CHAPTER TWO
REVIEW OF LITERATURE

2.1 INTRODUCTION

The ever increasing usage of synthetic chemicals and petroleum has resulted in large amounts of undesired industrial wastes being discharged into the biosphere (Ghisalba, 1983). The widespread pollution of the environment with industrial and domestic effluents containing toxic and recalcitrant organic compounds is a cause for great concern due to their potential hazard to the well being of plants and animals. Some of the environmental pollutants include compounds ranging from halogenated aliphatics, aromatics, nitroaromatics, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides and their metabolites. Due to their potential for causing adverse effects on humans, US EPA (Environmental Protection Agency) has initiated a comprehensive program of screening industrial effluents for the presence of organic priority pollutants. Some of the organic pollutants observed with highest frequency are given in Table 1.

PAHs are widespread environmental pollutants with toxic, mutagenic and carcinogenic properties and this has prompted considerable research on the sources, occurrence, bioaccumulation, metabolism and disposition of these pollutants in aquatic and terrestrial ecosystems (Pahlmann and Pelkonen, 1987). PAHs contain carbon and hydrogen with the carbon atoms arranged in a series of adjoining six-membered benzene rings in linear, angular and cluster arrangements. The structures of a few of the PAHs are shown in Figure 1. These PAHs are formed during the incomplete combustion of almost any organic material (Blumer and Youngblood, 1975; Hites et al., 1977) and have been isolated from air, water, soil and food (Sivaswamy et al., 1990). Some common sources are cigarette smoke, automobile exhaust, chimney soot, refuse burning and oil pollution (Ghisalba, 1983). PAHs also provide the starting materials for the production of some pharmaceuticals, agrochemicals, dyes, polymers and explosives (Smith, 1990). These are also considered potential carcinogens, mutagens and teratogens (Dipple et al., 1990). Many of the PAHs, due to their low water solubility, are usually bound to suspended particles in aquatic ecosystems and
### Table 1:

**Some of the organic chemicals on the EPA list of priority pollutants.**

<table>
<thead>
<tr>
<th>CHEMICAL CLASS</th>
<th>IMPORTANT REPRESENTATIVES AND THEIR FREQUENCY OF OCCURRENCE[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticides and metabolites</td>
<td>β-Hexachlorocyclohexane (0.8% Samples)</td>
</tr>
<tr>
<td></td>
<td>Aldrin (0.5%)</td>
</tr>
<tr>
<td>Polychlorinated biphenyls</td>
<td>Aroclor 1254 (0.6%)</td>
</tr>
<tr>
<td></td>
<td>Aroclor 1242 (0.8%)</td>
</tr>
<tr>
<td>Nitroaromatics</td>
<td>2-Nitrophenol (2%)</td>
</tr>
<tr>
<td></td>
<td>Nitrobenzene (2%)</td>
</tr>
<tr>
<td>Chloroaromatics</td>
<td>Dichlorobenzene (6%)</td>
</tr>
<tr>
<td></td>
<td>Pentachlorophenol (7%)</td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbons</td>
<td>Pyrene (8%)</td>
</tr>
<tr>
<td></td>
<td>Naphthalene (10%)</td>
</tr>
<tr>
<td></td>
<td>Phenanthrene/Anthracene (11%)</td>
</tr>
<tr>
<td>Aromatics</td>
<td>Benzene (26%)</td>
</tr>
<tr>
<td></td>
<td>Toluene (28%)</td>
</tr>
<tr>
<td>Phthalate esters</td>
<td>Di-«-butylphthalate (19%)</td>
</tr>
<tr>
<td></td>
<td><em>bis</em>- (2-Ethylhexyl)-phthalate (42%)</td>
</tr>
</tbody>
</table>

[^a]: Frequency of occurrence of these chemicals in 2600 waste water samples from different industrial categories.

* Adapted from Leisinger and Brunner (1986)
Figure 1:

The structures of some of the polycyclic aromatic hydrocarbons (PAHs) present in the environment.

Compounds shown are: A, naphthalene; B, anthracene; C, phenanthrene; D, fluorene; E, fluoranthene; F, pyrene; G, benzo(a)pyrene; H, benzo(a)anthracene
ultimately are deposited into sediments (Means et al., 1980). Various conventional physico-chemical methods used for decontamination of hydrocarbon-contaminated sites basically employ volatilization, evaporation, photooxidation and adsorption (Park et al., 1990). However, the disposal of volatile and other pollutants of significant toxicity is not preferred via these routes. Moreover, these methods of cleanup are generally less efficient and are very expensive (Morgan and Watkinson, 1989). Biological means in many cases are the only way to completely metabolize or mineralize a compound (Franklin et al., 1981; Keck et al., 1989; Atlas and Pramer, 1990). In order to achieve this goal, it is important to understand the biodegradation of the PAHs of interest at the molecular level. The varied mechanisms by which microorganisms utilize aromatic hydrocarbons as the carbon and energy sources have been reviewed (Cerniglia, 1984; Fewson, 1988; Smith, 1990; Cerniglia, 1991, 1993; Smith, 1994). Among others, three model classes of PAHs viz. naphthalene, phenanthrene and anthracene are important and are considered among the most acutely toxic compounds in the fractions of petroleum. The proposed research work is aimed to study the biodegradation of naphthalene and phenanthrene.

2.2 CENTRAL METABOLIC PATHWAYS FOR THE DEGRADATION OF PAHs

The degradation of different PAHs by microorganisms is generally initiated by the incorporation of both atoms of molecular oxygen (\(O_2\)) into the aromatic nucleus by the action of a dioxygenase enzyme (Patel and Gibson, 1974; Cerniglia and Heitkamp, 1989). Eventually, depending on the substitution in the original molecule undergoing oxidation, the two hydroxyl groups may be positioned either ortho to each other (as in catechol and protocatechuate) or para to each other (as in gentisate and homogentisate) (Bayly and Barbour, 1984).

