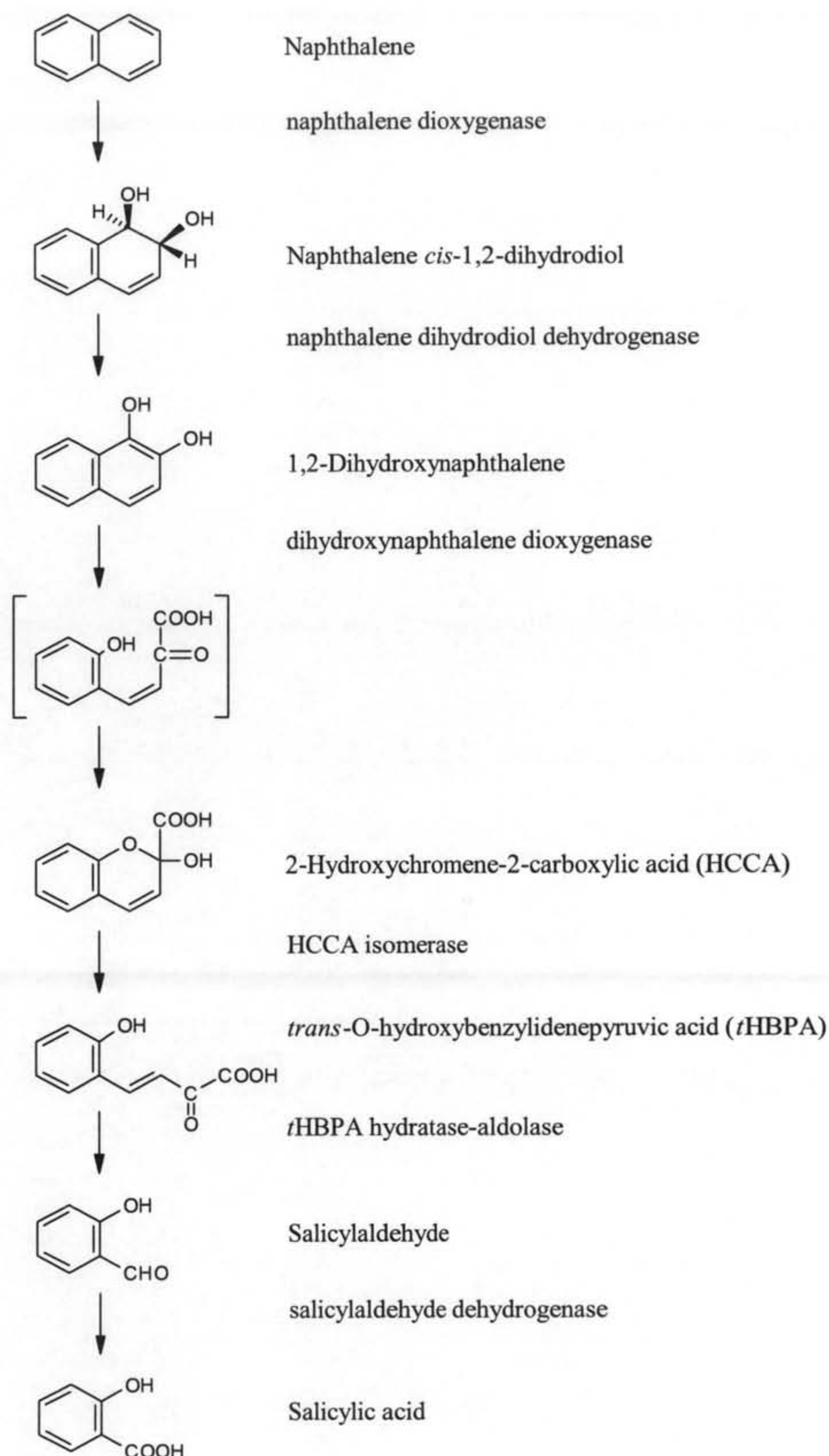

Fig. 2.2 Catabolic pathway for the degradation of naphthalene by *Sphingomonas yanoikuyae* B1 and *Pseudomonas putida* G7 (Kim et al., 1997; Yen and Gunsulas, 1982)

