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Environmental pollution has become a major threat to human life on earth. The factors responsible for the environmental pollution are named as environmental pollutants. Environmental Pollutants are compounds that are toxic to living organisms and are released into the ecosystem at high concentrations, usually as a consequence of human activities. Contaminants are either compounds of industrial origin that present chemical structures alien to the biosphere (Xenobiotics), e.g. polychlorobiphenyls (PCBs), polychlorodioxins, trinitrotoluene (TNT) and azo dyes, or natural compounds that have been mobilized to a bioavailable form that is toxic to organisms, e.g. hydrocarbons present in fossil fuels and heavy metals present in minerals (Fig.1.1).

Major sources of pollution are:

1. Chemical and pharmaceutical industries that produce a wide array of xenobiotics and synthetic polymers;
2. Pulp and paper bleaching industries, which are the main sources of chlorinated organic compounds in the environment;
3. Mining activities, which releases heavy metals into biogeochemical cycles;
4. Fossil fuels (coal and petroleum), which may be accidentally released in large amounts into the ecosystem (oil spills) and whose combustion increases significantly atmospheric levels of CO$_2$ (green-house effect) and causes depositions of nitric and sulfuric acids (acid rain and smog); and
5. Intensive agriculture, which releases massive amounts of fertilizers, pesticides, and herbicides.

1. **Major environmental pollutants**

   Next to glucosyl residues, the benzene ring is the unit chemical structure most widely spread in nature. Moreover the thermodynamic stability of the benzene ring
Fig. 1.1. Main sources of pollution in the ecosystem and the factors that influence bioremediation processes (Diaz, 2004)
increases its persistence in the environment; therefore, many aromatic compounds are major environmental pollutants (Dagley, 1986). The most important classes of organic pollutants in the environment are mineral oil constituents and halogenated products of petrochemicals. Aromatic hydrocarbons e.g. Benzene, Toluene, Ethylbenzene and Xylenes (BTEX compounds), and naphthalene belong to the large volume petrochemicals widely used as fuels and industrial solvents. Phenols and chlorophenols are released into the environment as products and waste materials from industry. Aromatic compounds are formed in large amounts by all organisms, e.g. as aromatic amino acids, phenols, or quinines.

1.1 BTEX compounds:

The BTEX chemicals (Benzene, Toluene, Ethylbenzene, and Xylenes) are volatile mono-aromatic hydrocarbons which are commonly found together in crude petroleum and petroleum products such as gasoline (Fig.1.2). They are also produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics, and synthetic fibers (Harwood & Gibson, 1997). They are considered one of the major causes of environmental pollution because of widespread occurrences of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals (Fries et al., 1994). Some estimates that 35% of the 1.4 million gasoline storage tanks in the United States are leaking (Harwood & Gibson, 1997).

(i) Benzene

Benzene is an aromatic hydrocarbon that is produced by the burning of natural products. It is a component of products derived from coal and petroleum and is found in gasoline and other fuels. Benzene is used in the manufacture of plastics, detergents,
Benzene
Ethylbenzene
Toluene
p-Xylene
m-Xylene
o-Xylene

Fig. 1.2. Structure of BTEX Compounds
pesticides, and other chemicals (Hazardous Substance Data Bank (HSDB), 1994). Research has shown benzene to be a carcinogen (cancer-causing) with exposures from less than five years to more than 30 years, individuals have developed, and died from, leukemia (Tsai et al., 1983; Cody et al., 1993). Long-term exposure may affect bone marrow and blood production (DeGowin, 1963). Short-term exposure to high levels of benzene can cause drowsiness, dizziness, unconsciousness, and death (Tsai et al., 1983; Cody et al., 1993).

(ii) Ethylbenzene

Ethyl benzene often enters the environment as a result of petroleum-based industrial discharges or spills; it can be a component of the “mixed Xylene” solutions contained therein. The compound is also used in the manufacturing of styrene and synthetic polymers.

(iii) Toluene

Toluene (methylbenzene) is an important aromatic hydrocarbon natural product of diagenic origin and an important commercial chemical. It is for example, commonly used as a paint thinning agent and in other solvent applications. Ethylbenzene has the potential to cause the following effects from a lifetime exposure at levels above the maximum contaminant level: damage to the liver, kidneys, central nervous system and eyes (Cirek, 1998).