Catechol and protocatechuate can be oxidized either via ortho- or via meta-cleavage pathway. The ortho-cleavage pathway (\(\beta\)-ketoadipate pathway) involves the cleavage of the aromatic ring between the carbon atoms bearing the hydroxyl groups (Figure 2). ortho-Cleavage of catechol and its analogues is mediated by catechol-1,2-dioxygenase to give rise to cis, cis-muconate which is converted to \(\beta\)-ketoadipate enol-lactone by a series of reactions. Hydrolysis of the enol lactone yields \(\beta\)-keto-
adipate which gives rise to succinate and acetyl-CoA. Protocatechuate is also degraded in a similar manner by protocatechuate-3,4-dioxygenase to cis, cis-β-carboxymuconate which is converted into (-)-β-carboxymuconolactone and then to oxoadipate (Rogoff, 1961; Cerniglia and Heitkamp, 1989) (Figure 2).

The meta-cleavage pathway (α-ketoacid pathway) yields α-ketoacids as a result of cleavage of the aromatic ring on one side of the neighbouring hydroxyl groups (Ornston, 1971; Dagley, 1986) (Figure 3). meta-Cleavage of catechol or its analogues occurs by catechol-2,3-dioxygenase which cleaves the bond between C2 and C3 of the ring giving rise to 2-hydroxymuconate semialdehyde which is then further metabolized. 2-Hydroxymuconate semialdehyde is hydrolyzed to 2-keto-4-enoate which is then hydrated to 2-keto-4-hydroxyvalerate and then to pyruvate and acetaldehyde which enter TCA cycle (Bayly and Barbour, 1984). The cleavage of protocatechuate at 4,5-position produces 4-carboxymuconate semialdehyde which is converted to 4-carboxy-2-hydroxymuconate by an NAD-dependent dehydrogenase and eventually to the formation of TCA intermediates by the action of other enzymes (Bayly and Barbour, 1984).

Murray et al. (1972) proposed that meta-cleavage pathway serves as a general reaction for the dissimilation of methyl-substituted aromatic compounds. Although the reactions of the two pathways (ortho- and meta-cleavage) are analogous, they appear to be mediated by physically separable enzymes that are subject to independent inductive control. Davies and Evans (1964) showed that a single Pseudomonas strain possessed the ability to form the enzymes of both meta- and ortho-cleavage pathway. When grown on naphthalene or salicylate, the organism converted the growth substrate to catechol which was dissimilated via the meta-cleavage pathway. On the other hand, growth of the same organism with benzoate elicited specifically the enzymes of the ortho-cleavage pathway (Murray and Williams, 1974). Barnsley (1976a) has reported that ortho-cleavage pathway plays the dominant role during naphthalene and salicylate metabolism in some pseudomonads whereas meta-cleavage pathway is dominant in others.

The para-dihydrich phenols such as gentisate and homogentisate are degraded via the gentisate pathway (Figure 4). Gentisate may be formed as an intermediate
during the degradation of compounds such as m-cresol, p-hydroxybenzoate, salicylate and anthranilate (Hopper and Chapman, 1971; Hopper et al., 1971). Tanaka et al. (1957) established a scheme for the catabolism of gentisate. The gentisate is acted upon by a gentisate-1,2-dioxygenase to form maleylpyruvate which is isomerized to form fumarylpyruvate in a reduced glutathione (GSH)-dependent reaction. The fumarylpyruvate is hydrolyzed to fumarate and pyruvate. The same pathway for the degradation of gentisate was also found in Alcaligenes eutrophus (Johnson and Stanier, 1971) and Moraxella osloensis (Crawford et al., 1975). An alternative pathway for the metabolism of maleylpyruvate was shown in Bacillus megaterium, where the isomerization of maleylpyruvate was catalyzed by a GSH-independent isomerase during the degradation of 3-hydroxybenzoate via gentisate pathway (Crawford and Olson, 1979). A third variation for gentisate degradation was described by Hopper et al. (1970) in which maleylpyruvate was hydrolyzed to maleate and pyruvate. This pathway was observed in different species of Pseudomonas for the catabolism of m-cresol and 2,5- and 3,5-xylenol (Poh and Bayly, 1980; Jain et al., 1984).

2.3 MECHANISMS OF DEGRADATION OF DIFFERENT PAHs

Due to the ubiquitous nature and increasing concentrations of PAHs, microorganisms can be found that have the ability to degrade these compounds. The microbiological degradation of some of the PAHs is described below.

2.3.1 Naphthalene

Naphthalene, the simplest fused polycyclic aromatic hydrocarbon, and the most toxic component in the water soluble fraction of crude and fuel oils (Gibson and Subramanian, 1984), is produced largely by petroleum refining and coal tar distillation. It is also used in the manufacture of a wide range of products including pesticides, fungicides, detergents, dyes, resins, and motor fuel (Gibson, 1971). It has long been used as an anthelmintic and as a household fumigant against cloth moths. A number of microorganisms have been isolated which can degrade naphthalene (Gibson and Subramanian, 1984; Cerniglia and Heitkamp, 1989; Smith, 1990). The metabolic sequence and enzymatic reactions leading to the degradation of naphthalene were first
presented by Davies and Evans (1964). Figure 5 shows the pathway proposed for the reactions involved in naphthalene metabolism.

