CHAPTER I

INTRODUCTION
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The production and use of phthalate esters has increased enormously with the growth of industrial activities and this has led to the accumulation of some of these compounds as environmental pollutants. Many of these chemicals and their biotransformation products are reported to be toxic, carcinogenic or teratogenic to man and animals. Indigenous microbial population present in soil and water possess versatile mechanism to degrade variety of phthalate esters of natural and synthetic origin. Microorganisms play a pivotal role in the biodegradation of many toxic and recalcitrant aromatic compounds which reach the soil as industrial effluents, sewage pollutants and secondary metabolites. Since the mineralization of chemical compounds in the environment is supposed to be primarily due to microorganisms, there has been considerable interest in the biodegradation of phthalate esters. The major aspects in these studies include isolation of suitable microbial strains exhibiting the required biodegradative capabilities as regard to the specific organic compounds and the elucidation of the intermediary metabolic pathways operating in the degradation of these compounds. The

Several compounds belonging to different classes of chemicals such as aromatics, haloaromatics, nitroaromatics, sulfoaromatics, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, aliphatics, halogenated aliphatics, nitroamines, etc. are found to be
environmental pollutants which have been placed on the list of the priority pollutants (Keith and Telliard, 1979). Among the aromatics, phthalate esters form the principal group of dicarboxylic aromatic compounds which are considered to be the highest frequency pollutants. There has been a continual accumulation of these compounds in the environment due to their large scale synthesis for use in plastics, plasticizers of vinyl products and manufacture of dacron. There are reports on the toxicity and carcinogenicity of phthalate esters. These compounds are considered to be potentially hazardous to life (Mayer et al. 1972; Autian, 1973; Marx, 1972; Peakall, 1975; Daniel, 1978; Lawrence, 1978; Thomas et al. 1978; Tomita et al. 1979; Gangolli, 1982; Kevy and Jacobson, 1982; Kluwe, 1982; Peck and Albro, 1982; Seth, 1982; Shiota and Nishimura, 1982; Warren et al. 1982; Wilbrown and Montesano, 1982). This has consequently provoked concern for understanding the metabolic fate of these compounds in the environment.

Studies of microbial degradation of a number of aromatic compounds over the past few decades have provided a wealth of knowledge. The aromatic compounds during microbial catabolism undergo intricate degradative
pathways before entering the central metabolic pathway that can yield energy or cellular constituents. The studies on aromatic metabolism are mainly concerned with the isolation and identification of the microbial strains capable of utilizing aromatic compounds and the elucidation of the intermediary degradative pathways which lead to the mineralization of these compounds. Many experimental approaches have been employed to investigate the degradative mechanisms adopted by microorganisms in the catabolic sequences of the aromatic substrates. These experiments are mainly concerned with the isolation and characterisation of the intermediary metabolites and also the identification of the microbial enzymes involved in the degradative processes by employing different physico-chemical methods. A powerful tool that also enables to unravel the metabolic pathways is that of demonstration of the sequential induction of enzymes to oxidise a specific substrate and the intermediary metabolites (Stanier, 1947; Suda et al. 1950). Further insight into the degradative pathways is also provided by assaying the probable key enzymes involved in the various metabolic reaction sequences. The studies on the biodegradation of a wide range of aromatic compounds adopting these methodologies over the past several

A survey of the studies reveals the underlying unity as well as the diversity in the microbial metabolism of the aromatic compounds. It may be observed that relatively a large class of compounds is metabolised through similar pathways by different microbial species, and also that a small class of compounds is metabolised through different pathways by a single microbial species. Under the influence of microbial enzymes, the peripheral
aromatic compounds by prior modification of the substituent group or by direct hydroxylation of the aromatic ring yield dihydroxyphenolic intermediates such as catechol, protocatechuate, gentisate, pyrocatechuate etc. These terminal metabolites produce smaller aliphatic fragments on subsequent fission of the aromatic ring. Ultimately this pathway enters the Kreb's Tricarboxylic acid cycle resulting in mineralization.

