CHAPTER 1.
GENERAL INTRODUCTION

In the last few decades, many hydrocarbon compounds especially aromatic hydrocarbons have been introduced in large quantity and accumulated in environment, anaerobic sediment, or even in deep-ground water (Mohn and Kennedy, 1992; Kuo and Genthner, 1996; Laine and Laine and Jergensen, 1996; Semple and Cain, 1996; Werwath et al., 1998). They become a serious problem since they are toxic and carcinogenic (Shimao et al., 1989; Leahy and Colwell, 1990; Dong et al., 1992; Valenzuela et al., 1997).

The persistence of the aromatic hydrocarbon compounds in environment depends on the structure and the complexity of the compounds. Haloaromatic and polycyclic aromatic hydrocarbon in general are relatively recalcitrant (Leahy and Colwell, 1990; Valenzuela et al., 1997). It is known that mineralization rate (degradation of the compounds to $\text{CO}_2$ and $\text{H}_2\text{O}$) of higher-molecular-weight complex aromatic hydrocarbon, such as resin, and asphalten is much slower than degradation of lower-molecule-weight aromatic hydrocarbon such as monocyclic aromatic compounds (Leahy and Colwell, 1990). However, previous studies showed that complex hydrocarbons were rapidly degraded in optimum condition (Leahy and Colwell, 1990).

One of the important monocyclic aromatic introduced to environment is benzoate. It is introduced through herbicide application or other industrial practices (Werwath et al., 1998). It is also encountered as an important intermediate in metabolic pathway of many aromatic hydrocarbon compounds
Metabolism of aromatic hydrocarbon compounds as well as other hydrocarbons in nature depends on the catabolic reaction of microorganisms (Semple and Cain, 1996). Degradation rate of the compound is affected by its properties, its concentration, and the microbial community in the environment (Leahy and Colwell, 1990; Nicholson et al., 1992).

Biodegradation process are versatile and can be utilized at various stages of treatment (Portier, 1991). However, introduced microorganisms may occasionally not work as they are supposed to (Nicholson et al., 1992). Lower resistance, predation, competition, inhibition by other toxic chemicals or much toxic intermediate, and other microbial contaminants are responsible for uncompleted biodegradation of organic compounds (Harwood et al., 1990; Hiepieper et al., 1992; Miethling and Karlson, 1996; Blasco et al., 1997). Since biotransformation products are not necessary safe, the complete mineralization of toxic organic substances to CO₂ and H₂O is the most desirable goal (Laine and Jørgensen, 1996; Blasco et al., 1997).

Utilization of microorganisms as bioremediation agents of hydrocarbon compounds has been plentifully reported (Blasco and Castillo, 1992; Lobos et al., 1992; Nicholson et al., 1992; Blasco et al., 1997). Furthermore, industrial devoted to the bioremediation of toxic organic pollutants are growing rapidly (Wyndham et al., 1994). Biotechnological approach in biodegradation process of complex hydrocarbons was established by choosing proper microorganisms or engineering degrading microorganisms. A knowledge of biotransformation pathways is
essential to assess risks at contaminated sites, implement biological treatment processes, or design effective bioremediation strategies (Nicholson et al., 1992).

Selection and characterization of new prospecting aromatic-utilizing organisms are still in need, while many workers are trying to optimize the extraction of the available strains. For the former groups, many restricted their efforts only on the ability of the bacteria to utilize the aromatic compounds. A knowledge of genetic, physiological, and ecological characterization in the screening and selection of bacteria are crucial in term of providing comprehensive information of the strains in order to determine and to establish proper technology for bioremediation (Leahy and Colwell, 1990; Nicholson et al., 1992; Wyndham et al., 1994).

Aerobic Degradation of Monocyclic Aromatic Hydrocarbon Compounds

Study on biodegradation of aromatic hydrocarbon compounds has primarily conducted for aerobic microorganisms (Genthner et al., 1989). Of these microorganisms, bacteria like Achromonas, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Pseudomonas, and Burkholderia spp. (Shimao et al., 1989; Leahy and Colwell, 1990; Shen and Wang, 1995; Kuo and Genthner, 1996; Blasco et al., 1997), and the fungi (Biebos et al., 1988; Bumpus, 1989; Spadaro et al., 1992; Gemble et al., 1996) are degradation organisms that have been reported most successful in catabolizing the aromatic compounds. Several other microbes (Grund et al., 1990; Lenke and Knackmuss, 1992; Lenke et al., 1992; Lobos et al., 1992; Schmidt et al., 1992; Behki et al., 1993; Allen et al., 1997; Miethling and Karlson, 1996) including algae (Semple and Cain, 1996) have also shown their ability to aerobically catabolize aromatic hydrocarbons.
Although a new oxidation pathway has been described via benzoyl-Coenzyme A and 3-hydroxybenzoyl-Coenzyme A in a denitrifying *Pseudomonas* sp. (Altenschmidt *et al.*, 1993), the most common routes for aerobic degradation of these compounds are usually through destabilization of the aromatic ring to form intermediate catechol (1,2 dihydroxybenzene) (Grund *et al.*, 1990; Blasco *et al.*, 1997). Protocatechuat (3,4 dihydroxybenzoate) and gentisate (2,5 dihydroxybenzoate) as intermediates were also postulated (Crowford, 1976; Fuenmayor *et al.*, 1998).