The initial reaction in the bacterial oxidation of naphthalene involves the enzymatic incorporation of both atoms of molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy-1,2-dihydroronaphthalene (Jerina et al., 1976; Jeffrey et al., 1975). The enzyme naphthalene dioxygenase catalyzing this reaction consists of three protein components, an iron-sulphur flavoprotein, a two iron-two sulphur ferredoxin and an iron-sulphur protein (Ensley and Gibson, 1983; Haigler and Gibson, 1990). The second step involving the conversion of cis-1,2-dihydroxy-1,2-dihydroronaphthalene to 1,2-dihydroxynaphthalene is catalyzed by naphthalene- (+)-cis-dihydrondiol dehydrogenase which requires nicotinamide-adenine-dinucleotide (NAD) as an electron acceptor (Patel and Gibson, 1974). Davies and Evans (1964) showed that 1,2-dihydroxynaphthalene is enzymatically cleaved by 1,2-dihydroronaphthalene oxygenase to form cis-2-hydroxybenzalpyruvate (cHBPA). However, Barnsley (1976b) suggested that 1,2-dihydroronaphthalene undergoes oxidation to form 2-hydroxychromene-2-carboxylate (HCCA) as the initial product and that an inducible isomerase is responsible for the formation of cHBPA. Patel and Barnsley (1980) later on proposed that 1,2-dihydroronaphthalene is cleaved to an unstable ring-fission product which spontaneously recyclizes to HCCA. This compound is subsequently converted by an isomerase to cHBPA yielding salicylaldehyde and pyruvate (Figure 5).

The above reactions have been reinvestigated by Eaton and Chapman (1992) using recombinant bacteria, carrying genes encoding the metabolism of naphthalene to salicylate, and a pathway is proposed on the basis of these results (Figure 5). 1,2-Dihydroronaphthalene is cleaved by a dioxygenase to give rise to an unstable compound 2-hydroxy-4-(2-oxo-cyclo-hexa-3,5-dienyl)-buta-2,4-dienoate which rearomatizes yielding another unstable intermediate, cHBPA. This compound is spontaneously converted to its hemiketal HCCA which isomerizes to an equilibrium mixture containing 45% trans-2-hydroxybenzalpyruvate (tHBPA) both spontaneously and by the action of HCCA-isomerase. The tHBPA is then metabolized by cHBPA hydratase-aldolase to salicylaldehyde and pyruvate. The salicylaldehyde is oxidized to salicylate by an NAD+-dependent dehydrogenase. In most cases, the salicylate undergoes oxidative decarboxylation with the help of salicylate hydroxylase (White-
Stevens and Kumin, 1972) to yield catechol which is the substrate for fission of the aromatic nucleus via meta- or ortho-cleavage pathway (Section 2.2). In case of *P. fluorescens* and *P. alcaligenes* (Buswell *et al.*, 1980; Monticello *et al.*, 1985), the metabolism of salicylate via gentisate has also been reported. Grund *et al.* (1992) reported that the oxidation of salicylate to gentisate takes place by the action of salicylate-5-hydroxylase which has an unusual cofactor requirement for NADPH, ATP and CoA. The gentisate is then cleaved by gentisate-1,2-dioxygenase (Section 2.2).

### 2.3.2 Phenanthrene

Phenanthrene, a tricyclic aromatic hydrocarbon, is widely distributed throughout the environment as a result of pyrolytic processes and as a minor contaminant in waste water effluents from coal gasification and liquefaction processes (Blumer, 1976). It is a common constituent of coal tar and crude petroleum (Cerniglia, 1984). Pure cultures of bacteria and microbial populations have been isolated from fresh water and marine environments having the ability to metabolize phenanthrene (Guerin and Jones, 1988; Weissenfels *et al.*, 1990; Bogardt and Hemmingsen, 1992). The complete biodegradation using [14C]-phenanthrene in mineral oil by various bacteria has been recently reported (Foght *et al.*, 1990; Keuth and Rehm, 1991).

Two reaction sequences have been proposed for the degradation of phenanthrene (Figure 6). Phenanthrene is first oxidized at 3,4-position to form dihydrodiol (Jerina *et al.*, 1976). *cis*-3,4-Dihydroxy-3,4-dihydrophenanthrene is then dehydrogenated to 3,4-dihydroxyphenanthrene (Patel and Gibson, 1974) by the action of an NAD-requiring dehydrogenase (Nagao *et al.*, 1988). Evans *et al.* (1965) provided evidence that several pseudomonads further oxidized this dihydroxylated derivatives to an orange ring-fission product, *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut3-enoate. This ring-fission product is further oxidized to 1-hydroxy-naphthaldehyde which in turn is converted to 1-hydroxy-2-naphthoate in an NAD- dependent reaction. It has been suggested that 1-hydroxy-2-naphthoate undergoes oxidative decarboxylation to form 1,2-dihydroxynaphthalene prior to its further metabolism by the reactions proposed for the degradation of naphthalene (Section 2.3.1).

Kiyohara and Nagao (1978) proposed an alternative pathway for phenanthrene
metabolism in an *Aeromonas* sp. They found that in some cases, 1-hydroxy-2-
napthoate is converted to 2-carboxybenzaldehyde (Barnsley, 1983a) and then to o-
phthalate via an intradiol cleavage (Kiyohara and Nagao, 1977). o-Phthalate is then
hydroxylated and decarboxylated to protocatechuate which undergoes either ortho- or
meta-cleavage pathway (Section 2.2) depending on the organism.