(iv) Xylenes

Xylenes are volatile organic compounds, common constituents of gasoline, and common ground water contaminants. Mixtures of o-, p-, and m-Xylenes are extensively used in the chemical industry as solvents for products including paints, inks, dyes, adhesives, pharmaceuticals, and detergents (HSDB, 1995). In the petroleum industry xylenes are used as antiknock agents in gasoline, and as an
intermediate in synthetic reactions. Of the three isomers, p-xylene is produced in the highest quantities in the U.S. for use in the synthesis of phthalic, isophthalic, and terephthalic acid used in manufacture of plastics and polymer fibers including Mylar and Dacron (HSDB, 1995). Xylene exposure has been associated with effects in a number of organ systems including the lungs, skin and eyes; neurological system; heart and gastrointestinal system; kidney; and possibly the reproductive system (Hipolito 1980; Roberts et al., 1988; Savolainen et al., 1985).

1.2 Phenol

Phenol is a natural as well as a man-made aromatic compound. Phenol is one of the most widely used organic compounds in existence. Annual production of phenol is approximately 1.25 billion kg (Stephen et al., 1983). Phenol is a white crystalline mass, which turns red or pink if exposed to air or light. It has a burning taste and a distinct aromatic, acrid odor. It is very soluble in liquid sulfur dioxide, acetic acid, carbon tetrachloride and alcohol, and soluble in chloroform, ether, glycerol, petrolatum, carbon disulfide, volatile and fixed oils, aqueous alkali hydroxides, and acetone. It is slightly soluble in mineral oil. It is almost insoluble in petroleum ether. Phenol is combustible when exposed to heat, flame, or oxidizers and emits toxic fumes when heated (Sax, 1989). It is incompatible with strong oxidizers and calcium hypochlorite (Sittig, 1985).

Probable routes of human exposure to phenol are inhalation, ingestion, and dermal contact. Phenol is a strong eye and respiratory irritant. It is corrosive to the eyes and skin upon direct contact. Acute inhalation exposure may cause nausea, vomiting, cardiac arrhythmias, circulatory collapse, convulsions, and coma (Juhl et al., 2003). Limited data are available on the chronic effects of phenol in humans from inhalation or oral exposure. Phenols are highly toxic. Although insufficient
information exists on the carcinogenicity of most phenols. 2, 4, 6-trichlorophenol has been shown to be an animal carcinogen based on mutagenicity screening (Stephen et al., 1983). Nitrophenols have also been recognized as the major toxicants to aquatic microorganisms besides posing odor problems to water bodies (Bruhn et al., 1987).

1.3 Nitrophenols

Nitroaromatic compounds are widely distributed in the environment. Most of the nitro aromatic compounds detected in the environment are anthropogenic and released because of their extensive use in the synthesis of drugs, explosives, dyes, plasticizers (Munnecke, 1976; Zylstra et al., 2000). Wide use of nitro aromatic compounds like nitrophenols, nitrobenzoates, and nitrotoluenes and their subsequent release leads to environmental pollution (Fig. 1.3). Due to this potential toxicity and persistence in the environment, rapid removal and detoxification of these compounds are necessary.

*p-Nitrophenol (PNP)* is priority environmental pollutant among such compounds found in many different environments. This compound is used on a large scale in the synthesis of the aspirin substitute acetaminophen and in the manufacture of pesticides such as parathion and methyl parathion (Spain and Gibson, 1991; Zylstra et al., 2000). PNP accumulate in the soil as a result of hydrolysis of several organophosphorus insecticides such as parathion or methyl parathion or from the use of other nitrophenolic herbicides and was listed as a priority pollutant in 1979 by the US-EPA (Munnecke and Hsieh, 1974; Munnecke and Hsieh, 1976; Sharmila et al., 1989; Spain, 1995). The toxicology and carcinogenicity of PNP have been studied and reviewed by the Agency for Toxic Substances and Disease Registry (ATSDR), 1992.
Fig. 1.3. Structure of some nitro aromatic compounds
1.4 Chlorophenols

Chlorophenols, one of the most dangerous classes of environmental pollutants, have been produced in thousands of tons annually by the pulp and paper and agrochemical industries (Harayama et al., 1987). Chlorophenols are a group of chemicals in which chlorines (between one and five) have been added to phenol. There are five basic types of chlorophenols mono[one]chlorophenols, di[two]chlorophenols, tri[three]chlorophenols, tetra[four]chlorophenols, and penta[five]chlorophenols. Except for 2-chlorophenol, which is a liquid at room temperature, all of the chlorophenols are solids. The chlorophenols have a strong medicinal taste and odor; small amounts (at parts per billion [ppb] to parts per million [ppm] concentrations) can be tasted in water. Very small amounts of chlorophenols can also make fish taste bad ATSDR, 1999). All the compounds discussed are produced commercially.