The conversion of the monocyclic aromatic to intermediate catechol involves ring-dioxygenases. However, in some bacteria ring-monoxygenases are the common enzymes for the conversion (Powlowski and Shingler, 1994; Williams and Sayers, 1994; Shield *et al.*, 1995). The catechols are substrates for the second stage of catabolism which is performed by the actions of ring-cleavage dioxygenases that break one of the carbon-carbon bonds of the ring by addition of molecular oxygen. This reaction produces an unsaturated aliphatic acid (William and Sayers, 1994). The ring cleavage usually occurs through ortho cleavage (intradiol) which produced cis,cis muconic acid (or a derivative) and through meta cleavage (extradiol) which produces 2-hydroxymuconic semialdehyde (or a derivative) (Williams and Sayers, 1994; Laine and Laine and Jørgensen, 1999; Blasco *et al.*, 1997). The enzyme systems resemble each other, even though many different metabolic pathways have been identified (Williams and Sayers, 1994; Kudo *et al.*, 1998).

The biochemistry of the two reaction sequences appears to be conserved (Figure 1) in all bacteria in which they are found. Therefore, aerobic aromatic
Catabolism consists of a variety of pathways that converge on a common intermediate (catechols) which are further assimilated by a common pathway (Williams and Sayers, 1994). The pathway itself has undoubtedly been in existence for a considerable period of evolutionary time (Williams and Sayer, 1994).

Phenol, benzoate, and their derivatives have generally been a subject of extensive study on aerobic biodegradation of monocyclic aromatic compounds (Gurujeyalakshmi and Oriel, 1989; Williams and Sayer, 1994; Shen and Wang, 1995; Semple and Cain, 1996; Valenzuela et al., 1996). Phenol is found as natural phenolic compounds in plant materials as well as in the effluents of oil refineries, petrochemical plants, pesticide application, and other industrial processes (Gurujeyalakshmi and Oriel, 1989; Lenke et al., 1992; Werwath et al., 1998). It is also found as an important intermediate in the anaerobic degradation of many complex and simple aromatic compounds (Zhang and Wiegel, 1990). Like phenol, benzoate and its derivatives are often encountered as intermediate of complex aromatic hydrocarbon catabolism including biphenyl (Williams and Sayers, 1994; Arendorf et al., 1995), chlorophenol (Williams and Sayers, 1994), cinnamate, mandelate, 5-phenylvalerate, 3-phenyl propionate, and benzoylformate (Harwood and Gibson, 1988). Thus, the factors that influence the rate and extent of benzoate degradation may also influence biodegradation of other aromatic compounds (Hopkins et al., 1995; Warikoo et al., 1996). It was also introduced through pesticide applications or other industrial processes (Shimao et al., 1989).
Figure 1. General pathway of aerobic catabolism of aromatic compounds (Williams and Sayers, 1994).
Anaerobic Catabolism of Monocyclic Aromatic Hydrocarbons

Anaerobic degradation pathway of aromatic hydrocarbon (Figure 2) has not been fully understood (Coschigano and Young, 1997; Harwood and Gibson, 1988; Madsen and Licht, 1998), but the process has been reported (Genthner et al., 1989; Madsen and Licht, 1992; Nicholson et al., 1992; Hopkins et al., 1995).