2.3.3 Anthracene

There have been only a few reports on the biodegradation of anthracene and
little is known about the enzymes involved and the modes of ring-fission (Evans
*et al.*, 1965; Cerniglia, 1984; Gibson and Subramanian, 1984; Bauer and Capone, 1985). The
proposed biodegradative route for anthracene is shown in Figure 7. A *Beijerinckia*
sp. B-836 and *P. putida* 119 oxidized anthracene to form (+)cis-1,2-dihydroxy-1,2-
dihydroanthracene (Gibson *et al.*, 1973; Jerina *et al.*, 1976) which is further oxidized
via an NAD*-dependent dihydrodiol dehydrogenase to form 1,2-dihydroxyanthracene
(Patel and Gibson, 1974). 1,2-Dihydroxyanthracene is further metabolized to the ring-
fission product cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoate with subsequent
conversion to 2-hydroxy-3-naphthaldehyde (Gibson and Subramanian, 1984) and
2-hydroxy-3-naphthoate. This ring-fission product (2-hydroxy-3-naphthoate) is fur-
ther metabolized to salicylate and catechol which undergo ring-fission by a sequence
similar to that described for the bacterial oxidation of naphthalene (Evans *et al.*, 1965)
(Section 2.3.1).

2.3.4 Other higher PAHs

The biodegradation of higher molecular weight PAHs is one of the least
understood aspects of aromatic hydrocarbons-degrading bacteria. However, there are
now reports on mineralization of higher polycyclic aromatic compounds beyond ring-
fission by bacterial strains. Some of these compounds include fluorene (Weissenfels
*et al.*, 1990; Monna *et al.*, 1993; Pothuluri *et al.*, 1993), fluoranthene (Mueller *et al*.,
et al.*, 1988; Wunder *et al.*, 1994) and PCBs (Smith, 1990; Barriault and Sylvestre, 1993).

It is hoped that the promising reports on the isolation of bacteria capable of
growing on complex polycyclic aromatic compounds are followed up by detailed studies into the exact nature of the pathways involved.

2.4 CATABOLIC PLASMIDS

The pathways for the catabolism of various aromatic hydrocarbons are often specified by extrachromosomal genetic elements called plasmids. These catabolic plasmids usually expand the metabolic potential of the host bacteria and thus allow their respective host strains to utilize hydrocarbon(s) as the sole source of carbon and energy (Chakrabarty, 1972; Rheinwald et al., 1973; Dunn and Gunsalus, 1973; Wong and Dunn, 1974; Don and Pemberton, 1985). Most of these plasmids are high molecular weight plasmids often greater than 50kb in size (Haas, 1983). The majority of the degradative plasmids are conjugative, that is, they can be transferred among populations of different bacteria permitting the rapid spread of catabolic ability among different members (Haas, 1983). Some of the well known naturally-occurring degradative plasmids have been listed in Table 2. Among these plasmids, TOL (toluene/xylene degradation), SAL (salicylate degradation), NAH (naphthalene degradation) and PHN (phenanthrene degradation) are being described here.

2.4.1 Toluene Catabolic Plasmids (TOL plasmids)

At present, the best understood catabolic plasmid is the TOL plasmid which encodes the enzymes that degrade toluene/xylene. The archetype TOL plasmid, pWW0, was originally described in P. putida (arvilla) mt-2 (Williams and Murray, 1974; Wong and Dunn, 1974). The molecular size of pWW0 is about 117kb (Duggleby et al., 1977; Downing and Broda, 1979) out of which approximately 41kb is needed for the catabolic pathway and the regulatory genes (Bayley et al., 1977; Nakazawa et al., 1978). The plasmid is self-transmissible (Williams and Murray, 1974) and belongs to the incompatibility group Inc P9 (White and Dunn, 1977). Worsey and Williams (1975) discovered that TOL specified the activities for the oxidation of not only toluene but also of m- and p-xylene, benzoate and m- and p-toluate. Degradation of these compounds is initiated by progressive oxidation of the methyl side chain followed by oxygenative cleavage of the aromatic ring of the carboxylic acid thereby formed (Williams and Murray, 1974; Worsey and Williams, 1975).
### Table 2:

Some of the naturally-occurring catabolic plasmids.

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>HOST STRAIN</th>
<th>RELEVANT CHARACTERISTICS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOL</td>
<td><em>P. putida</em></td>
<td>Toluene degradation, 117kb, Inc P9</td>
<td>Williams and Murray (1974); Wong and Dunn (1974)</td>
</tr>
<tr>
<td>NAH7</td>
<td><em>P. putida</em> G7</td>
<td>Naphthalene degradation, 83kb, Inc P9</td>
<td>Dunn and Gunsalus (1973); Johnston and Gunsalus (1977)</td>
</tr>
<tr>
<td>pDTG1</td>
<td><em>P. putida</em> NCIB 9816</td>
<td>Naphthalene degradation, 81kb</td>
<td>Serdar and Gibson (1989a, b)</td>
</tr>
<tr>
<td>SAL</td>
<td><em>P. aeruginosa</em> PAC</td>
<td>Salicylate degradation, 68kb, Inc P9</td>
<td>Lehrbach <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>SAL1</td>
<td><em>P. putida</em> G1</td>
<td>Salicylate degradation, 85kb, Inc P9</td>
<td>Chakrabarty (1972)</td>
</tr>
<tr>
<td>CAM</td>
<td><em>P. putida</em></td>
<td>Camphor degradation</td>
<td>Rheinwald <em>et al.</em> (1973)</td>
</tr>
<tr>
<td>NIC</td>
<td><em>P. convexa</em></td>
<td>Nicotine degradation, Inc C</td>
<td>Thacker and Gunsalus (1979)</td>
</tr>
<tr>
<td>pMWD-1</td>
<td><em>P. putida</em> PMD-1</td>
<td>Salicylate degradation, 165kb</td>
<td>Zuniga <em>et al.</em> (1981)</td>
</tr>
<tr>
<td>pKG2</td>
<td><em>Beijerinckia</em> sp.</td>
<td>Phenanthrene degradation, 31.2kb</td>
<td>Kiyohara <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>pJP4</td>
<td><em>A. eutrophus</em></td>
<td>2,4-Dichlorophenoxyacetate degradation</td>
<td>Don and Pemberton (1985)</td>
</tr>
</tbody>
</table>