Chlorophenols with at least two chlorines have been used either directly as pesticides or converted into pesticides. Also, chlorophenols, especially 4-chlorophenol, have been used as antiseptics (Bollag et al., 1986). In addition to being produced commercially, small amounts of chlorophenols, especially the mono- and dichlorophenols, may be produced when waste water or drinking water is disinfected with chlorine, if certain contaminants are present in the raw water. They are also produced during the bleaching of wood pulp with chlorine when paper is being produced. One of the most important halogenated insecticides is hexachlorocyclohexane (HCH), a homocyclic (alicyclic) chlorinated hydrocarbon popularly called benzenehexachloride (BHC). This compound has been used extensively worldwide for mosquito control. BHC is highly toxic to insects, birds, mammals and other non-target organisms.
1.5 Cresols

Cresols are natural products that are present in many foods and in animal and human urine. They are also present in wood and tobacco smoke, crude oil, and coal tar. In addition, cresols also are man-made and used as disinfectants and deodorizers, to dissolve substances, and as starting chemicals for making other chemicals.

Three types of closely related cresols exist: ortho-cresol (o-cresol), meta-cresol (m-cresol), and para-cresol (p-cresol) (Fig. 1.4). Pure cresols are colorless chemicals, but they may be found in brown mixtures such as creosote and cresylic acids (e.g., wood preservatives). Cresol compounds (mixtures of the ortho-, meta- and para-isomers) can be obtained from coal tar and petroleum or synthesized by sulfonation or oxidation of toluene (HSDB, 1995). Crude cresol (commercial grade) contains approximately 20% o-cresol, 40% m-cresol, and 30% p-cresol.

Phenol and xyleneols are present in small amounts as contaminants. Cresylic acid compounds are called cresol when the boiling point is below 204°C. Cresols have a wide variety of uses including the manufacture of synthetic resins, tricresyl phosphate, salicylaldehyde, coumarin, and herbicides. Cresols also serve as components of degreasing compounds in textile scouring and paintbrush cleaners as well as fumigants in photographic developers and explosives. Cresols also function as antiseptics, disinfectants, and parasiticides in veterinary medicine. An approximate breakdown of cresol and cresylic acid use is 20% phenolic resins, 20% wire enamel solvents, 10% agricultural chemicals, 5% phosphate esters, 5% disinfectants and cleaning compounds, 5% ore flotation, and 25% miscellaneous and exports.
Fig. 1.4. Structure of cresol compounds
1.6 Polycyclic aromatic compounds

A wide variety of polycyclic aromatic hydrocarbons (PAHs) (Fig. 1.5) are found in the environment as a result of the incomplete combustion of organic matter, emission sources, automobile exhausts, stationary matter (e.g. coal-fired, electricity-generating power plants), domestic matter (e.g. forest fires and agricultural burning) and also in food (Finlayson and Pitts, 1997). Massive relocation of natural materials to different areas of the ecosystem has taken place during the past several decades as a result of human activity, thus exposing living systems to these different compounds (Sudip et al., 2002).

Bicyclic aromatic compounds: carbazole, dibenzofuran and biphenyl

Carbazole (CAR) is a heterocyclic aromatic compound containing a dibenzopyrrole system, being derived from coal tar and shale oil (Nestler, 1974). As CAR is known to possess mutagenic toxic activities and also to be a recalcitrant molecule. Some PAHs (e.g. naphthalene and phenanthrene) have also been used in the synthesis of different organic compounds in pesticides, fungicides, detergents, dyes and mothballs (Cerniglia, 1984).

(i) Carbazole

Carbazole (CAR), a heterocyclic aromatic compound containing a dibenzopyrrole system, is produced during coal gasification and in cigarette smoke. Coal tar produced at high temperature contains an average of 1.5% carbazole. Several thousand tons of carbazole are produced each year from coal tar and crude oil. It is used widely in synthesis of dyes, pharmaceuticals, and plastics and is a suspected carcinogen.
Fig. 1.5. Structure of some abundant polycyclic aromatic hydrocarbons (PAHs) in the environment.
(ii) Biphenyl and polychlorinated biphenyls:

**Biphenyl**

Biphenyl (or diphenyl or 1,1'-biphenyl or lemonene) is a solid organic compound that forms colorless to yellowish crystals. Biphenyl occurs naturally in coal tar, crude oil, and natural gas and can be produced from these sources by distillation. Biphenyl is insoluble in water, but soluble in typical organic solvents. Biphenyl prevents the growth of molds and fungus, and is therefore used as a preservative, particularly in the preservation of citrus fruits during transportation. Biphenyl is most notable as a starting material for the production of polychlorinated biphenyls (PCBs), which were once widely used as dielectric fluids and heat transfer agents. Biphenyl is also used as an intermediate for the production of a host of other organic compounds such as emulsifiers, optical brighteners, crop protection products, and plastics.