Madsen and Licht (1992) isolated and characterized an anaerobic bacterium from municipal sludge. The bacterium, related to Clostridium, was able to dechlorinate chlorophenol. Anaerobic biodegradation of atrazine by the facultatively anaerobic bacterium M9-1-3 has been studied (Crawford et al., 1998). The isolate was capable to utilize atrazine as its sole C and N source under anaerobic conditions (Radosevich et al., 1995). Nicholson et al. showed that pentachlorophenol-acclimated methanogenic consortium dechlorinated pentachlorophenol to 4-chlorophenol, although not all routes produce this intermediate. Kuo and Genthner (1996) isolated a new bacterium, strain SB, that degrades benzoate only when coculture with an H₂ or formate-utilizing bacterium such as Desulfovibrio sp. strain G-11.
Figure 2. General pathway of anaerobic catabolism of aromatic compounds (Pelletier and Harwood, 1998).
The effect of electron acceptors and electron donors availability in anaerobic degradation of aromatic compounds might differ. Mohn and Kennedy (1992) reported that addition of electron donors such as sucrose and some potential products of sucrose fermentation, H₂, formate, acetate and propionate had negligible effect on dehalogenation of chlorophenol. Added elemental S, sulfate, nitrate, and ferric ions inhibited dehalogenation. However, Heindriksen et al. (1992) demonstrated that the addition of glucose in a glucose-amended reactor stimulated dechlorination rate of pentachlorophenol. This might be due to a higher concentration of the biomass. Häggblom et al. (1993) showed that the process depended on the availability of electron acceptor, and on the position of the chlorine substituent. In anoxic sediments, nitrate, sulfate, or carbonate may serve as terminal electron acceptors (Kohring et al., 1989; Häggblom et al., 1993). When sulfate concentration tend to be low, such as in anaerobic freshwater environment, carbonate reduction to methane serves as the predominant electron sink. On the other hand, in marine systems, sulfate concentration tend to be high, and sulfate reduction or sulfidogenesis serves as the major electron accepting process (Häggblom et al., 1993).

The effect of heavy metal ions like Cd(II), Cu(II), Cr(VI), or Hg(II) on biodegradation of 2-chlorophenol, 3-chlorobenzoate, phenol, and benzoate in anaerobic bacterial consortia has been examined. Although the effect of the ions was different in different aromatic compounds, increasing degradation rate was observed in benzoate with 0.01 ppm Cr(IV), Cd(II) and Cu(II), in phenol with 0.01 ppm Cr(IV), and in 2-chlorophenol and 3-chlorobenzoate with 1.0 to 2.0 ppm Hg(II) after an extended acclimation period (Kuo and Genthner, 1996).
Although previous works showed anaerobic degradation of many monocyclic aromatic (Genthner et al., 1989; Madsen and Licht, 1992; Mohn and Kennedy, 1992; Kuo and Genthner, 1996; Crawford et al., 1998), anaerobic benzoate has gotten more attention. Häggblom et al. (1993), Mohn et al. (1995), Kuo and Genthner (1996), and Warikoo et al. (1996) observed anaerobic benzoate degradation in various bacteria. Harwood and Gibson (1988), Kamal and Wyndham (1990), Wright and Madigan (1991), Blasco and Castillo (1992), Gibson and Gibson (1992), Sasikala et al. (1994), and Shoreit and Shaheb (1994) saw that anoxygenic phototrophic bacteria photo-anaerobically catabolize benzoate and its derivatives or homologs. Since the anoxygenic photosynthetic bacteria demonstrate biochemical versatility, they are relatively easier to study rather than any other obligately anaerobic bacteria. A complete pathway of the anaerobic degradation of aromatic compound has been regulated from anoxygenic photosynthetic bacteria (Figure 2) (Pelletier and Harwood, 1998).

Genetic and Biochemistry of Aromatic Hydrocarbon Catabolism

Genetic and biochemical analysis of aerobic degradation has been done primarily in Pseudomonas (Altenschmidt et al., 1993; Dunaway-Mario and Babbitt, 1994; Powloski and Shingler, 1994; William and Sayers, 1994; Shield et al., 1991; Souza et al., 1995; Blasco et al., 1997; Fuenmayor et al., 1998). Degradation of aromatic compound is encoded in plasmids or chromosome (Harayama et al., 1991; Jeffrey et al., 1992; Brenner et al., 1993). Some transposable elements such as Tn4651 and Tn4653, the toluene transposons, and Tn4655, the naphthalene transposon also carry the degradative genes (Wyndham et al., 1994). Shield et al.
(1995) found that TOM plasmid, a 108 kb degradative plasmid, are responsible for toluene and phenol catabolism. This plasmid possesses genes coding for toluene ortho monooxygenase and catechol 2,3-dioxygenase. Large plasmid collectively called the TOL plasmids carrying xyl gene for toluene/xylene has been subject of intensive study (Assinder and Williams, 1990). Several other degradative genes have also been identified. These include bph, dmp, nab and tod (Williams and Sayers, 1994), gtd (Werwath et al., 1998), ben (Jeffrey et al., 1992), and nag (Fuenmayor et al., 1998).