(a) Inc, plasmid incompatibility group
On the basis of the regulatory data, Worsey et al. (1978) reported that the catabolic genes of pWWO are organized into two distinct operons conveniently referred to as upper- and lower-pathway operons. Cloning of the genes into an expression vector and the analysis involving identification of their protein products in maxi-cells of E. coli revealed that the upper-pathway operon contains genes in order xylCMABN (Harayama et al., 1989) encoding for degradation of toluene and xylenes to benzoate and tolulates, respectively and the lower-pathway operon consists of xylXYZLTEGFJQKIH (Harayama and Rekik, 1989) encodes the degradation of benzoate and tolulates to acetaldehyde and pyruvate, respectively. Other TOL plasmids have also been described (Friello et al., 1976; Worsey and Williams, 1977; Yano and Nishi, 1980; Assinder and Williams, 1990; Zylstra and Gibson, 1991).

2.4.2 Salicylate Catabolic Plasmids (SAL plasmids)

Chakrabarty (1972) observed that P. putida strain R1 degraded salicylate (Sal+) via catechol which is cleaved through meta-cleavage pathway. He also showed that the Sal+ phenotype of P. putida R1 could be transferred by conjugation to different Pseudomonas spp. or eliminated by mitomycin C treatment. This phenotype was attributed to the presence of a plasmid designated as SAL1 plasmid (Chakrabarty, 1972). The plasmid is self-transmissible and was shown to have a size of about 85kb (Yen et al., 1983). When SAL1 plasmid was transferred to P. aeruginosa PAC, it was shown to have a size of about 68kb (Lehrbach et al., 1983) and was termed as SAL. Further analysis of the restriction fragments of SAL plasmid suggested that this plasmid has been derived from a 22kb deletion of the NAH7 plasmid (naphthalene-degrading plasmid, Section 2.4.3.1) followed by an 8 to 9kb insertion of some unknown DNA (Lehrbach et al., 1983). The available evidence strongly suggests that the SAL1 is completely homologous to NAH7 (Yen and Gunsalus, 1982) with the exception of a 2.5kb segment (including part of nahA genes and their promoter) which is replaced by 4.6kb of nonhomologous DNA.

Zuniga et al. (1981) reported a 165kb plasmid, designated as pMWD-1, responsible for the degradation of salicylate via the meta-cleavage pathway in a P. putida strain NP. However, marked differences were observed in endonuclease
restriction patterns of SAL1 and pMWD-1 which showed that these two plasmids are dissimilar (Zuniga et al., 1981).

2.4.3 Naphthalene Catabolic Plasmids (NAH plasmids)

Naphthalene catabolic plasmids either belong to incompatibility group Inc P7 or Inc P9. Most of them are self-transmissible and are quite large in size. The best studied naphthalene catabolic plasmid is NAH7.

2.4.3.1 NAH7 plasmids

In 1973, Dunn and Gunsalus reported that the naphthalene-utilizing phenotype of \textit{P. putida} strain PpG7 was specified by a catabolic plasmid NAH. This study was mainly based on the observations that the naphthalene-degrading phenotype could be cured by mitomycin C and that the same phenotype was transferable back to cured strains and to other heterologous fluorescent pseudomonads by conjugation and pI6-mediated transduction. During subsequent detailed analysis in the derivatives of \textit{P. putida} strain G1 the plasmid was renamed as NAH7. On the basis of endonuclease restriction patterns, Lehrbach et al. (1983) calculated the plasmid size to be approximately 83kb. Recently, Menn et al. (1993) and Sanseverino et al. (1993) observed that the organisms containing NAH7-like plasmids may be involved in the degradation of high molecular weight PAHs other than naphthalene.

Physical Map of Plasmid NAH7

The organization of genes encoding the enzymes responsible for naphthalene degradation has been determined by analyzing a series of NAH7 polar mutations generated by the transposon Tn5 (Yen and Gunsalus, 1982; Yen and Serdar, 1988; Figure 8). The enzyme patterns revealed that the genes were organized in two physically separated clusters which were also functionally distinct. The first cluster which includes genes \textit{nahABCDEFE} encodes the conversion of naphthalene to salicylate and pyruvate. The second operon includes \textit{nahGHIJK} which encodes genes for the oxidation of salicylate via catechol meta-cleavage pathway. The genes \textit{nahA,B,C,D,E} and \textit{F} code for naphthalene dioxygenase, cis-naphthalenedihydrodiol dehydrogenase,
1,2-dihydroxynaphthalene oxygenase, 2-hydroxychromene-2-carboxylate isomerase, 2-hydroxybenzalpyruvate aldolase and salicylaldehyde dehydrogenase, respectively. Genes nahG,H,I,J and K code for salicylate hydroxylase, catechol-2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxymuconate tautomerase and 4-oxalocrotonate decarboxylase, respectively. Based on polarity studies, it was shown that the transcription is from nahA towards nahE-F in the first gene block (nah1 operon) and from nahG towards nahK in the second gene block (nah2 operon) (Yen and Gunsalus, 1982; Yen et al., 1983). However, recently Eaton and Chapman (1992) established a revised gene order to be ABFCED on the basis of the enzymes encoded by various subclones (Figure 9). Various nah genes in NAH7 have been sequenced. Ghosal et al. (1987) determined the nucleotide sequence and deduced the amino acid sequence and expression of nahH gene of NAH7. The nucleotide sequence of the DNA segment encoding 2-hydroxychromene-2-carboxylate isomerase (nahD) from upper operon of plasmid NAH7 has recently been determined (Eaton, 1994). Simon et al. (1993) sequenced the genes encoding naphthalene dioxygenase in P. putida PpG7. Lehrbach et al. (1983) reported the molecular relationships between pWW0, NAH7 and SAL1 plasmids on the basis of DNA : DNA hybridization studies. They identified six regions on pWW0 which shared varying amount of homologies with NAH7 and SAL1. They suggested that out of these six regions, three regions are related to the transfer and replication of plasmids and a fourth region related to the meta-cleavage pathway for catechol metabolism. The function for the other two regions were not identified.