The microbial degradation of peripheral aromatic compounds essentially involves the formation of catechol, protocatechuate, gentisate, pyrocatechuate etc. as the terminal metabolites which are further degraded to cellular components through ortho or meta cleavage pathways. The study on degradation of these terminal aromatic metabolites in several microorganisms has been made in detail by many investigators (Dagley et al. 1960, 1964, 1968; Ribbons and Evans, 1962; Ornston and Stanier, 1966; Ribbons, 1966; Dagley, 1971; Crawford et al. 1979). The degradation of protocatechuate follows both ortho and meta-fissions. The nucleus of protocatechuate in case of ortho-fission is cleaved between C₃ and C₄ positions by the action of protocatechuate 3,4-dioxygenase to yield β-carboxy cis-cis-muconate. The muconate is finally transformed under the successive influence of β-carboxy
lactonizing enzyme, carboxymuconolactone decarboxylase and enol-lactone hydroxylase to \( \beta \)-ketoadipate which is eventually converted to succinate and acetoacetate.

The nucleus of protocatechuic acid in Pseudomonas putida (Ornston and Stanier, 1966) and via meta-cleavage through \( C_2 \sim C_3 \)
fission in *Bacillus macerans* (Crawford, 1979) and through \( C_4 - C_5 \) fission in *Pseudomonas testosteroni* (Dennies et al. 1973) are illustrated in Figure 1.1.

1.1 BIODEGRADATION OF PHTHALATE AND PHTHALATE ESTERS

Phthalate and phthalate esters are most often encountered as environmental pollutants and this has aroused much enthusiasm for understanding the biodegradation of these compounds. There seemed to be initially, however, two schools of thought, one understanding that phthalates and their esters are relatively biodegradable and another considering that the evidence for biodegradation of these compounds is inconclusive (Marx, 1972). But a number of subsequent investigations have not only revealed that microorganisms which degrade various phthalates and phthalate esters are widely found in nature but have also led to an elucidation of the biodegradative pathways of some of these compounds. The isolation of a wide variety of microbial strains capable of utilizing phthalate and phthalate esters necessarily indicates that the extent of exposure of the environment to these compounds and the detailed degradative considerations help to comprehend the basic principles of phthalate and phthalate ester
Figure 1.1: Degradative pathways of protocatechic acid

a: Ortho-cleavage
b: Distal meta-cleavage
c: Proximal meta-cleavage.
1.1.1 Survey of Isolation of Microbes Utilizing phthalates and phthalate esters

Ribbons and Evans (1960) isolated a soil Pseudomonas sp. capable of utilizing o-phthalate by enrichment culture technique. Stanier et al. (1966) isolated a few strains of Pseudomonas capable of growing on o-phthalate and terephthalate. Stevenson (1967) found a strain of Arthrobacter utilizing o-phthalate. Engelhardt et al. (1976) isolated a few strains of Nocardia, Arthrobacter and Pseudomonas, metabolising o-phthalate and observed that only the strains belonging to Nocardia were able to utilize terephthalate. Harada and Koiva (1977) isolated several strains of Alcaligenes, Corynebacterium and Arthrobacter growing on phthalate, isophthalate and terephthalate. Aftring et al. (1981) isolated several strains of Acinetobacter and Pseudomonas capable of growing on all the phthalate isomers from aquatic sediments under anaerobic conditions. Elmorsi and Hopper (1981) isolated a strain of Pseudomonas sp. utilizing isophthalate and terephthalate.