Several study on homology of the degradative genes has been carried out. Kim et al. (1996) has conducted homology study of degradative genes in Sphingomonas. Harayama et al. (1991) observed that xylXYZ of Pseudomonas putida and benABC of Acinetobacter calcoaceticus shared a common ancestry. Bundy et al. (1998) saw the similarity between the antABC-encoded anthranilate dioxygenase and the benABC-encoded benzoate dioxygenase of Acinetobacter sp. strain ADP1.

Substitution of antC of Acinetobacter mutants by benC during growing in anthranilate suggesting relatively broad substrate specificity of the BenC reductase. In contrast, the benAB genes did not substitute for antAB (Bundy et al., 1998) indicating a narrow substrate specificity (Harayama et al., 1991; Bundy et al., 1998). The genes responsible in conversion of naphthalene to gentisate, nag, from Pseudomonas sp. strain U2 isolated from oil-contaminated soil have been sequenced. Sequence comparisons suggested that the novel genes represented the archetype for naphthalene strains which use the gentisate pathway rather than the meta cleavage pathway of catechol (Fuenmayor et al., 1998).
Comparative study on enzyme responsible for degradation of aromatic compounds were conducted by Dong et al., (1992) and Neidle et al. (1991). Catechol 2,3 dioxygenase of *B. stearothermophilus* was functionally the same as the enzyme encoded by *xylE* in *P. putida*, although their thermostability and homology between the two genes were rather different (Dong et al., 1992). Neidle (1991) demonstrated that the comparison of the deduced amino acid sequences of *BenABC* of *A. calcoaceticus* with relative sequences including those of the multicomponent toluate, toluene, benzene, and naphthalene 1,2 dioxygenase enzymes indicated that the similar size of the hydroxylase component subunits were derived from a common ancestor.

Study on genetic of anaerobic catabolism of aromatic compounds was almost limited on anoxic photosynthetic bacteria. Anaerobic catabolism of benzoate by anoxic photosynthetic bacteria involves *bad* genes. For the ring-cleavage of benzoate, *badl* that codes for Bad1, a 2-ketocyclohexanecarboxyl Coenzyme-A hydrolase, are seemingly responsible (Palletier and Harwood, 1998).

Biochemical analysis of the anaerobic monocyclic aromatic hydrocarbon catabolism showed the possible pathways (Palletier and Harwood, 1998) with cyclohex-1,5-diene-1 carboxyl-CoA and 3-hydroxypimelil Co-A as a common intermediate before separating to their specific pathway and entering TCA cycle, respectively.

Cloning of degradative genes has been reported. Kim and Oriel (1995) successfully cloned *pheA* and *pheB* from *B. stearothermophilus* BR219 to *Escherichia coli*. The genes are coding for the conversion phenol to catechol and catechol to 2-hydroxymuconic semialdehyde, respectively. Cloning and mapping of phenol
Degradative genes for meta pathway from B. stearothermophilus FDTP-3 to E. coli was also carried out by Dong *et al.* (1992). Springael *et al.* (1994) reported a transfer of degradative genes into heavy metal resistant *Alcaligenes eutrophus* strains. Zylstra (1996) cloned degradative genes that distinct from the classical gene *nah* from *Comamonas testosteroni* GZ39, capable of degradation of polycyclic aromatic hydrocarbon. Cloning and partial sequence of atrazine degradative gene from *Pseudomonas* sp. strain ADP have been conducted (de Souza *et al.*, 1995).

Only limited studies on degradative genes of anaerobic degradation of aromatic compounds has been conducted. Coschigano and Young (1997) carried out cloning and sequencing of *tut* genes which involved in anaerobic toluene degradation pathway of a denitrifying bacterium.

**1. General Objectives of The Research**

The objective of this study is to select and characterize bacterial isolates capable of utilizing monocyclic aromatic hydrocarbon as their C-source. The study of examination of anaerobic degradation of aromatic compounds was restricted only on anoxygenic photosynthetic bacteria. To achieve this aim, we utilized a number molecular techniques including analysis of 16S-rRNA genes of selected isolates, Macro Restricted Fragment Length Polymorphism (MFLP) for DNA profiling analysis and spectral analysis for the anoxygenic photosynthetic bacteria, transposition and mutation by transconjugation, and Southern hybridization. Bacterial identification for selected aerobic bacteria was performed using Microbact kit (Medvet Science PTY Ltd., Adelaide, Australia). Physiological
Properties were determined by growing the isolates in different monocyclic aromatic compounds, different concentrations of benzoate and NaCl, and different initial pH of the medium as well as non-aromatic C-sources. Microscope examination was employed to examine morphological properties including cell motility, colony appearance, and Gram staining.