Regulation of Naphthalene Catabolic Genes

Activation of the nah operons from NAH7 requires both an inducer and the product of a regulatory gene. Salicylate and its structural analog, 2-aminobenzoate, were initially shown to induce all the enzymes of the naphthalene oxidation pathway (Barnsley, 1975; 1976a). Induction by salicylate acts at the transcriptional level resulting specifically in the increased synthesis of mRNA from the two nah operons (Schell, 1985). Although both nah operons of the NAH7 plasmid are induced during growth in the presence of naphthalene, this compound is not an inducer of the NAH7-encoded naphthalene oxidation pathway. Connors and Barnsley (1980) observed that in mutants which had a block in the metabolic pathway preceding the reaction catalyzed by salicylaldehyde dehydrogenase, the enzymes of naphthalene-degrading pathway were
not induced during growth in the presence of naphthalene. This observation suggested that the formation of salicylaldehyde was an absolute pre-requisite for induction of *nah* enzymes which was, therefore, identified as the first possible inducer. However, this study did not rule out salicylate as an inducer of naphthalene pathway. Therefore, it is quite likely that both salicylate and salicylaldehyde could act as effective inducers (Connors and Barnsley, 1980). Thus, the regulation of naphthalene metabolism contrasts with that of other aromatic hydrocarbons in the sense that a relatively large number of enzyme-catalyzed steps are required to produce the first inducer.

Yen and Gunsalus (1985) characterized the mutants resulting from NAH7::Tn5 mutations that affected the regulation of the naphthalene oxidation pathway. The pleotropic, recessive and negative nature of the *nahR* mutations indicated that the *nahR* gene specifies a regulatory element which is required to activate both *nah* operons (Yen and Gunsalus, 1985). Further study of a *nahR::Tn5* mutant revealed that the mutation blocked the salicylate-induced synthesis of mRNA from the *nahA* and *nahG* regions of NAH7 (Schell, 1985). However, *nahR* gene is transcribed in a direction opposite to that of *nahG* gene (Schell, 1986). All these studies suggested that coordinate induction of salicylate is mediated at the transcriptional level by *nahR* (Schell and Wender, 1986) which encodes a salicylate-dependent activator of the transcription of both operons (You and Gunsalus, 1986; You *et al.*, 1988). Further, the genes for naphthalene degradation have been located on 25kb and 5.9kb *EcoRI* fragments by cloning studies (Grund and Gunsalus, 1983; Schell, 1983).

### 2.4.3.2 Other naphthalene catabolic plasmids

Relatively less information is available regarding the other reported naphthalene catabolic plasmids. Cane and Williams (1982) revealed that a naphthalene-utilizing *Pseudomonas* sp. NCIB 9816 contained two plasmids; pWW60 (NAH2), an Inc P9 plasmid of 87kb encoding genes for the catabolism of naphthalene and, pWW61, a smaller cryptic plasmid. pWW60 was transferred by conjugation into a plasmid-free new host PaW340 and was then designated as pWW60-l. It was shown that the plasmid pWW60-l carried genes for the conversion of naphthalene to catechol and that further degradation of catechol requires a functional chromosomal *ortho*-cleavage pathway.

The plasmid pDTG1, a conjugative plasmid involved in naphthalene utilization,
is 83kb in size (Serdar and Gibson, 1989a). Restriction endonuclease digestion, hybridization and mutation studies indicated that a 15kb EcoRI fragment encodes the enzymes of the upper-pathway. This 15kb EcoRI fragment was cloned into the broad-host-range vector pKT230 to construct a recombinant plasmid designated pDTG113 (Serdar and Gibson, 1989b) which was then used as probe to identify the homologous regions on NAH7 plasmid.

The role of some other naturally-occurring plasmids has also been suggested in naphthalene degradation. These include NPL1 (Boronin et al., 1980), pND140 and pND160 (Austen and Dunn, 1980; Dunn et al., 1980), pNB33 (Day et al., 1988a, b), pDBT2 (Monticello et al., 1985). Kochetkov and Boronin (1984) and Ferrer et al. (1986) have also suggested the role of plasmids in naphthalene degradation in some of the organisms isolated by them.

2.4.3.3 Applications of nah genes

Several bacteria that oxidize aromatic hydrocarbons to cis-dihydrodiols also oxidize indole to indigo. Ensley et al. (1983) reported the construction of a strain of E. coli by cloning a fragment of NAH7 that encodes enzymes responsible for the conversion of naphthalene to salicylate. They found that the growth of a recombinant E. coli in nutrient medium results in the formation of indigo. The metabolic interactions that produce indigo appear to involve the NAH7-encoded naphthalene dioxygenase and indole which is produced during normal metabolic processes of E. coli by the activity of the enzyme tryptophanase on tryptophan. This recombinant E. coli can be used to synthesize the textile dye, indigo. Moreover, a blue colony phenotype also makes screening for stable variants very simple.