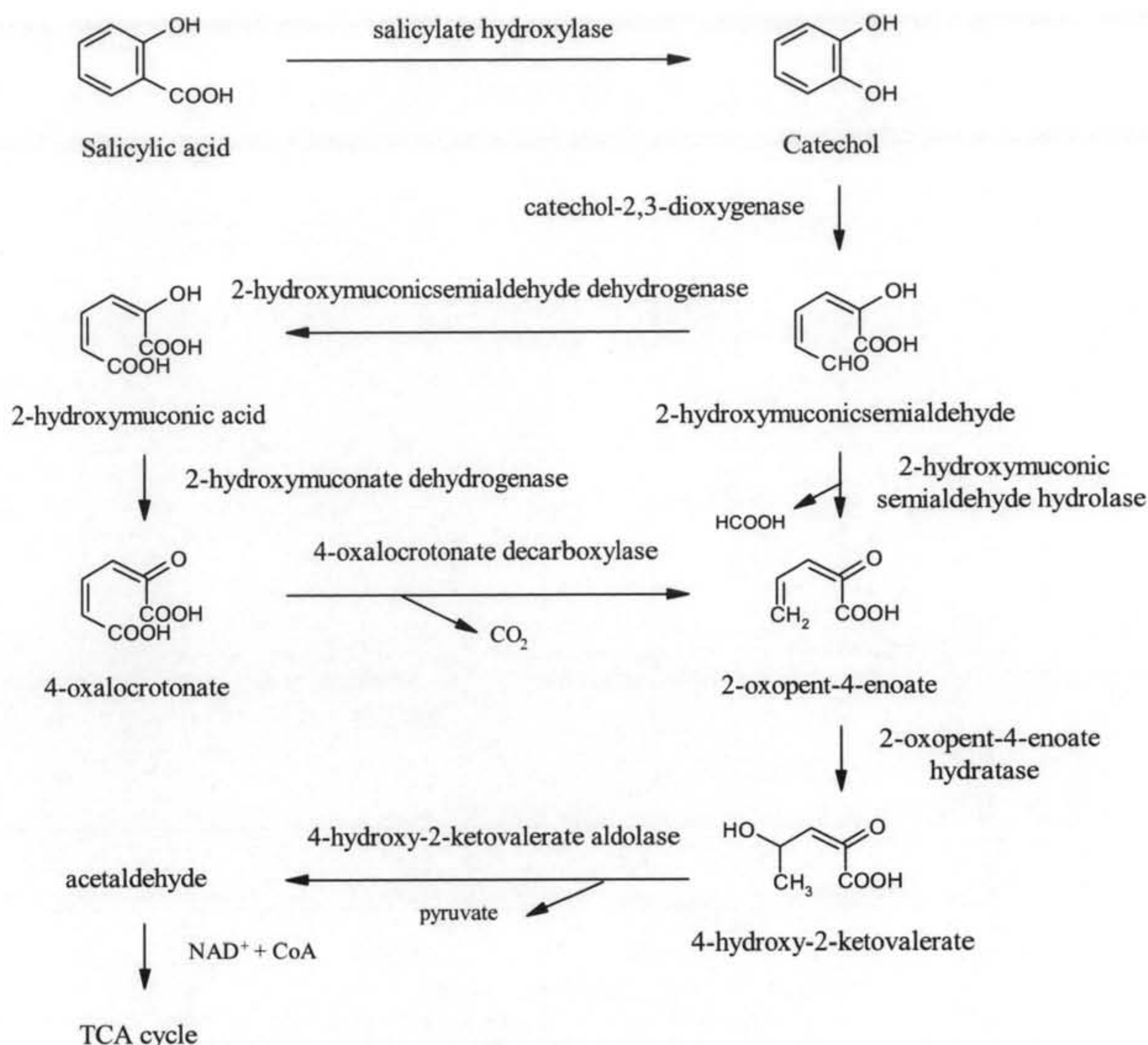

Fig. 2.3 Salicylic acid catabolic pathway via catechol in *Pseudomonas putida* and *Pseudomonas* sp. ND-6 (Meer et al., 1992 and Zhao et al. 2005)

The other pathway is by hydroxylation in the benzene ring of salicylic acid at C-5 position to form gentisic acid. The metabolism of salicylic acid to gentisic acid has been reported in *Pseudomonas* sp. U2 (Fuenmayor et al. 1998), *Rhodococcus* sp. B4 (Grund et al., 1992), *Pseudomonas* sp. TA-2 (Ohmoto et al., 1991) and in the degradation of naphthalene by *Pseudomonas fluorescens* (Starovoitov, 1975). Zhou reported that *Ralstonia* U2, formerly known as *Pseudomonas* sp. U2, could metabolize salicylic acid to gentisic acid, which was further oxidized to maleylpyruvate, fumarylpyruvate by gentisate-1,2-dioxygenase and maleylpyruvate isomerase, respectively. Finally, fumarylpyruvate would be oxidized to pyruvate and fumarate as shown in Fig. 2.4 (Ning et al., 2002).

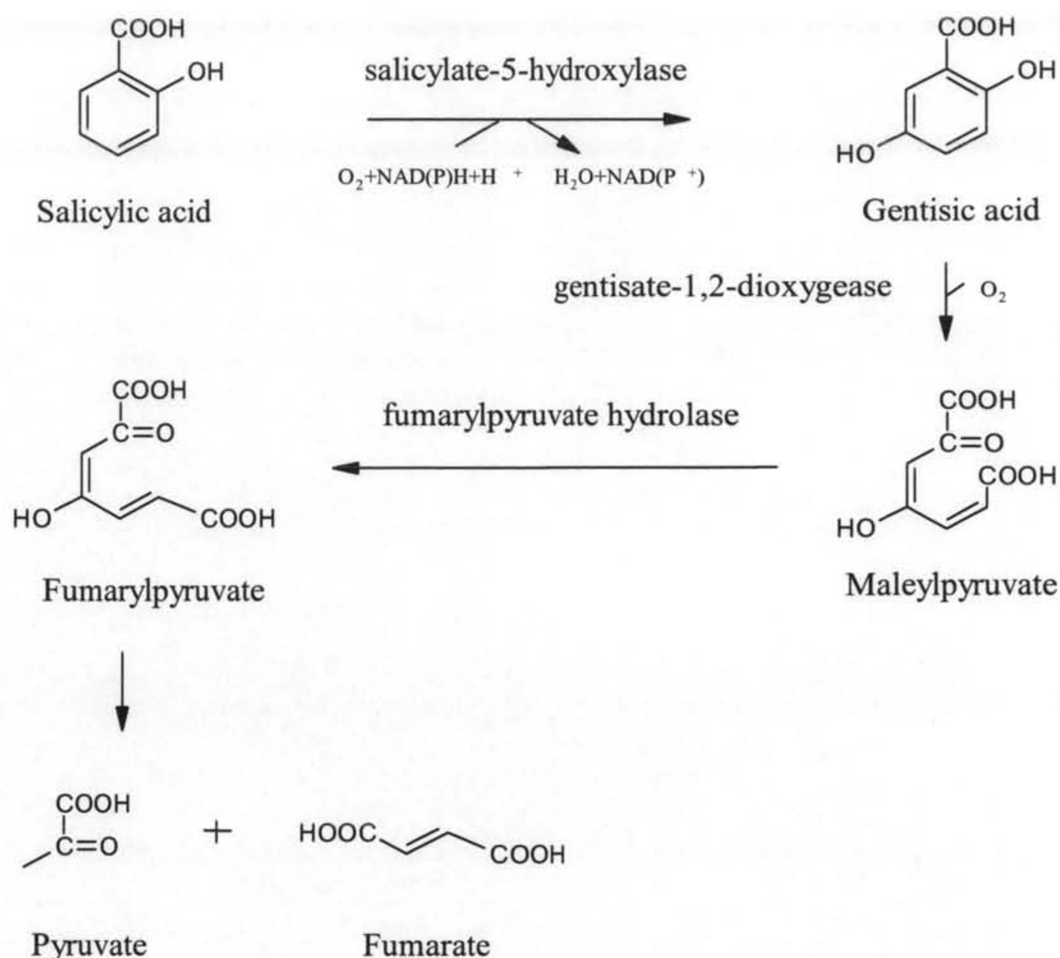

Fig. 2.4 Salicylic acid catabolic pathway in *Ralstonia* U2 (Ning et al., 2001 and 2002)

It is known that dioxygenation, the enzymatic incorporation of two atoms of molecular oxygen into the aromatic structure, is normally occured as the initial step in the aerobic degradation of PAH by most bacteria that have ability to degrade PAHs. The reaction occurs via oxidation of the PAHs to dihydrodiol, which catalyzes by multicomponent enzyme system, termed dioxygenases. The oxidized intermediate is then proceeded through ring cleavage leading to central intermediates such as catechol, gentisic acid and protocatechuic acid, which are further oxidizd to the TCA cycle intermediates. For example, pyrene was initially oxidized to *cis*-4,5-pyrenedihydrodiol, and *cis*-2,3-pyrenedihydrodiol by *Mycobacterium* sp. AP1 and *Rhodococcus* sp. UW1 (Heirkamp et al., 1988 and Walter et al., 1991), and benzo[*a*]pyrene was also dioxygenated to *cis*-4,5-benzo[*a*]pyrenedihydrodiol, *cis*-9,10-benzo[*a*]pyrenedihydrodiol and *cis*-7,8-benzo[*a*]pyrenedihydrodiol by

Mycobacterium RJGII 135 (Schneider et al., 1996) as shown in Fig. 2.5. Moreover, fluoranthene, anthracene, phenanthrene and fluorene could be initially dioxygenated by dioxygenases, resulting in the formation of dihydrodiol products by *Alcaligenas denitrificans* WW1 (Grifoll et al., 1992), *Mycobacterium* sp. PYR-1 (Moody et al., 2001) and *Arthrobacter* sp. F101 (Weissenfels et al., 1991), respectively.