**Polychlorinated biphenyls (PCBs)** are a class of organic compounds with 1 to 10 chlorine atoms attached to biphenyl and a general structure of \( \text{C}_{12}\text{H}_{10-x}\text{Cl}_x \). They are soluble in most organic solvents, oils, and fats. PCBs are very stable compounds and do not degrade easily. PCBs were commercially produced as complex mixtures containing multiple isomers at different degrees of chlorination. PCB mixtures have been used for a variety of applications, including dielectric fluids for capacitors and transformers, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in pesticides, paints, carbonless copy ("NCR") paper, adhesives, sealants, plastics, reactive flame retardants, and as a fixative for microscopy. The most commonly observed health effects in people exposed to large amounts of PCBs are skin conditions such as chloracne and rashes (ATSDR, 2001). Studies in exposed workers have shown changes in blood and urine that may indicate liver damage.
PCB exposures in the general population are not likely to result in skin and liver effects (ATSDR, 2001).

(iii) Naphthalene

Naphthalene, the first member of the PAH group, is a common micro pollutant in potable water. The toxicity of naphthalene has been well documented and cataractogenic activity has been reported in laboratory animals (Goldman, 2001; Mastrangela, 1997). Naphthalene binds covalently to molecules in liver, kidney and lung tissues, thereby enhancing its toxicity; it is also an inhibitor of mitochondrial respiration (Falahatpisheh, 2001). Acute naphthalene poising in humans can lead to haemolytic anaemia and nephrotoxicity (International Agency for Research for Cancer (IARC), 2002). In addition, dermal and opthalmological changes have been observed in workers occupationally exposed to naphthalene (IARC, 2002). Phenanthrene is known to be a photosensitizer of human skin, a mild allergen and mutagenic to bacterial systems under specific (Mastrangela, 1997). It is a weak inducer of sister chromatid exchanges and a potent inhibitor of gap junctional intercellular communication (Weis, 1998). Little information is available for other PAHs such as acenaphthene, fluranthene and flourene with respect to their toxicity in mammals. However, the toxicity of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluranthene, dibenza(a,h)anthracene and indenol(1,2,3-c,d)pyrene has been studied and there is sufficient experimental evidence to show that hey are carcinogenic (Mastrangela, 1972; Liu, 2001; Bucker, 1979).

2. Microbial utilization of aromatic compounds

The removal of pollutants from the environment via natural physico-chemical and biological processes (natural attenuation) is, in general a slow and unpredictable
way of counteracting anthropogenic pollution and irreversible damage to the biosphere. Therefore, the main, if not the only, successful strategy to fight pollution as shown in Fig.1.1, is the use and manipulation of the detoxification abilities of living organisms (bioremediation) (Dua et al., 2002; Lovley, 2003; Wackett, 2000; Wackett and Bruee, 2000; Watanabe, 2001). Microorganisms play a major role in the breakdown and mineralization of these pollutants (Alexander, 1981). Over billions of years, microorganisms have evolved an extensive range of enzymes, pathways and control mechanisms in order to degrade an array of aromatic compounds. As a result they can make use of most of the biochemical sequences and cycles those occupy a central position in the human metabolic maps and are generally found in other living forms (Widdel and Rabus, 2001). In addition, microbes have the unique biochemical asset of being able to use molecular oxygen to catalyze the oxidation of numerous natural and man-made chemicals thereby initiating reaction sequences that enter the central pathway of metabolism such as the Kreb’s cycle or the fatty acid spiral (Widdel and Rabus, 2001; Riser-Robert, 1998).

When a xenobiotic compound is exposed to a microbial species, there are four major possibilities of its transformations or inactivation (Bollag, 1974)

1. The xenobiotic can serve as substrate for growth and energy
2. The xenobiotic compound can undergo co-metabolism, i.e., transform it but cannot derive from it energy for growth.
3. The entire xenobiotic molecule or an intermediate of it can be conjugated with naturally occurring compounds, and
4. The xenobiotic compound is incorporated and accumulated within the organism.
Microbial transformations have long been beneficial in many ways. One important activity by which microbes assist mankind is through their active participation in the degradation of many natural and man-made toxicants such as the xenobiotics.