Burlage et al. (1990) described the construction of a bioluminescent reporter plasmid, pUTK9, for naphthalene catabolism. The promoter for the upper-pathway of naphthalene degradation was transcriptionally fused to the genes of Vibrio fischeri. For resting cell cultures in naphthalene minimal medium, light production was directly correlated with naphthalene catabolism (King et al., 1990). Therefore, this system can be used to examine catabolic activity in a unique manner under a variety of growth conditions. This bioluminescence technology can be used in environmental simulations.
and for on-line monitoring of the microbial biodegradative activity. This type of monitoring system has distinct advantages as it is noninvasive, non-destructive, rapid and population-specific. Such a bioluminescent reporter strain has also been used to develop and standardize assay procedures for monitoring the presence and bioavailability of contaminants in soil slurries (Heitzer et al., 1992).

2.4.4 Phenanthrene Catabolic plasmids (PHN plasmids)

There are only a few reports on the genetics of bacterial degradation of phenanthrene. Kiyohara et al. (1981) isolated a Beijerinckia sp. capable of growth on phenanthrene and later on suggested the involvement of a small plasmid designated pKG2 in phenanthrene degradation (Kiyohara et al., 1983). A strain of Alcaligenes faecalis (AFK2) was isolated from soil which was able to grow on phenanthrene as the sole source of carbon and energy (Kiyohara and Nagao, 1978; Kiyohara et al., 1982). On the basis of curing and conjugation studies, it was confirmed that pHK2 present in A. faecalis is a transmissible plasmid and was involved in phenanthrene degradation. Guerin and Jones (1988) also suggested the involvement of a plasmid in phenanthrene degradation in Mycobacterium sp. Kiyohara et al. (1994) showed that P. putida OUS28 utilizes naphthalene and phenanthrene using a single system of enzymes that has broad-substrate specificity for the upper-catabolic pathways. They also characterized the chromosomal gene cluster responsible for the upper-pathway enzymes and designated it as pah.

There have been virtually no reports on the genetics of anthracene degradation pathway. However, it has recently been observed that the organisms containing NAH7 and NAH7-like plasmids are involved in the degradation of phenanthrene and anthracene (Menn et al., 1993; Sanseverino et al., 1993). A mutant strain defective in nahG gene (salicylate hydroxylase), produced by transposon insertion, resulted in accumulation of metabolites when grown in presence of anthracene or phenanthrene thus implying that plasmid-encoded nah genes are involved in the degradation of these compounds.
2.5 CONSTRUCTION OF GENETICALLY-MANIPULATED ORGANISMS (GMOs)

The rapid development of industrial activities has resulted in the introduction of enormous quantities of chemical pollutants into the biosphere. Most of these toxic compounds are xenobiotics, that is, they are catabolized slowly or incompletely and are accumulated in the environment. Although a number of physico-chemical processes including absorption, chemical oxidation, incineration are available to transform these chemical pollutants (Park et al., 1990), technologies based on microbial processes receive much attention as they consume less energy and are very efficient (Morgan and Watkinson, 1989; Singleton, 1994). Moreover, microorganisms can be subjected to genetic manipulations to improve their degradation abilities. In many cases, degradative genes responsible for xenobiotic metabolism are present on plasmids or are grouped in clusters on chromosomes (Haas, 1983). This makes the genetic manipulation of the existing microorganisms easier in order to develop novel organisms capable of degrading pollutants efficiently. Such manipulations can be achieved either in vivo or in vitro in a number of ways, either by modifying the existing enzymes in terms of specificity and activity (Fisher et al., 1978), or by evolving hybrid pathways (Ghosal et al., 1985; Bruhn et al., 1988). Therefore, evolution of new biodegradative pathways to remove environmental pollutants can be accelerated by genetic engineering.

2.5.1 Objectives

The metabolic pathways may be modified in order to accomplish the following objectives:

2.5.1.1 To evolve multidissimilatory traits

Industrial wastes often contain mixtures of organic compounds. Although different organism(s) may be available capable of degrading different organic compound(s), it is desirable to construct organism(s) capable of catabolizing more than one compound at the same time. Furukawa et al. (1983) isolated a P. putida strain capable of degrading m-xylene, m-toluate and salicylate from mixed chemostat culture. They constructed a SAL-TOL cointegrate plasmid (termed pKF439) in vivo. The
plasmid contained a 57kb TOL fragment with the 40kb dissimilatory region fused with the SAL replicon (Furukawa et al., 1985). Similarly, in order to construct a strain capable of degrading toluene, naphthalene, camphor and other short chain alkanes, Friello et al. (1976) transferred TOL, NAH, CAM-OCT (a plasmid cointegrate carrying genes for catabolizing both camphor and short chain alkanes) plasmids to a *P. aeruginosa* strain. Although this strain represents an interesting model capable of removing a range of substrates in crude oil, it requires rigorous field testing as to whether it would be able to compete with the natural flora once released into the environment.

### 2.5.1.2 To evolve hybrid pathways

In order to construct strains with new catabolic abilities, it is possible to combine dissimilatory gene fragments present on different plasmids or chromosomes into a single strain. The catabolic pathway(s) can be extended to process a number of compounds. The genes from one organism can be added to another organism so as to completely degrade a given compound. For example, *Pseudomonas* sp. B13 metabolizes 3-chlorobenzoate to 3-chlorocatechol which is further degraded by a modified *ortho*-cleavage pathway (Reineke et al., 1978). However, it is not able to degrade 4-chlorobenzoate and 3,5-dichlorobenzoate due to narrow substrate specificity of its benzoate-1,2- dioxygenase. Reineke et al. (1982) cloned the genes for analogous enzymes with broader specificities for benzoate derivatives into *Pseudomonas* sp. B13 and selected 4-chlorobenzoate-degrading derivatives defective in catechol-2,3-dioxygenase so that 4-chlorobenzoate was catabolized via the *ortho*-cleavage pathway. A similar strategy was adopted to extend the catabolic activities of B13 to 3-, 4- and 5-chlorosalicylates by cloning the *nahG* gene which encodes the broad substrate-specific salicylate hydroxylase into vector pKT231 (Lehrbach et al., 1984).

