CHAPTER V

CONCLUSION

The complete catabolic pathways for the degradation of PAHs such as naphthalene (Gibson and Subramanian, 1984), phenanthrene (Moody et al., 2001), fluorene (Casellas et al., 1997), fluoranthene (Kelley et al. 1993) and pyrene (Vila et al., 2001) are well documented. However, only a few number of bacteria capable of degrading acenaphthylene have been reported and only two acenaphthylene-utilizing bacterial isolates have been reported (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). Moreover, the complete catabolic pathway of acenaphthylene has never been reported. Bacterial strain CU-A1 was isolated from petroleum contaminated soil in Thailand by growing on acenaphthylene as a sole carbon source and was identified by 16 S rDNA sequence analysis as *Rhizobium* sp. (Paengthai, 2000). As *Rhizobium* sp. CU-A1 is capable of mineralizing 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 incapable of utilizing this compound as a sole carbon source were constructed by using transposon mutagenesis via transposon Tn*5*. These mutants accumulated some acenaphthylene catabolic intermediates (Kriangkripipat, 2001). From the previous study, the accumulated intermediates of acenaphthylene degradation from those mutants were purified and identified to be acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, and 2,5-dihydroxybenzoic acid (gentisic acid) (Poonthrigpun, 2002).

The present work further identified additional intermediates of acenaphthylene produced by *Rhizobium* sp. CU-A1 and its mutants. At present, nine major metabolites: acenaphthenediol, acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, 1-naphthoic acid, 1,2-dihydroxynaphthalene, salicylic acid, gentisic acid, and maleylpyruvate and/or fumarylpyruvate, were detected. As a result, almost a complete acenaphthylene catabolic pathway in *Rhizobium* sp. CU-A1 was proposed.

The proposed catabolic pathway of acenaphthylene in *Rhizobium* sp. CU-A1 has shown that the initial reaction in the degradation of this compound involves an incorporation of two oxygen atoms into the cyclopentene ring of the substrate to form acenaphthenediol which is catalyzed by dioxygenase. This step was commonly found as the first step in the aerobic degradation of a wide variety of aromatic compounds in bacteria (Bunz and Cook, 1993; Divari et al., 2003). Because of the extreme instability of dioxygenase when exposed to air, up to date only one dioxygenase involving PAHs degradation has been well characterized which was naphthalene dioxygenase from *Pseudomonas putida*. The CU-A1 was shown to have acenaphthylene dioxygenase activity in the cell free extract prepared from acenaphthylene induced cells as shown in Fig. 4.33. Although acenaphthenediol which was expected to be an initial reaction product was not found as a hydroxylation product from the transformation of acenaphthylene by cell free extract of *Rhizobium* sp. CU-A1, however identification of acenaphthenediol from acenaphthylene grown mutant B1 for 12 h as shown in Figs. 4.13 and 4.14 ensured that the first step in the catabolism of acenaphthylene was catalyzed by dioxygenase. This result was similar to those found in the transformation of phenanthrene by whole cell and cell free extract of *Mycobacterium* sp. in which the first hydroxylation product was rapidly oxidized (Moody et al., 2001). However, the present work found that formation of acenaphthenediol could be detected when reconstituted partially purified acenapthylene dioxygenase complex after gel filtration step was used (Fig. 4.45). This result suggested that dehydrogenase activity might have been removed from the dioxygenase complex during the purification.

Dioxygenases are highly sensitive to oxygen in general and completely inactivated in the presence of NEM, a sulfhydryl blocking agent, which indicated that the enzymes might have thiol groups at the catalytic site. Due to the extremely unstable of acenaphthylene dioxygenase in the crude cell extract during the preliminary study, the catalytic site of this enzyme might consist of thiol groups. To determine whether thiol group is involved in the catalytic mechanism of this enzyme, NEM was introduced to the cell free extract. Treatment of the enzyme with NEM resulted in the loss of its activity which could be restored in the presence of DTT. Moreover, DTT also stabilized the enzyme similarly to those found with naphthalene dioxygenase systems (Haigler and Gibson, 1990; Fetzner et al., 1992).

In the preliminary study, acenaphthylene dioxygenase activity was not linearly related to the protein concentration and it could be stimulated by the addition of reduced pyridine nucleotides which NADH was in preference to NADPH as external electron donor. The presence of FAD, FMN and exogenous iron also stimulated the enzyme activity. These results showed similarity of acenaphthylene dioxygenase to those of benzene dioxygenase (Axcell and Geary, 1975), toluene dioxygenase (Suramanian et al., 1985), biphenel-1,2-dioxygenase (Haddock and Gibson, 1995) and naphthalene dioxygenase with the exception that naphthalene dioxygenase was not stimulated by exogenous iron (Ensley et al., 1982).