Although most organisms are endowed with detoxification abilities, i.e. mineralization, transformation and/or immobilization of pollutants, particularly bacteria, have been the well-studied and the most frequently used for bioremediation strategies (Dua et al., 2002; Lovley, 2003; Wackett, 2000; Wackett and Bruee, 2000; Watanabe, 2001). Bacteria are omnipotent and omnivorous. Their combined ability to consume natural waste products as their food stuffs (growth substrates) and thereby recycle them has been a critical factor upon which all life has depended. The general ability of bacteria to use such compounds is related to the fact that most of these compounds are commonly present in the environment as a result of the recycling of plant-derived materials (Harwood and Parales, 1996). Human-made xenobiotic compounds, by contrast, have been in contact with the micro biota only for about 100 years, therefore, some of them are still poorly degraded, if at all. Bacteria have developed strategies for obtaining energy from virtually every compound. They play a crucial role in sustainable development of the biosphere and in biogeochemical cycles. The abundance of microorganisms, together with their great ability for horizontal gene transfer and their high growth rates, allows them to evolve quickly and to adapt to environmentally changing conditions, even to extreme environments that do not allow proliferation of other living organisms. However, the kinetics of the biodegradation process may be much slower than desired from public health or environmental considerations. The slow biodegradation of these compounds in the natural environment may be caused by unfavorable physicochemical conditions (such
studies have focused on aerobic pathways, and many details of these metabolic routes have been described (Chaudhry and Chapalamadugu, 1991; Clarke, 1982 & 1984; Commandeur, and Parsons, 1990; Gibson and Subramanian, 1984; Rangnekar, 1988; Reineke and Knackmuss, 1988). A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that structurally diverse aromatic compounds were transformed to a limited number of key intermediates, which were then metabolized further by the central pathways. For example, a large proportion of different aromatic compounds are converted to one of a few aromatic ring cleavage substrates, such as catechol, gentisate, protocatechuate, and derivatives thereof (Fig. 1.7) (Chaudhry and Chapalamadugu, 1991; Clarke, 1982 & 1984; Fewson, 1988; Commandeur, and Parsons, 1990; Reineke, 1984; Reineke and Knackmuss, 1988). The benefits of channeling diverse compounds into a few central pathways, namely, a reduced genetic load, the simplification of regulatory circuits and the economization of energy are clearly or major advantage to soil microbes, which often find themselves in unfavorable environments containing low concentrations of carbon sources suitable for growth.

These dihydroxylated intermediates are channeled into one of two possible pathways, either *meta*-cleavage-type pathway or an *ortho* cleavage-type pathway (Harayama and Rekik, 1989). These intermediate compounds are the substrates of ring-cleavage enzymes that use molecular oxygen to open the ring between the two hydroxyl groups (*ortho*-cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (*meta*-cleavage, catalyzed by extradiol dioxygenases (Fig. 1.8) (Vandermeer et al., 1992). Central pathways involve a series of reactions leading to the formation of Krebs cycle intermediates (central metabolism). This generalized scheme of catabolic pathways for aromatic compounds suggests that
Fig. 1.7. Degradation of various aromatic compounds into central intermediates: Catechol and substituted catechols.
Fig. 1.8. Extradiol and intradiol dioxygenases enzymes. Catechol 2,3-dioxygenases XylE, NahH and DmpB catalyze meta-cleavage of catechol as indicated by the arrow. The superfamily of extradiol enzymes also includes TodE, NahC, BphC and CbpC. The preferential substrates and the sites of cleavage are indicated. Ortho-cleavage is catalyzed by intradiol dioxygenases. The superfamily of intradiol dioxygenases includes protocatechuater 3,4-dioxygenases (PcaGH), Catechol 1,2-dioxygenase (CatA) and chlorocatechol 1,2-dioxygenases (TcbC, TfdC and ClcA). The catechol 1,2-dioxygenase activity which converts 3-methyl-6-chlorocatechol was detected in a mutant of *Pseudomonas* sp. strain JS6 (Vander meer et al., 1992).
microorganisms have extended their substrate range by developing peripheral enzymes, which are able to transform initial substrates into one of the central intermediates (Fig. 1.9).