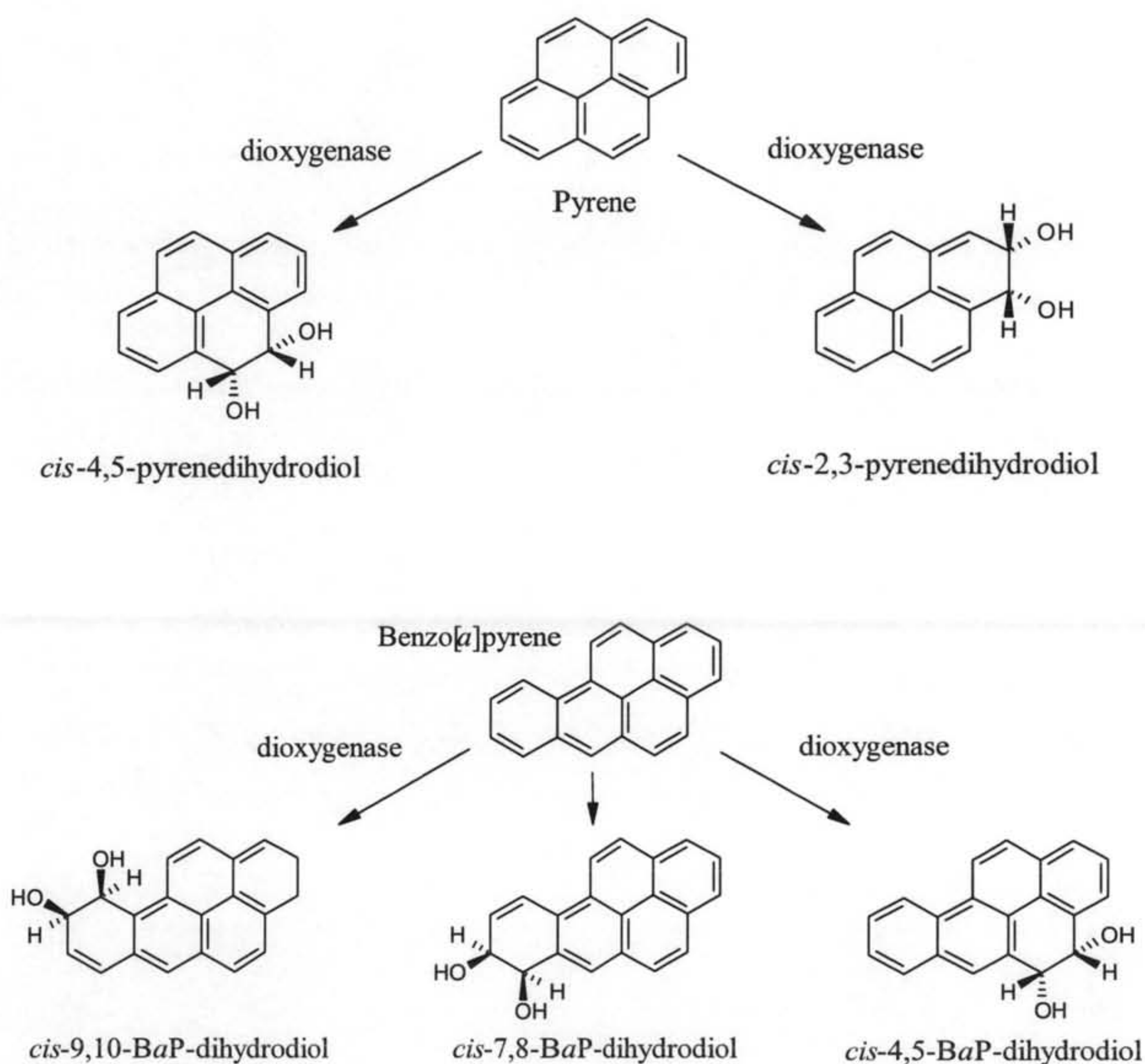

Fig. 2.5 Initial step of catabolic pathways proposed for the metabolism of pyrene and benzo[*a*]pyrene by bacteria

Biodegradation of Acenaphthylene

Isolation and characterization of a wide variety of bacteria from the environmental samples possessing ability to metabolize acenaphthylene and PAHs makes bioremediation of acenaphthylene contaminated sites possible (Muller et al., 1991). The metabolism of acenaphthylene by cultures of organisms have been reported for many years. A number of reports revealed the transformation of acenaphthylene to various metabolites by mammals and various strains of fungi and bacteria. A few reports indicated the complete mineralization of acenaphthylene by bacteria (Barclay et al., 1995; Michiel et al., 1998; Haemmerli et al., 1986; Shocken and Gibson, 1984; Komatsu et al., 1993 ; Grifoll et al., 1995; Selifenov et al., 1996).

Biodegradation of Acenaphthylene by Mammals

The catabolic pathway of acenaphthylene in mammal principally involved sequences of reactions initiated by oxidation of cyclopentene ring to form acenaphthylene epoxide which catalyzed by cytochrome P_{450} monooxygenase. Then, hydrogen and hydroxyl group are added to acenaphthylene epoxide, giving *cis*- and *trans*-acenaphthenediol. The resulting acenaphthenediol are oxidized to acenaphthenequinone and naphthalic acid.

Biodegradation of Acenaphthylene by White Rot Fungi

Lignolytic white rot fungi have ability to degrade lignin, an aromatic compound with complex structure, by peroxidase enzyme including lignin peroxidase, manganese peroxidase and laccase. Broad substrate specificity is now a well-recognized property of the enzymes catalyzing lignin degradation. Therefore, these enzymes are also capable of oxidizing polycyclic aromatic hydrocarbons which have the structure similar to lignin (Barclay et al., 1995). The metabolism of PAHs by lignolytic fungi has been investigated. For example, the degradation of benzo[*a*]pyrene by *Bjerkabdera* sp. strain BOS55 was studied and the results showed that the combination of lignin peroxidase, manganese peroxidase and laccase oxidized benzo[*a*]pyrene to various metabolites (Michiel et al., 1998). Haemmerli et al. (1986) revealed that lignin peroxidase prepared from *Phanerochaete chrysosporium* oxidized

benzo[*a*]pyrene to 6-hydroxybenzo[*a*]pyrene, and the subsequent reactions to form quinones occur rapidly and spontaneously.

The catabolic pathway of acenaphthylene by white rot fungus *Trametes versicolor* has been proposed. Acenaphthylene is initially oxidized to acenaphthene-1-one, which could be further transformed to 1-hydroxy-2-acenaphthenone or cis-1,2-dihydroxyacenaphthene and its tautomer, trans-1,2-dihydroxyacenaphthene which could then be oxidized to 1,8-naphthalic aldehyde. These metabolites including 1,2-dihydroxyacenaphthylene, its tautomer, would subsequently be converted to acenaphthenedione and 1,8-naphthaldehydic acid (Johannes et al., 1998) as shown in Fig. 2.6.