Engelhardt et al. (1975) isolated a few strains belonging to genera Corynebacterium, Arthrobacter and Mycobacterium, metabolising dibutyl phthalate. Keyser et

1.1.2 Degradative routes of phthalates

Ribbons and Evans (1960), studied the metabolism of o-phthalate by a soil Pseudomonas and suggested on the basis of metabolite characterisation, oxygen uptake and enzymatic investigation that phthalic acid by initial dioxygenation yielded 4,5-dihydro 4,5-dihydroxylphthalate
which on dehydrogenation was converted into 4,5-dihydroxyphthalate. The dihydroxyphthalate was subsequently decarboxylated to protocatechuate which on ortho-cleavage yielded $\beta$-keto-adipate via $\beta$-carboxy cis-cismuconate. Engelhardt et al. (1976) proposed the same pathway for the degradation of o-phthalate in Pseudomonas, Nocardia and Orthobacter on the basis of successive adaptation studies. Nakazawa and Hayashi (1977) isolated protocatechuate 4,5-dioxygenase deficient mutant strain of _Pseudomonas testosteroni_ which helped to confirm the degradation pattern of o-phthalate. Harada and Koïwa (1977) studied the metabolism of o-phthalate by Corynebacterium and proposed on the basis of metabolite characterisation, that phthalic acid was degraded through 3-hydroxyphthalate instead of 4,5-dihydroxyphthalate. The 3-hydroxyphthalate was found to be subsequently converted to protocatechuate. Aftring et al. (1981), on the basis of sequential induction, showed that _Bacillus_ sp. metabolised phthalic acid anaerobically by initial decarboxylation to benzoic acid which on successive hydroxylation formed gentisic acid, a substrate for ring fission. Taylor and Ribbons (1983), studying the metabolism of phthalic acid and a few of its derivatives by _Bacillus, Pseudomonas_ and a marine mixed culture, observed that the anaerobic degradation of phthalic acid
by *Bacillus* sp. involved an initial reduction to 1,2-dihydropthalic acid which on oxidative dicarboxylation yielded benzoic acid to form catechol. Whereas, the degradation of phthalic acid by *Pseudomonas testosteroni* and marine mixed culture, involved the formation of 4,5-dihydroxyphthalate which was decarboxylated to yield protocatechuate. Hari Babu and Vaidyanathan (1982), investigating the metabolism of isophthalate by a soil bacterium, proposed that the degradative pathway of isophthalic acid involved the formation of 1,5-cyclohexadiene 3,4-diol 1,3-dicarboxylic acid. Further, it was observed that the dihydrodiol was dehydrogenated to protocatechuate. Karegoudar and Pujar (1985), studying the metabolism of terephthalic acid by a soil bacterium, observed on the basis of oxygen uptake studies and enzymatic investigation, that the degradative pathway of terephthalate involved the formation of terephthalate dihydrodiol which was dehydrogenated to protocatechuate. The protocatechuate was found to be degraded through ortho-fission by *Bacillus* sp.

1.1.3 Degradative Routes of Phthalate Esters

Engelhardt *et al.* (1975) investigated the metabolism of dibutylphthalate in various strains of
coryneform bacteria and proposed a pathway on the basis of growth and oxidation studies. Dibutylphthalate on successive hydrolysis was converted to monobutylphthalate and phthalate. It was further observed that monobutylphthalate was formed only as a transient intermediate and that phthalic acid was degraded via 4,5-dihydroxyphthalate to protocatechuic. Engelhardt and Wallnofer (1978), studying the metabolism of dibutylphthalate by different strains of Nocardia sp., observed that the same pathway was operating in the degradation of dibutylphthalate. It was also observed that the dibutylphthalate esterase activity was inducible in the case of Nocardia sp. Kurane et al. (1980), investigating the metabolic pathway of various phthalate esters by Nocardia erythropolis by employing metabolite characterisation, observed that metabolism of phthalate esters proceeded via the conversion phthalate esters to phthalic acid and protocatechuic. Nocardia erythropolis degraded protocatechuic via \( \beta \)-ketoadipate by an intradiol fission. Eaton and Ribbons (1982a) studied the metabolism of dibutylphthalate by Micrococcus sp. with the help of metabolite characterisation and enzyme investigation. It was observed that dibutylphthalate was sequentially hydrolysed to o-phthalate. The phthalate on initial
dioxygenation was further degraded to 3,4-dihydroxyphthalate which on subsequent dehydrogenation was converted to 3,4-dihydroxyphthalate. The 3,4-dihydroxyphthalic acid in turn was decarboxylated to protocatechuate which was degraded both by a meta-cleavage pathway via \( \gamma \)-carboxy \( \alpha \)-hydroxy muconic semialdehyde and by an ortho-cleavage pathway via \( \beta \)-ketoadipate. Further, it was found that dibutylphthalate hydrolysing esterase and 3,4-dihydroxyphthalate decarboxylase activities were constitutive, whereas, phthalate 3,4-dioxygenase and protocatechuate dioxygenases were inducible in \textit{Micrococcus} sp.