Despite the tendency to converge in catabolic pathways, divergence is nevertheless observed, reflecting the fact that the substituents on some substrates are incompatible with one or more enzymes of particular catabolic routes (Vandermeer et al., 1992). Catechol, the prime intermediate formed during the degradation of various aromatic compounds as shown in Fig. 1.5, undergoes degradation via either ortho-cleavage pathway or meta-cleavage pathway (Wigmore et al., 1974; Williams and Murray, 1974; Worsey et al., 1978; Murray et al., 1972; Nakazawa and Yokota, 1973). Chlorocatechols are generally degraded by an ortho-fission pathway; if they are subjected to meta-cleavage, toxic or dead-end intermediates are formed (Reineke et al., 1982). On the other hand, methylcatechols are usually degraded by meta-cleavage: the ortho-cleavage enzyme catechol 1, 2-dioxygenase has a very low affinity for methylcatechols or produces dead-end intermediates.

3.1 Ortho-cleavage pathway

Ortho-cleavage pathways are involved in the degradation of catechol and protocatechuate. These compounds are transformed to a common intermediate, 3-oxoadipate, which is further converted to succinate and acetyl coenzyme A (Fig. 1.10). Catechol 1, 2-dioxygenase (C12O) catalyzes the intradiol cleavage (ortho-cleavage) of catechol and generates cis, cis-muconic acid (Eltis, 1993; Gibson, 1993). The family of intradiol dioxygenases includes three subgroups of enzymes: catechol 1, 2-dioxygenase, protocatechuate 3, 4-dioxygenase, and chlorocatechol 1, 2-dioxygenase. These enzymes cleave the aromatic ring between two adjacent hydroxyl groups of catechol or protocatechuate (Fig. 1.8). These enzyme contains a ferric ion
Fig. 1.9. The catabolic funnel for the aerobic degradation of aromatics. White arrows indicate peripheral pathways, Black arrows show ring-cleavage. Central pathways are indicated by gray arrows.
Fig. 1.10. ortho-cleavage pathway map

Catechol 1, 2-dioxygenase

O$_2$

2H$^+$

Muconate lactonizing enzyme

Muconolactone

Muconolactone isomerase

3-oxoadipate enol-lactone

Oxoadipate enol-lactone hydrolase

3-oxoadipate

Krebs cycle
(Fe$^{+3}$) as the prosthetic group. The product of the ortho-cleavage, cis, cis-muconic acid is transferred to the instable enol-lactone, which is in turn hydrolyzed to oxoadipate.

This dicarboxylic acid is activated by transfer to acetyl-CoA and succinate (Fig.1.10). These compounds were fed into the TCA cycle for further metabolism. Chlorocatechols are generally degraded by an ortho-fission pathway; if they are subjected to meta-cleavage, toxic or dead-end intermediates are formed (Reineke et al., 1982). Ortho-cleavage pathway plays a key role in the degradation of chlorinated aromatic compounds (Harayama et al., 1989). Chlorinated aromatic compounds is considered one of the most persistent and toxic pollutants of the environment (Chaudhry and Chapalamadugu, 1991).