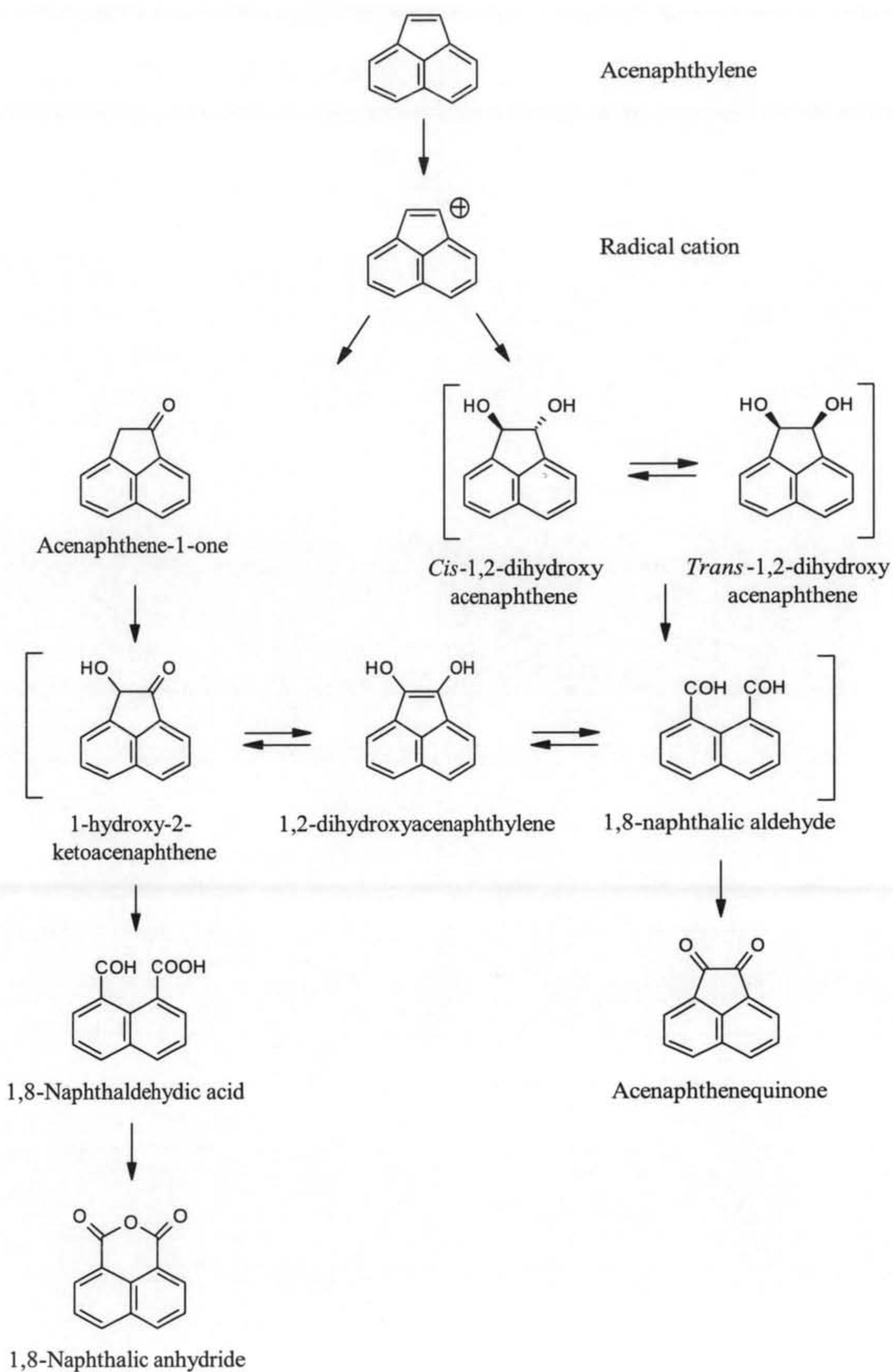

Fig. 2.6 Acenaphthylene catabolic pathway by *Trametes versicolor* (Johannes et al., 1998)

Biodegradation of Acenaphthylene by Bacteria

The bacterial catabolic pathways for acenaphthylene have been investigated for more than 30 years. Dean-Raymond and Bartha (1975) firstly reported that the bacterium strain isolated from oil contaminated estuarine water was capable of cooxidizing acenaphthylene in the presence of naphthalene. This strain could cooxidize acenaphthylene to an unidentified quinone intermediate.

Chapman (1979) reported that naphthalene-grown bacterium could cooxidize acenaphthylene to *cis*-1,2-acenaphthenediol and some unidentified metabolites.

Shocken and Gibson (1984) isolated and identified intermediates from acenaphthylene degradation by *Beijerinkia* sp., a strain capable of cooxidizing acenaphthylene when grown on medium containing succinate. The intermediates were identified to be *cis*-acenaphthene-1,2-diol and 1,2-dihydroxyacenaphthylene. Whereas acenaphthenequinone was found to be a dead-end product which could not be further oxidized. *Beijerinkia* sp. B8/36, a diol dehydrogenase defective mutant, accumulated *cis*-acenaphthene-1,2-diol when grown on acenaphthylene.

Sphingomonas sp. A4, formerly known as *Pseudomonas* sp. A4, a bacterium isolated from soil samples of an industrial waste deposit could utilize acenaphthylene as a sole source of carbon and energy giving the main intermediate as 1,8-naphthalenedicarboxylic acid. The strain A4 could utilize acenaphthylene and 1,8-naphthalenedicarboxylic acid as a sole source of carbon and energy. No metabolite from the oxidation of 1,8-naphthalenedicarboxylic acid was reported (Komatsu et al.,1993).

Grifoll et al. (1995) revealed that *Pseudomonas cepacia* F297 could transform acenaphthylene after inducing with fluorene. The intermediates from the transformation of acenaphthylene were identified to be 1-acenaphthenone, acenaphthenequinone, 1,8-naphthalic anhydride and 1,8-naphthalenedicarboxylic acid. The transformation of acenaphthylene was catalyzed by naphthalene dioxygenase in the same manner as that of naphthalene by this strain.

Selifenov et al. (1996) reported that *Pseudomonas aeruginosa* PA01 (pRE695), a recombinant strain harboring plasmid pRE695 containing naphthalene dioxygenase gene (*nahA*) could transform acenaphthylene to *cis*-1,2-acenaphthenediol. Then, the nonspecific dehydrogenase activities present in the host

strain further catalyzed the oxidation of *cis*-1,2-acenaphthenediol to acenaphthenequinone prior to spontaneous ring fission to form naphthalene-1,8-dicarboxylic acid as shown in Fig. 2.7. However, further oxidation of naphthalene-1,8-dicarboxylic acid had not been reported.