Eaton and Ribbons (1982b), investigating the metabolism of dimethylphthalate by a \textit{Micrococcus} sp., observed on the basis of metabolite characterisation that dimethylphthalate was hydrolysed to monoethylphthalate which was subsequently degraded either to 3,4-dihydroxyphthalate-2-methyl ester or to phthalic acid. The phthalic acid was degraded to protocatechuate via 3,4-dihydroxyphthalate. However, 3,4-dihydroxy-2-methyl ester was not degraded further because the 2-methyl ester was not a substrate for decarboxylase activity found in extracts of \textit{Micrococcus}.
The minor pathway leading to the 3,4-dihydroxyphthalate-2-methyl ester has been particularly useful for the easy procurement of 3,4-dihydroxyphthalate, as well as providing evidence that Micrococcus sp. metabolises phthalates by pathways different from those used by pseudomonads. Eaton and Ribbons (1982c), studying the utilization of several phthalate esters by Micrococcii, observed that a strain of Micrococcus sp. grown on octyldecylphthalate, decarboxylated 3,4-dihydroxyphthalic acid to form protocatechuic acid. Whereas, most of the other strains grown on various phthalate esters formed protocatechuic acid. The pyrocatechuic acid was observed to be subsequently degraded to L-hydroxymuconic semialdehyde via a meta-cleavage pathway. Karegoudar and Pujar (1984), studying the metabolism of diethylphthalate by Micrococcus sp., proposed on the basis of metabolite characterisation and oxidation studies, that diethylphthalate on successive hydrolysis was converted to monoethylphthalate and phthalate. It was observed that monoethylphthalate was formed only as a transient intermediate and that phthalic acid was degraded via protocatechuic acid by 3,4-dioxygenative cleavage. Kurane (1986), studying degradation of various phthalate esters by Nocardia erythropolis, proposed that
Phthalate esters were metabolised in *N. erythropolis* by hydrolysis of esters of free phthalic acid. Phthalic acid was then metabolised via protocatechuic acid by ortho-cleavage. The metabolite from protocatechuic acid by *N. erythropolis* was identified as β-ketoadipic acid.

From the preceding considerations it is evident that some general features are associated with phthalate and phthalate ester metabolism. Several schemes of degradation are given in Fig. 1.2 and 1.3. It appears that the formation of protocatechuate as a terminal aromatic intermediate is a common feature in the microbial degradation of phthalate and phthalate esters. Further it seems that accumulation of phthalic acid through monoester formation is the common mode in the overall reaction sequence of phthalate mineralization.

1.2 *Microbial Transformation of Aromatic Compounds by Fungi*

Microbial transformation of organic compounds by fungi has been known in an empirical way from the dawn of history. Extensive work has been done on the biotransformation of steroids, alkaloids, aromatic and aliphatic compounds including hydrocarbon, carbohydrate
Figure 1.2: Degradative pathways of phthalic acids

- a. Phthalic acid
- b. Isophthalic acid
- c. Terephthalic acid

Chemical structures of:

- Benzoic acid
- Hydroxybenzoic acid
- Phthalic acid
- Isophthalic acid
- Terephthalic acid
- Gentisic acid
- Hydronbenzoic acid
- 3,4-Dihydroxy phthalic acid
- Pyrocatecholic acid
- 1.5-Cyclohexadiene
- Protocatechuic acid
- 1,5-Cyclohexadiene
- 3,4-diol 1,5-dicarboxylic acid
- 3,4-diol 1,4-dicarboxylic acid
Figure 1.3: Degradative pathways of phthalate ester.