3.2 Meta-cleavage pathway

The meta-cleavage pathway is versatile, being used in the dissimilation of a range of substituted catechols. The initial step in the meta-pathway involves the fission of catechol and substituted catechol by catechol 2,3-dixoygenase (Fig.1.8). Catechol 2,3-dixoygenase (C23O) catalyzes the extradiol ring-cleavage (meta-cleavage, proximal to one of the two hydroxyl groups) of catechol and substituted catechols (Eltis, 1996; Harayama and Rekik, 1987). This enzyme contains a ferrous ion (Fe$^{+2}$) as the prosthetic group. Meta-fission of catechol and 4-methylcatechol by C23O produces aldehyde compounds, 2-hydroxymuconic semialdehyde (HMSA) and 2-hydroxy-5-methylmuconic semialdehyde (HMMSA) whereas meta-fission of 3-methylcatechol by C23O produces a ketone compound, 2-hydroxy-6-oxo-hepta-2,4-dienoate (HOHD) (Fig. 1.11). Further the meta-cleavage products were catabolized via two pathways due to the specificities of primary enzymes towards the ring-cleavage products (Harayama et al., 1987; Sala-Trepat et
Fig. 1.11. Primary steps in the oxidation of catechol and substituted catechols.
One of the pathways is hydrolytic pathway and the other one is 4-oxalocrotonate / dehydrogenase pathway. Dehydrogenase pathway consists of three enzymes, hydroxymuconic semialdehyde dehydrogenase (HMSD), isomerase and decarboxylase (Murray et al., 1972). The hydrolytic pathway consists of single enzyme, hydroxymuconic semialdehyde hydrolase (HMSH). However, both pathways convert the substrates to the same end product, 2-oxopent-4-enoate (Fig.1.12). Further the formed product subsequently channeled into the Kreb’s cycle intermediates and serves as energy to the cell (Fig.1.12). The metabolic roles of these two branches of the meta-pathway have been elucidated. The ring-fission products of catechol and 4-methylcatechol are metabolized primarily by the 4-oxalocrotonate branch because the affinity of the dehydrogenase (HMSD) towards these compounds, containing an oxidizable aldehyde group, is much higher than that of the hydrolase (HMSH) (Harayama et al., 1987; Sala-Trepat et al., 1972). The hydrolytic pathway is devoted to the catalysis of the ring-fission product of 3-methylcatechol (a ketone); the rate of this reaction is significantly greater than that occurring with the alternative aldehyde substrates (Duggleby and Williams, 1986; Sala-Trepat et al., 1972). It therefore appears that the enzymes of the 4-oxalocrotonate pathway are functional in the metabolism of catechol, 4-methylcatechol and their metabolic precursors (phenol or p-cresol) and that the physiological role of the hydrolytic activity is essentially limited to the dissimilation of 3-methylcatechol and its metabolic precursors, namely o- and m-cresol. The hydrolytic cleavage of carbon-carbon bond by meta-cleavage hydrolases without any cofactor is a rare class of enzymatic reaction (Lam and Bugg, 1997). Some of the hydrolases involved in the degradation of mono-aromatic compounds were purified and characterized from different bacterial strains (Bayly and Berardino, 1978; Duggleby and Williams, 1986; Nordlund and Shingler, 1990; Menn
Fig. 1.12. *Meta*-fission pathway of catechol and substituted catechols. Name of intermediate compounds of each substrate were not shown (Murray et al., 1972).
et al., 1991; Lam and Bugg, 1997; Shin et al., 1997; Yamada et al., 1998; Hatta et al., 1998). Sequence analysis of these hydrolases revealed their relation to an alpha/beta hydrolase fold family of protein (Ollis et al., 1992).

4. Alpha/beta hydrolase fold enzymes

The alpha/beta (α/β) hydrolase fold family of enzymes is rapidly becoming one of the largest groups of structurally related enzymes with diverse catalytic functions (Ollis et al., 1992). Members in this family include acetylcholinesterase, dienelactone hydrolase, lipase, thioesterase, serine carboxypeptidase, proline iminopeptidase, proline oligopeptidase, haloalkane dehalogenase, haloperoxidase, epoxide hydrolase, hydroxynitrile lyase and others (Holmquist, 2000). As members of this family, meta-fission product hydrolases share a modest sequence similarity and contains a similar three dimensional structure, the α/β hydrolase fold. The core of the enzymes contain 3 layers, alpha/beta/alpha. The number of β-sheets varies from 5 to 8 strands in order 12435678. The strand 2 is antiparallel to the rest (Fig. 1.13).

The canonical α/β hydrolase fold has been described as consisting of a mostly parallel, eight-stranded β sheet surrounded on both sides by α helices. The topology of the central beta sheet is $+1, +2, -1x, +2x$, $(+1x)_{3}$, and it displays a left-handed super helical twist, with the first and last strands crossing each other at an angle of approximately 90°. The degree of twisting can show significant differences, however, with the largest deviations usually localized between strands β5 and β6. The first and last helices αA and αF pack on one side of the sheet while the rest of the helices are located on the opposite side of the sheet. Differences may be also present in the spatial position of the α helices connecting β-strands of the central β-sheet. In some cases, one or more of these helices may even be completely absent (Ollis et al., 1992). Only αC appears to be well conserved; it has a strategic positon in the centre of the β-
Fig. 1.13. A schematic diagram of the α/β hydrolase fold (as proposed by Ollis et al., 1992). The naming scheme has been chosen to emphasize the similarity between different members of the α/β hydrolase fold family. Consequently, β-strands before start of the eight-stranded α/β hydrolase fold domain are labeled -n, ..-2,-1, and β-strands after the α/β hydrolase fold are labeled 9, 10... The broken lines indicate places where some of the structures have excursions. The excursion between strands 3 and 4 is the A' excursion, between strands 4 and 5, the B' excursion, and so on. The crossover helices are part of the hydrolase domain and are called A, B, C, D, E and F. The helices in the excursions between the strands are primed (') and numbered sequentially (Ollis et al., 1992).
beta sheet and plays a key role in the correct positioning of the nucleophilic residue in the active site.