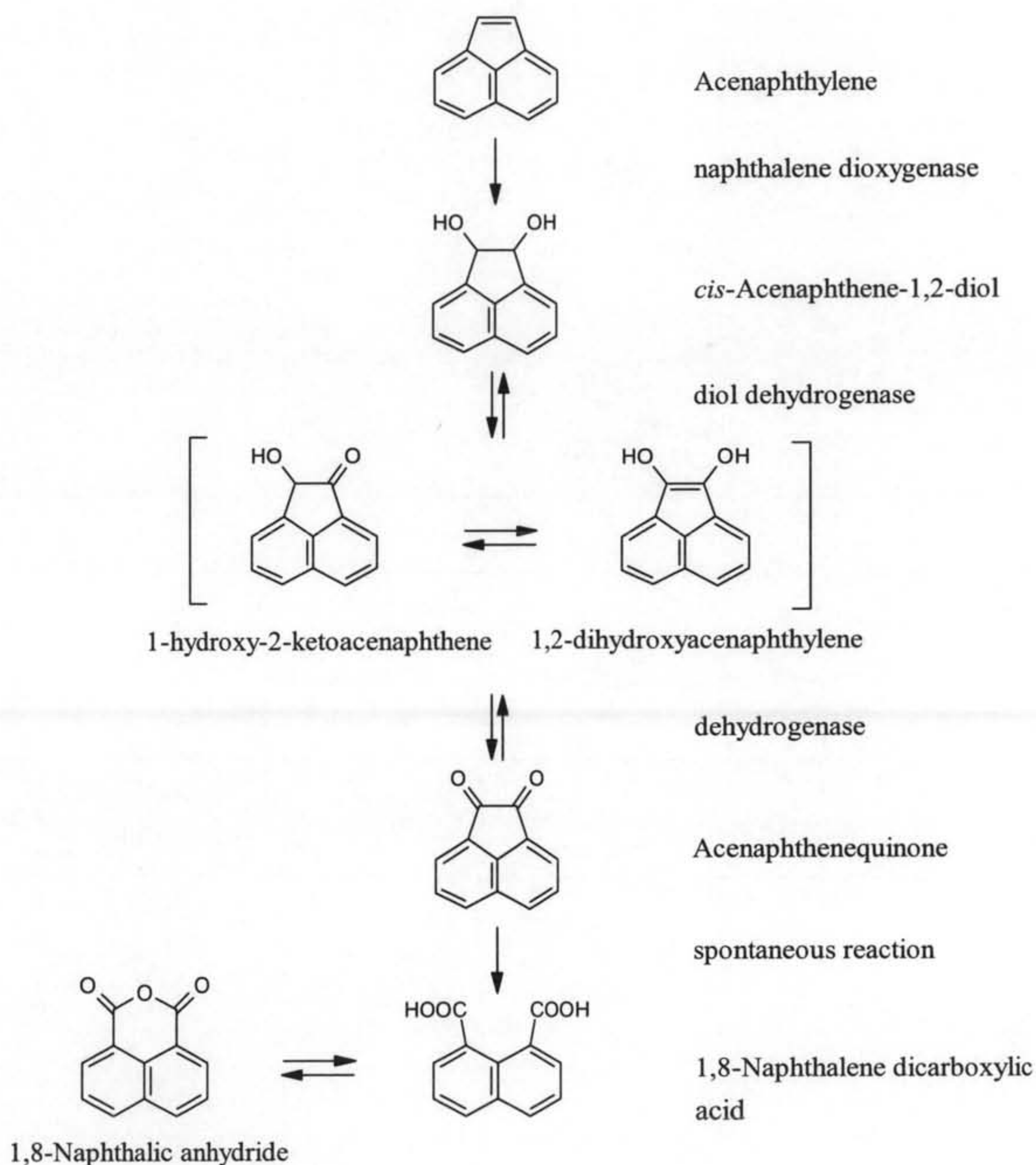

Fig. 2.7 Proposed pathway for the oxidation of acenaphthylene by *Pseudomonas aeruginosa* PA01 (Selifenov et al., 1996)

The previous documents have suggested that the initial reaction for acenaphthylene degradation involved the incorporation of two oxygen atoms into cyclopentene ring of acenaphthylene to form *cis*-acenaphthene-1,2-diol which was catalyzed by the enzyme termed dioxygenase. Subsequent dehydrogenation by diol dehydrogenase would then result in the formation of 1,2-dihydroxyacenapthylene and its tautomer, 1-hydroxy-2-ketoacenaphthene. The resulted 1,2-dihydroxyacenaphthylene and 1-hydroxy-2-ketoacenaphthene would then be oxidized or reduced to acenaphthenequinone by dehydrogenase and reductase enzyme. The resulting acenaphthenequinone is then spontaneously cleaved the cyclopentene ring to form naphthalene-1,8-dicarboxylic acid.

Biodegradation of Acenaphthylene by *Rhizobium* sp. CU-A1

A novel bacterium strain CU-A1, identified as a member of genus *Rhizobium*, was isolated from petroleum contaminated soil in Thailand. This strain was capable of growing on acenaphthylene and naphthalene as a sole source of carbon and energy and cometabolically transformed a wide variety of PAHs and heterocyclic compounds such as acenaphthene, fluorene and phenanthrene (Paengthai , 2000). The results showed, for the first time, the ability of degradation of acenaphthylene by a rhizobial strain, *Rhizobium* sp. strain CU-A1. The pathways for the biodegradation of some PAHs such as naphthalene and pyrene are well documented. However, only a few numbers of bacteria capable of utilizing acenaphthylene and the catabolic pathway involving acenaphthylene degradation have been reported, but none from genus *Rhizobium*. As *Rhizobium* sp. strain CU-A1 can mineralize acenaphthylene completely, it is interesting to elucidate the acenaphthylene catabolic pathway in this strain.

To elucidate the degradation pathway of acenaphthylene by this organism, blocked mutants of this organism incapable of utilizing this compound as a sole carbon source were constructed by using transposon mutagenesis via transposon Tn*5*. Transposon Tn*5*, a DNA fragment that can transfer itself from one position and randomly insert into another position catalyzing by enzyme transposase, was introduced to this strain via conjugation with *Eschericia coli* strain S17-1 harboring suicide plasmid pSUP2021. The highest transposition efficiency of 4.75×10^{-5} per recipient was achieved by mixing donor and recipient at their early log phase with the

ratio of 1:1, followed by incubating at 30°C for 24 h and selecting of transconjugants on mineral medium containing protocatechuic acid and kanamycin (Kriangkripipat, 2001). The intermediates of acenaphthylene degradation formed from those mutants were purified and identified to be acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, and 2,5-dihydroxybenzoic acid (gentisic acid). Based on these results, a complete pathway for the degradation of acenaphthylene is tentatively proposed as follow: acenaphthylene is oxidized to acenaphthenequinone, naphthalene-1,8-dicarboxylic acid and gentisic acid, respectively (Poonthrigpun, 2002).