For purification of acenaphthylene dioxygenase from *Rhizobium* sp. CU-A1, ammonium sulfate precipitation was applied in the first step. The result showed that the enzyme was precipitated between 40-60% salt saturation. Then, it was applied to Q Sepharose fast flow column and eluted from the column between 0.45-0.60 M NaCl in phosphate buffer pH 7.5. Subsequent gel filtration chromatography resolved acenaphthylene dioxygenase into three protein components, designated as component A, B and C. All components were essential for the enzyme activity. Component A, separated after gel filtration, was further purified by another two chromatographic steps, hydroxyapatite and hydrophobic chromatography using phenyl Sepharose column, to near homogeneity. Component B was further purified by Blue Sepharose chromatography whereas component C was found to be homogeneity after gel filtration.

Components A, B and C were identified to be terminal oxygenase, reductase and ferredoxin, respectively. The estimated molecular masses of component A; α subunit, β subunit, B and C by SDS-PAGE were 45, 22, 48.2 and 9.8 kDa, which were closely related to 48, 20, 45 and 11 kDa predicted from the deduced amino acid sequence reported by Thupmongkhon (2003) and Nintanawongsa (2002). The optimal pH and temperature for acenaphthylene dioxygenase activity were 7.5 and 35°C, respectively. The enzyme complex was stable at pH ranging from 6.5 to 9.5 and at temperature up to 45°C. The pH and temperature stability range of the individual component were similar to those of the enzyme complex, except for component B. Component B was stable at temperature up to 60°C. This enzyme complex was

strongly inhibited by metal ions which included Ni^{2+}, Zn^{2+}, $Co^{2+}$and Cu^{2+} in which $Zn^{2+}$and Cu^{2+} were the most potent inhibitors similar to that of 2-halobenzoate dioxygenase from *Pseudomonas cepacia* 2CBS (Fetzner et al., 1992). Chemicals such as diphenyliodium, a flavoprotein inhibitor, and EDTA inactivated the enzyme activity which were similar to those of phthalate dioxygenase from *Pseudomonas cepacia* (Batie et al., 1987). Similarly, iodoacetate and N-ethylmaleimide completely inactivated the enzyme activity as reported with naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816 (Haigler and Gibson, 1990). The properties of acenaphthylene dioxygenase complex were summarized in Table 5.1.

Table 5.1 Properties of acenaphthylene dioxygenase enzyme complex from *Rhizobium* sp. CU-A1

Properties	Component A (oxygenase)		Component B (reductase)	Component C (ferredoxin)
Molecular masses (gel filtration)	129.6 kDa		42.7 kDa	9.3 kDa
Molecular masses (SDS-PAGE)	45 kDa	22 kDa	48.2 kDa	9.8 kDa
Optimal temperature	35°C			
Optimal pH	7.5			
pH stability	6.5-9.5 for the enzyme complex			
	6.5-9.5		6.5-9.5	6.5-9.5
Temperature stability	up to 45°C for the enzyme complex			
	up to 45°C		up to 60°C	up to 45°C
K_m for acenaphthylene	0.59 mM			
V_{max} for acenaphthylene	4.5×10^{-2} μmol/min/mg			

Bacterial dioxygenases such as naphthalene dioxygenase, toluene dioxygenase and biphenyl dioxygenase had ability to oxidize many related aromatic compounds to dihydrodiol products. Similarly, acenaphthylene dioxygenase from *Rhizobium* sp. CU-A1 was capable of oxidizing a wide variety of PAHs including naphthalene,

acenaphthene, phenanthrene, fluorene, anthracene, fluoranthene, pyrene, and 1-naphthoic acid. From the K_m values, they suggested that the enzyme is more specific to acenaphthylene (0.59 mM) than 1-naphthoic acid (0.70 mM) and naphthalene (1.27 mM). However, from the literature review, only K_m value of naphthalene dioxygenase from *Corynebacterium renale* for naphthalene was reported to be 2.9 mM (Dua and Meera, 1981) which was higher than that of the acenaphthylene dioxygenase reported here. Moreover, the acenaphthylene dioxygenase is also capable of oxidizing acenaphthenequinone, 1,2-dihydroxynaphthalene and gentisic acid by ring cleavage activity. Although, to date, the occurrence of many aromatic compound dioxygenases has been reported, acenaphthylene dioxygenase is the only polycyclic aromatic hydrocarbon dioxygenase which can catalyze dihydroxylation of acenaphthylene to form diol product moreover it also catalyzes ring fission of acenaphthenequinone to naphthalene-1,8-dicarboxylic acid in the acenaphthylene catabolic pathway. Our finding indicated that conversion of acenaphthenequinone to naphthalene-1,8-dicarboxylic acid involves enzyme catalysis not just spontaneous conversion as suggested by Selifenov et al. (1996). Therefore, further research focusing on the catalytic mechanism and molecular structure of this enzyme should be of interest.