### 2.5.1.3 To enhance enzyme activity and stabilisation

Generally, plasmids encoding aromatic hydrocarbon degradation are in low copy number and may be lost spontaneously in non-selective conditions (Fisher et al., 1978; Harayama and Don, 1985). The enzyme synthesis may be augmented by cloning the dissimilatory trait to a high-copy-number plasmid (Franklin et al., 1981), or in
expression vectors, and by optimising the promoter location of the degradative trait (Shine and Dalgarno, 1975). Franklin et al. (1981) showed that the expression of catechol-2,3-dioxygenase activity could be substantially enhanced by cloning the genes into a high-copy-number plasmid vector carrying active constitutive promoters. Furthermore, in general, cloned genes may be more stable if they are inserted into the host chromosome (Harayama and Don, 1985).

2.5.1.4 To enhance bioaccumulation

The inactivation of one or more gene(s) of a pathway may provoke accumulation of a specific intermediate so as to channel it into another pathway. An example is the use of cloned xylD gene (coding for toluate-1,2-dioxygenase) of TOL plasmid in E. coli cells to effect biotransformation of benzoates and accumulation of cis-dihyrdioisls which are useful educts for the production of synthetic polymer, polyphenylene (Zeyers et al., 1985). Similarly, genetically-manipulated P. putida MW1000 has been used to convert toluene to muconate which could subsequently be chemically hydrogenated to adipate for the synthetic polymer industry (Sinclair et al., 1987).

2.5.2 Release of Genetically-Manipulated Organisms

There is general agreement that genetic and molecular manipulations have great potential for developing modified or engineered microorganisms for use in agricultural, medical and environmental applications. These organisms can be constructed by either in vivo or in vitro techniques. Although the use of GMOs provides exciting alternative technology for the treatment of industrial wastes and other recalcitrant chemicals, it also raises a number of new uncertainties (Jain et al., 1988). Various uncertainties regarding the fate of the released GMOs and mobility of the recombinant genes in the environment include transfer of genes among natural populations of microorganisms by conjugation, transduction and transformation (Henschke and Schmidt, 1990); transportation of GMOs from initial site of release; proliferation, colonization and establishment of recombinant strains; and disappearance of GMOs due to the competition with natural microflora (Jain et al., 1988; Stotzky et al., 1993).
2.5.3 Detection of Genetically-Manipulated Organisms

Effective and safe application of GMOs will require development of rapid, sensitive and selective detection methods to monitor the environmental impact of released organisms. Current regulatory standards strongly recommend research with recombinant bacteria only within contained settings. Thus, laboratory-contained microcosms can serve as standard test systems to properly evaluate and assess the potential impact of recombinant strains on representative ecosystems (Bentjen et al., 1989; Walter et al., 1991).

Both microbiological and molecular techniques are available to permit analysis of GMOs and/or recombinant DNA sequences in the environment. These techniques include use of different genetic markers, use of immunological techniques, use of nucleic acid hybridization and conventional microbiological techniques. There are detailed reviews regarding usefulness and drawbacks of all these techniques (Sayler et al., 1987; Jain et al., 1988; Pickup and Saunders, 1990). However, the use of nucleic acid hybridization technique is described in brief since this technique has proved to be reliable for the detection of specific nucleic acid sequences. A gene probe which is specific for complementary DNA sequences present in GMOs provides reliable, powerful and specific detection technique.

Nucleic acid hybridization involves the detection of target nucleic acid sequences by the binding of nucleotide sequences with a homologous complementary probe sequence. The target DNA can either be transferred to a filter membrane directly after extraction and purification or transferred following restriction analysis and electrophoretic separation. Colony hybridization is the simplest application of nucleic acid hybridization and the easiest way to integrate with conventional environmental microbiological sampling and analysis (Sayler and Layton, 1990; Walia et al., 1990). However, its sensitivity depends upon the relative abundances of the target and the background non-target population that form colonies on the plating media (Jain et al., 1987; Steffan et al., 1989a, b). Polymerase chain reaction may also be used in combination with solution hybridization as a highly sensitive detection technique for target organisms that occur in extremely low numbers (Sayler et al, 1985; 1987).
Although nucleic acid hybridization technique is probably one of the better technologies available to date for detection of the recombinant organisms/genes in the environment, it is unlikely that any one system will be suitable for monitoring recombinant bacteria in all environmental locations (Pickup and Saunders, 1990). A detailed study will be required for specific cases in order to determine the most suitable and accurate method for monitoring specific organisms. In many cases, multiple methodological approaches will be needed to ensure adequate detection sensitivity and accurate quantitative determination of population levels.

2.6 OBJECTIVES OF THE PRESENT STUDY

Polycyclic aromatic hydrocarbons (PAHs) are one of the most acutely toxic compounds found ubiquitously in the environment (Dipple et al., 1990). The present research work has been undertaken to study the biodegradative pathways for the degradation of naphthalene and phenanthrene at the molecular level. The main objectives of the present work are:

a. Isolation, identification and characterization of the organisms capable of utilizing either naphthalene or phenanthrene as the sole source of carbon and energy.

b. Identification of location of structural/regulatory genes responsible for degradation of above mentioned compounds through biochemical and genetic manipulations.

c. Cloning and expression of genes responsible for naphthalene degradation using broad-host-range vectors.

d. Attempts to construct genetically-manipulated organisms capable of degrading naphthalene and phenanthrene/o-phthalate simultaneously and monitoring the stability of the recombinant plasmid(s) in non-selective conditions.