Dioxygenases

Many reports revealed that the initial reactions of PAHs metabolism including acenaphthylene involved the enzymatic incorporation of two oxygen atoms into the structure of the compounds catalyzed by dioxygenase. Dioxygenase could be classified into two types by its catalytic mechanisms:

1. Ring cleavage dioxygenase

This dioxygenase catalyzes the ring cleavage of hydroxyl derivatives of aromatic compounds with no requirement of any co-factor. The examples of this type of dioxygenase are catechol-2,3-dioxygenase and gentisate-1,2-dioxygenase with their mechanisms presented in Fig. 2.3 and 2.4, respectively.

2. Ring hydroxylating dioxygenase

Dioxygenases that catalyse the incorporation of oxygen atoms into the aromatic structure to form diol compounds require NAD(P)H as an electron donor. Naphthalene dioxygenase is an example of this type. It has a mechanism as shown in Fig. 2.2. Most ring-hydroxylating dioxygenases are non-heme iron proteins consisting of two to three protein components, a ferredoxin, a ferredoxin reductase and a terminal oxidase, in an electron transport system. Ferredoxin reductase acts as an initial electron acceptor and transfer electron from NAD(P)H to ferredoxin which acts as an intermediate electron transfer protein transferring electron to catalytic site of terminal oxidase. Ring-hydroxylating enzyme could be classified by its components into 3 types (Bertini et al., 1996) as follows;

Class I : Dioxygenase that consists of two protein components, a plant type iron sulfur [2Fe-2S] center ferredoxin reductase and a Rieske type iron sulfur [2Fe-2S] center terminal oxygenase. Moreover, it could be divided by its prosthetic group of the reducatse into two sub-classes as follow;

Class IA : Dioxygenase with reductase containing FMN.

Class IB : Dioxygenase with reductase containing FAD.

Class II : Dioxygenase that consists of three protein components, an FAD ferredoxin reductase, a Rieske type iron sulfur [2Fe-2S] center terminal oxygenase, and a ferredoxin. This class is classified into two sub-classes.

Class IIA : Dioxygenase with ferredoxin containing Plant type iron sulfur [2Fe-2S] center.

Class IIB : Dioxygenase with ferredoxin containing Rieske type iron sulfur [2Fe-2S] center.

Class III : Dioxygenase that consists of three protein components, a Rieske type iron sulfur [2Fe-2S] center ferredoxin, an FAD iron sulfur [2Fe-2S] center ferredoxin reductase, and a Rieske type iron sulfur [2Fe-2S] center terminal oxygenase.

Naphthalene Dioxygenase

Of all the enzymes involved in the microbial metabolism of PAHs, the enzymes for naphthalene metabolism produced by bacteria in genus *Pseudomonas* have been studied most extensively. Up to date, only one naphthalene dioxygenase has been well characterized which was from *Pseudomonas putida* NCIB 9816. Naphthalene dioxygenase, a multicomponent enzyme system belongs to bacterial ring hydroxylating family of sixteen known members, containing three proteins, a ferredoxin, a ferredoxin reductase and a terminal oxidase. It catalyses the initial reaction in the metabolism of naphthalene by transforming naphthalene to naphthalene dihydrodiol in the presence of oxygen and NADH (Ensley et al., 1982).

A ferredoxin, purified by Blue Sepharose CL-6B column, DEAE-cellulose column, gel filtration and DEAE-cellulose column (Ensley et al., 1983) from cell free extract of *Pseudomonas putida* NCIB 9816, acts as an intermediate electron transfer protein in the dioxygenase system transferring two electrons to iron-sulfur center of terminal oxygenase subunit. It was specifically required for the function of the

dioxygenase system as substitution with ferredoxins from other sources could not restore the activity (Haigler and Gibson, 1990). It was a red-brown iron-sulfur protein (Rieske [2Fe-2S]) with average molecular weight of 13,600 and an isoelectric point of 4.6. It had maximal UV/visible absorption spectrum at 280, 325 and 460 nm.

Ferredoxin reductase, an NADH-dependent oxidoreductase functions as the initial electron acceptor, transfers electrons from NADH to ferredoxin. NADPH could substitute for NADH but resulting in the decreasing of the activity (Haigler and Gibson, 1990). This enzyme was purified by using the same procedure as for ferredoxin. From native PAGE and SDS-PAGE analyses indicated that the enzyme was a single polypeptide with a molecular weight of 36,300. The UV/visible absorption spectrum and determination of the metal compositions revealed that this enzyme was a red iron-sulfur flavoprotein with 1 mole of FAD bound per 1 mole of protein, but was readily lost during purification process, and approximately 1.8 g of iron and 2.0 g of sulfur per mole. The isoelectric point of ferredoxin reductase was 6.3. This enzyme has cytochrome *c* reductase activity, which could be significantly induced by the addition of FAD. Although, NADH-dependent ferredoxin reductase contained a flavin co-factor and an iron-sulfur center, this enzyme could not directly transfer electrons to the terminal oxygenase (Haigler and Gibson, 1990).

A terminal oxygenase was purified from *Pseudomonas putida* NCIB 9816 or a recombinant strain containing naphthalene terminal oxygenase gene. The enzyme was purified to homogeneity by three step chromatography on Q Sepharose FF column, Octyl Sepharose CL-4B, and Sephacryl S-300, respectively. The purified enzyme required ferredoxin, ferredoxin reductase and NADH for its activity. It catalyzed the incorporation of two oxygen atoms into one benzene ring of naphthalene (Ensley et al., 1983). Its estimated molecular weight of 158,000 was obtained by gel filtration, and SDS-PAGE showed the presence of two subunits with molecular weight of 55,000 and 20,000 indicating an $\alpha_2\beta_2$ quaternary structure. Absorption spectrum of the oxidized enzyme showed maxima at 566, 462 and 344 nm. The UV/visible absorption spectrum characteristic of terminal oxygenase was similar to those of ferredoxin which indicated that this enzyme might be an iron-sulfur protein. The determination of metal content showed that it had 6.0 g of iron and 5.0 g of acid-labile sulfur per mole of the enzyme. Three-dimensional structure of this enzyme has been elucidated by x-ray crystallography and shown to be a 3α and 3β structure (Kauppi et

al., 1998). Crystal structure of the enzyme indicated that the molecule was tightly packed $\alpha_3\beta_3$ hexamer arranging in mushroom shape (Suan and Gibson, 1993).

In summary, the transformation of naphthalene to naphthalenediol was catalyzed by an enzyme system as follows: an iron-sulfur flavoprotein reductase initially transfers electrons from reduced nucleotide NADH via FAD to Rieske iron-sulfur center of ferredoxin. The electrons are then transferred to the catalytic component of the enzyme to facilitate the addition of two oxygen atoms to naphthalene as shown in Fig. 2.8. NADH-dependent ferredoxin reductase and ferredoxin are necessary for the function of naphthalene dioxygenase. Moreover, the electron transport system is not interchangeable among other dioxygenases by using electron transport system of other bacteria (Laurie and Lloyd-Jones, 1999).