With the sound understanding of microbiology and to integrate this knowledge to the engineering principles that have governed industrial wastewater treatment, environmental scientists and engineers can design and operate the treatment system with high efficiency for hazardous wastes treatment. Successful biological treatment projects require a basic understanding of the microbiology involved as well as the engineering factors required for the design, implementation and operation. With this in mind, the results obtained from this work will be useful to facilitate the application and the improvement of bioremediation in the actual contaminated environment and resources. From the identification of acenapthylene degrading intermediates, it suggested that all identified intermediates were less toxic and less persistant than the parent compound, acenaphthylene (Roy, 1997). Moreover, this strain is capable of mineralizing acenaphthylene completely without any accumulated intermediates. Although, this strain is capable of utilizing only acenaphthylene or naphthalene as a sole source of carbon and energy but it can co-metabolically transform a wide varity of aromatic compounds (Paengthai, 2000). Therefore this strain can be useful to remediate the acenaphthylene contaminated sites as well as other aromatic compounds

in the presence of acenaphthylene. However, the experiments on the application of this strain in the actual contaminated sites are still needed to be conducted prior to the application.

Moreover, the acenaphthylene catabolic pathway and identification of acenaphthylene dioxygenase can be used for understanding of basic biochemistry, biocatalysis leading to specialty chemical manufacture, and biodegradation of environmental pollutants. These information can be used to support pure and applied research such as study on genes and enzymes involving the degradation and study on the bioremediation of contaminated sites by using this bacterial strain. Recently, industries increasingly need to know the environmental fate of their commercial chemicals which is largely governed by the metabolism of the chemicals by microorganisms. Commercial users can use our information as part of EPA reports. Recently, biotechnology companies used new advanced technology in biocatalysis in specialty chemical manufacture. For example, using of naphthalene dioxygenase to produce blue jean dy indigo (Bialy, 1997). The knowing of acenaphthylene catabolic pathway can also be used to predict biodegradation reaction of other aromatic pollutants that might occur during co-metabolic degradation by this strain, both practically and theoretically. The theoretically prediction of metabolism is also very important to industry and require such information. In most case, information on the biodegradative pathways of new compounds is lacking and too expansive and time-consuming to obtain experimentally. Therefore, it is important to predict the biodegradative metabolism of new organic compounds based on known catabolic pathways. As a result, acenaphthylene catabolic pathway can also be used as a guidance for prediction of metabolism of other PAHs or related compounds.

Acenaphthylene dioxygenase from *Rhizobium* sp. CU-A1 has a broad substrate spectrum and remained effective in a wide range of pH and temperature. Because *Rhizobium* sp. CU-A1 can only degrade acenaphthylene and naphthalene as a sole carbon source and can not initially attacked aromatic ring of many aromatic compounds. In contrast, acenapthylene dioxygenase showed ability to oxidize many aromatic compounds including acenaphthene, phenanthrene, fluorene, anthracene, fluoranthene, and pyrene. Therefore, the addition of this enzyme into contaminated sites as a bioremediation agent in combination with the using of whole cell of CU-A1

or indigenous organisms might facilitate the mineralization of these polycyclic aromatic contaminants.

Suggestions for future work

For purification of acenaphthylene dioxygenase, conventional chromatography techniques were applied. However, the results showed that the yields after purification was very low. Therefore, the use of recombinant strain that produce high level of the enzyme are of interest.

Because of the unusual mechanism of the enzyme including its catalysis of acenaphthenequinone transformation by ring fission reaction, the detailed biochemical characterization and function of each component such as iron-sulfur content, N-terminal amino acid sequence, reaction stoichiometry and anaerobic catalyzed reduction are also of interest.

Rhizobium sp. CU-A1 and its enzyme involved in the initial degradative step of acenaphthylene are capable of degrading or catalyzing a variety of PAHs. However, there are many biochemical and environmental factors that affect the activities when introduced into the contaminated environment. As a result, additional research on the degradation of PAHs in the environment should be studied not only by whole cell of *Rhizobium* sp. CU-A1 but also by using the enzyme.