The α/β hydrolase fold is able to provide a stable scaffold for the active sites of a wide variety of enzymes. The catalytic residues always constitute a highly conserved triad: a nucleophile (serine or cysteine or aspartic acid) positioned after strand β5, an acidic residue almost always positioned after strand β7 and an absolutely conserved histidine residue located after the last β strand. However, recent mutational and crystallographic studies have shown that the acidic residue can be relocated to after β6 (Krooshor et al., 1997; Copley, 1998; Liu et al., 1998; Hynkova et al., 1999; Bourne, Isupov and Littelchild, 1999; Rink and Janssen, 1998). Members in this enzyme family contain loop insertions that fold into sub-domains on the carboxyl edge of the principal sheet. These secondary structure elements from what has been called caps, lids or flaps which have important roles in defining, the shape of, and regulating accessibility to the substrate binding crevices of the enzymes. The insertions occur in several locations, often in the loop that follows strand β6. The spatial positions of the catalytic triad atoms involved in catalysis are remarkably well conserved in all enzymes in the family.

The nucleophile is always located in a very sharp γ-like turn (Matthews, 1972), called the ‘nucleophile elbow’, where it can be easily approached by the substrate, as well as by the hydrolytic water molecule. The nucleophile elbow is identified by the consensus sequence Sm-X-Nu-Sm-Sm (Sm=small residue, X=any residue, Nu=nucleophile). The sharpness of the turn results in the nucleophile backbone phi and psi angles being in an unfavorable region of the Ramachandran plot. The strand-nucleophile-helix feature the ‘nucleophile elbow’ is the most conserved structure within the alpha/beta hydrolase fold. An important part of the
active site of all alpha/beta hydrolases is an oxyanion hole that stabilizes the oxyanion transition state of the catalyzed reaction. The geometry of the nucleophile elbow makes the amide proton of the residue following the active nucleophile residue, a conserved part of oxyanion hole. The rest of the oxyanion hole is created by one or more protein back bone amide hydrogen atoms and amino acid side-chains usually located between strand β3 and helix αA. The acid member of the catalytic triad is located in a reverse turn usually following strand β7. The second member of the triad, which can either be aspartic acid or glutamate. While the sequence of the aspartic acid turns varies, the local structure (from the end of seven strands to two residues past the acid) is almost identical (Ollis et al., 1992). The histidine is the only residue of the catalytic triad that is absolutely conserved. The his peptide, which lies at the end of strand eight, comprises a turn, one amino acid and then the histidine which is the first residue in a reverse turn, but the structural conservation only extends up to the histidine. The extended loops present after strand eight bring the histidine to a position appropriate for forming the triad, and similar to that in the other proteins.

As members of this superfamily, meta-fission product hydrolases (MFP-hydrolases) exhibit very low sequence similarity. Surprisingly all hydrolases contains conserved motif’s characteristics of alpha/beta hydrolase fold (Diaz and Timmis, 1995; Fischer et al., 1999).

5. Origin of the present work

Our laboratory has been working on bioremediation of organophosphorus pesticides (op-pesticides) such as parathion and methyl parathion with an objective of designing novel bioremediation strategies for safe removal of this class of neurotoxicants. Before developing such strategies it is necessary to study the basic aspects, such as genetics and physiology of op-pesticide degradation. As part of this
objective our laboratory has cloned and analyzed the sequence of 7.1 kb conserved region found flanking the plasmid (pPLD2) encoded organophosphorus pesticides degrading (opd) gene of *Flavobacterium* sp. ATCC27551 (Siddavattam et al., 2003). The sequence analysis revealed existence of eight open reading frames (ORF’s). Among these eight open reading frames, downstream of the *opd* gene and transcribed in the opposite direction an open reading frame designated as *orf243* was observed. The sequence analysis of the deduced amino acid sequence showed no strong homology to the known proteins. However a very low homology was found towards a class of proteins designated as aromatic compound hydrolases (Siddavattam et al., 2003). Interestingly these hydrolases found to be the members of the alpha/beta hydrolase fold superfamily, catalyze variety of reactions including those involved in degradation of aromatic compounds (Ollis et al., 1992).

During the process of hydrolytic cleavage of parathion, methyl parathion and fenitrothion, nitro-aromatic compounds are generated (Munnecke and Hseih, 1976; Rani and Lalithakumari, 1994; Hayatsu et al., 2000). In view of its close linkage with *opd* gene and weak homology to aromatic hydrolases, the Orf243 was assumed to play either direct or indirect role in degradation of nitro-aromatic compounds such as *p*-nitrophenol. In the present study the author has made an attempt to elucidate the function of *orf243* in degradation of op-compounds.