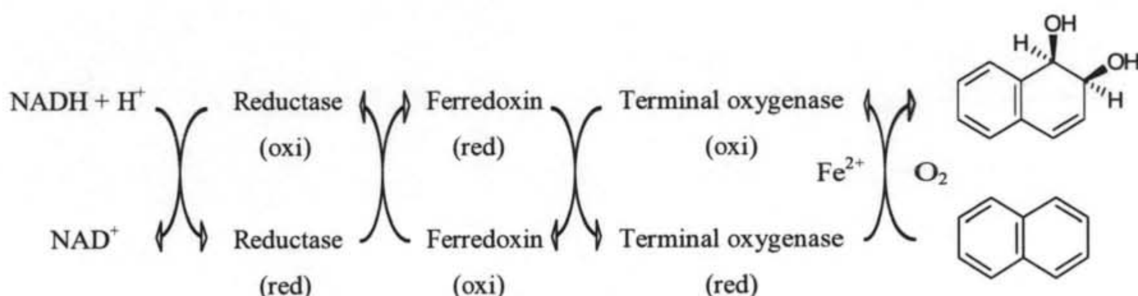

Fig. 2.8 Oxidation of naphthalene by a multicomponent enzyme system, naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816 (Haigler and Gibson, 1990; Suen and Gibson, 1993)

The catalytic component of the enzyme consists of α and β subunits. The role of the β subunit has not been determined, however, in the absence of β subunit, naphthalene could not be oxidized. The result suggested that β subunit plays a role in a structural aspect of naphthalene dioxygenase whereas the α subunit is directly involved in catalysis. Each α subunit contains a Rieske iron-sulfur center and mononuclear iron at the active site. The quaternary structure of the enzyme revealed

that distance between iron-sulfur center and mononuclear iron in the same α subunit is too far to facilitate the electron transfer while the distance between iron-sulfur center of one α subunit and the adjacent mononuclear iron is shorter. Therefore, electrons are passed to an iron-sulfur center of one α subunit and then to a mononuclear iron at the active site in an adjacent α subunit (Parales et al., 1999; Parales et al., 2000).

The catalysis cycle of naphthalene dioxygenase is shown in Fig. 2.9. Firstly, the irons of iron-sulfur center are in ferric state and the mononuclear iron is in ferrous state. When an electron is introduced to a ferric iron of iron-sulfur center, the enzyme is changed from resting form to active or reduced form. In the presence of naphthalene and atmospheric oxygen, one molecule of oxygen binds to a mononuclear iron using two electrons, one from the active enzyme and the other from mononuclear iron, resulting in the formation of (hydro)peroxo-iron(III), an intermediate in the reaction pathway leading to dihydrodiol. One electron is required to reduce the active mononuclear ferric iron to ferrous state and with the incorporation of H^+, naphthalene dihydrodiol is finally released (Wolfe et al., 2001; Karlsson et al., 2003).

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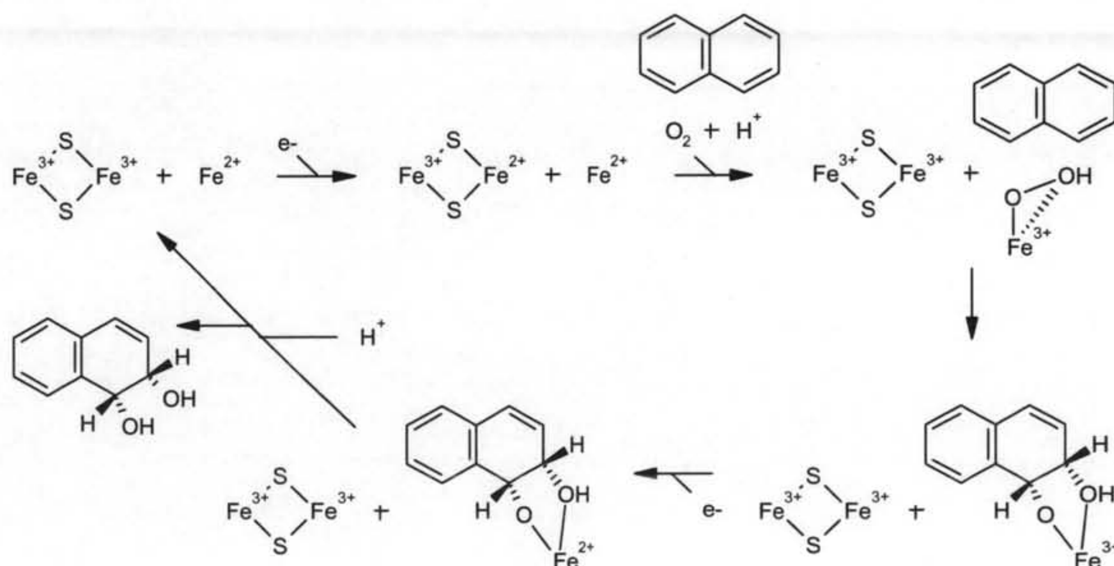

Fig. 2.9 Catalytic cycle of naphthalene-1,2-dioxygenase (Bassan et al., 2004)

Naphthalene dioxygenase catalyzed the first step in the degradation of naphthalene. Moreover, the enzyme also catalyzed dihydroxylation of a wide variety of substrates and yields dihydrodiol products such as biphenyl, anthracene, phenanthrene (Jerina et al., 1976), fluorene (Resnick and Gibson, 1996), acenaphthene and acenaphthylene (Selifonov et al., 1996). It also showed broad versatility in its action on substituted aromatic compounds such as 2-methoxynaphthalene (Whited et al., 1994), dimethylnaphthalene, trimethybenzene (Selifonov et al., 1996), 2-methylnaphthalene (Mahajan et al., 1994) and 6-Amino-2-naphthalenesulfonic acid (Haug et al., 1991), and on heterocyclic aromatic compounds such as dibenzothiophene and dibenzofuran (Resnick and Gibson, 1996). Beside naphthalene dioxygenase, other known three components of aromatic dioxygenases are summarized in Table 2.1.

Table 2.1 Properties of some aromatic dioxygenases

Enzymes	Sources	Reductase			Ferredoxin	Oxygenase			References
		Mw	Flavin cofactor	Fe-S center	Mw	Mw	Mw subunit	Subunit structure	
Benzene dioxygenase	*Pseudomonas putida*	60,000/ 81,000	FAD	-	21,000/ 12,300	168,000/ 215,300	54,500; 23,500	$\alpha_2\beta_2$	Zamanina and Mason, 1987; Axcell and Geary, 1975
Toluene dioxygenase	*Pseudomonas putida*	46,000	FAD	-	15,300	151,000	52,500; 20,800	$\alpha_2\beta_2$	Suramanian et al., 1981; Suramanian et al., 1985
Biphenyl dioxygenase	*Pseudomonas* sp. LB400	43,600	FAD	-	41,500	209,000	53,300; 27,300	$\alpha_3\beta_3$	Haddock and Gibson, 1995; Haddock and Gibson, 1997; Broadus and Haddock, 1998
Naphthalene dioxygenase	*Pseudomonas* sp. NCIB 9816	36,300	FAD	[Fe-S]	13,600	158,000	55,000; 20,000	$\alpha_2\beta_2$	Ensley and Gibson, 1983; Haigler and Gibson, 1990; Haigler and Gibson, 1990;
	Rhodococcus sp. NCIMB12038	n/a	n/a	n/a	n/a	155,000	55,000; 23,000	$\alpha_3\beta_3$	Larkin et al., 1999
Dibenzofuran dioxygenase	*Sphingomonas* sp. RW1	44,000	FAD	-	12,000	120,000	45,000; 23,000	$\alpha_2\beta_2$	Bunz and Cook, 1993
Pyrazon dioxygenase	Pyrazon-degrading Bacteria	67,000	FAD	-	12,000	180,000	n/a	n/a	Sauber et al., 1977

Benefits of Understanding Biochemical Pathway

1. Metabolic engineering

Bioremediation offers several advantages over other remediation techniques. However, bioremediation also has its limitation. The most important major microbial factors affecting bioremediation are toxic intermediates may be produced during remediation. There are some concerns that the products of biodegradation may be more persistent or toxic than the parental compounds thus understanding of the biochemical pathway by which the xenobiotic compounds are degraded will lead to the improvement of the bioremediation process of those compounds. Metabolic engineering is a new approach to understanding and using metabolic processes. This technique employs the application of genetic engineering and protein engineering to improve the efficiency and cost, which are key factors in the future widespread application of microorganisms to degrade the toxic substances (McClure et al., 1991). As the name implies, metabolic engineering is purposeful alteration of metabolic pathways found in an organism in order to better understand and use cellular pathways for chemical transformation. It typically involves the redirection of cellular activities by the rearrangement of the enzymatic functions of the cell through the use of recombinant DNA and other techniques. Moreover, the construction of hybrid pathways will lead to the complete degradation of persistant compounds. For example, the assembled pathway includes one catabolic segment encoding the toluene dioxygenase of *Pseudomonas putida* F1, which affords the bioconversion of 2-chlorotoluene into 2-chlorobenzaldehyde. A second catabolic segment encoded the entire upper toluene pathway from *P. putida* mt-2, which further transforms 2-chlorobenzaldehyde to 2-chlorobenzoate which is further oxidized completely (Haro and Lorenzo, 2001). Knowledge from the study of biological pathway will benefit society in a number of ways including the ability to modify metabolic pathways for better remediation of the toxic compounds. Up to present, bacteria possessing ability to degrade acenaphthylene has been rarely reported. The present work showed that *Rhizobium* sp. CU-A1 could mineralize acenaphthylene as its sole carbon source for growth. The knowledge of the acenaphthylene metabolic pathway in this bacteria will lead to its successful application for remediation of acenaphthylene contaminated sites. Furthermore, introduction of multiple or heterologous catabolic pathways into

this strain or a suitable candidate organism will provide a strain with broad substrate spectrum capable of degrading a wide variety of toxic contaminants.

2. Enzymatic bioremediation

An imerging concern about the adverse effect of PAHs has led to requirements for effective remediation technologies in a range of industries and other applications. Many of these applications require rapid action and thus traditional bioremediation techniques are inadequate. As a result, this has led to an interest in the use of enzymes instead of live bacteria as bioremediation agents, known as enzymatic bioremediation. The conventional bioremediation processes are considerable slow, taking weeks to months to achieve the goal of remediation and bacteria normally do not resist to the environmental changes. Unlike microorganisms, enzymes remain effective in a wide range of pH and temperature, particularly if they are immobilized on some carriers. Currently, many researches are focused on enzymes which have broad spectrum of substrates for the bioremediation such as dioxygenases. For example, organophosphate pesticide degrading enzymes produced from a bacterium isolated from soil in Australia, could degrade organophosphate pesticide residues in more than 80,000 litres of contaminated water by 90% in 10 minutes (Sutherland et. al., 1994). However, to fulfill the remediation performance, criteria for good enzymes such as function rapidly in a wide range of contaminant concentrations, high environmental stability, low specificity and cost effective are required. Therefore, for enzymes from new isolated organisms they must be studied extensively in details prior their application.

3. Biosensor

Determination of the presence of hazardous toxic substances in a contaminated site is required during the preliminary site assessment in order to reduce the risk of human exposure. However, conventional sampling, handling and laboratory analysis may be slow and expensive. The enzyme electrode for detection and determination of contaminants is an emerging technology. This method is faster, more cost effective than traditional sampling and laboratory analysis. It can rapidly provide information concerning the contaminated location, source and concentration of pollutants which

may have impact on human health and the environment. For instance, tyrosinase enzyme electrode could be used to measure the presence of phenolic compound in aqueous and organic media. Nevertheless, several technical challenges remain to be solved before this biosensor can be really applied in an actual environment (Hristoy et al., 2003).

In the other way, when assessing a site for potential bioremediation, it is neccessary to determine the capability of indigenous microorganisms in producing enzymes that catalyze the degradation of toxic contaminants. Under such conditions, genes involving the degradation are being expressed and the gene products are actively catalyzing the desired reactions. As a result, the method to determine gene expression as well as direct enzyme activity are very useful for monitoring the bioremediation process. Enzyme activity-dependent probe could be developed if the data on catabolic pathway and enzymes calatyzing the desired reactions are sufficient. For example, the probe for determination of toluene degrader consists of synthetic substrate which after being catalyzed by the enzyme will yield easily detectable fluorescent and/or colored products. The use of these probes is straightforward and rapid, and this approach has the potential to assess actual enzyme activity in situ (Clingenpeel et al., 2004).

4. Green chemistry

The transformation of compounds by enzymes, generally referred as biocatalysis, has evolved as a trend-section of organic synthesis. As a result, a number of biochemical techniques have been developed to obtain a powerful technique for the organic synthesis. Because of the limitation of using molecular oxygen in chemical oxidation process, which generally generate an unstable peroxocompounds, halogenated compounds have been used as intermediates, which has led to problems in waste treatment due to recalcitrant organohalogen compounds (Holland, 1992). Since oxygenation is a normal reaction in environment, dioxygenase is one of enzyme that could be use to synthesize the compounds that could not be produced by chemical synthesis. For example, the production of *cis*-glycol by dioxygenase of *Pseudomonas* sp.. Because of the extremely high stereoselectivity of the reaction, the *cis*-glycol product has become very popular as highly functionalized starting material for a wide variety of chemical synthesis (Hudlicky et al., 1996). The

chemical compound production by dihydroxylation of aromatic compounds is highly flexible as the functional groups of starting material can be varied, and heterocyclic derivatives may be employed.