Fungi oxidise unsubstituted aromatic hydrocarbons by the incorporation of one atom of molecular oxygen into the aromatic ring to form arene oxides. The arene oxides can isomerise to phenols, or undergo enzymatic hydration by the enzyme epoxide hydratase to yield trans-dihydrodiols. The formation of arene oxides in fungi has been shown to be catalysed by cytochrome P450 enzyme system. Fungi, in general, degrade benzoate through monohydroxylation reactions to yield 4-hydroxybenzoate, catalysed by benzoate-4-hydroxylase. The monohydroxy-benzoic acids are further metabolised through the introduction of another hydroxyl group at the
ortho or para position to the existing hydroxyl group. Most fungi degrade 4-hydroxybenzoate to protocatechuic acid.

Moore et al. (1968) studied the metabolism of aromatic amino acids, phenylalanine and tyrosine by Sporabolomyces roseus. They observed that the initial step involved the formation of corresponding cinnamic acid. Tracer studies showed that these compounds were further metabolised to protocatechuic acid through benzoic acid and p-hydroxybenzoic acid as intermediates in this pathway. The protocatechuic acid was found to be the terminal aromatic compound formed during the metabolism of these compounds. Yuasa et al. (1975) investigated the metabolic pathway of phenylalanine in Aspergillus sojae and found that a new compound, 2-hydroxy-3-phenylpropenoic acid and phenylacetic acid were formed in the culture medium containing phenylalanine by A. sojae. 2-Hydroxy-3-phenyl-propenoic acid was formed from phenylalanine via phenyllactic acid and was degraded to benzoic acid, p-hydroxybenzoic acid, protocatechuic acid and catechol. Phenylacetic acid was degraded to homogentisic acid via o-hydroxyphenylacetic acid by A. sojae. Jamaluddin and Vaidyanathan (1970) have reported the metabolism of mandelic acid by
Aspergillus niger and observed that mandelic acid was degraded to p-hydroxybenzoic acid and protocatechuic acid via benzaldehyde. The mandelate pathway in A. niger deviated completely from the bacterial pathway at the benzoate level. In the fungus, benzoate was metabolised via the protocatechuate pathway, whereas in bacteria catechol was the terminal aromatic compound derived from mandelate.

Dodge et al. (1979) studied the metabolism of biphenyl in Cunninghamella elegans. They observed that fungi oxidised biphenyl to 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl as the major metabolites. The major site of hydroxylation was at the 4-position, with significantly less hydroxylation occurring at the 2- and 3-positions. Wiseman et al. (1975) presented evidence that microsomal preparations from Candida tropicalis oxidised biphenyl to 4-hydroxybiphenyl.

There are hardly any reports on conversions of phthalate esters by fungi.
1.3 SCHEME OF PRESENT INVESTIGATION

It is evident from the previous survey of literature on phthalate and phthalate ester metabolism that, among the principal group of the hazardous phthalate esters, the metabolism of dimethylterephthalate still awaits a detailed study. Dimethylterephthalate is extensively used in textile industry. Thus because of their abundant production, this compound is frequently encountered as an environmental pollutant that is considered to be potentially hazardous to life.

The present study concerns with the investigations on the degradation of dimethylterephthalate by Bacillus species and fungal transformation of dimethylterephthalate by Sclerotium rolfsii. We have isolated a bacterial strain from garden soil by enrichment culture on dimethylterephthalate as sole source of carbon and energy. The isolated organism was identified as Bacillus sp. on the basis of its cultural, morphological and physiological characteristics. We have studied the utilization of various aromatic compounds by Bacillus sp. The metabolic pathway of dimethylterephthalate in Bacillus sp. was elucidated by metabolite characterisation, growth studies, sequential induction,
oxygen uptake studies and enzymatic investigations adopting various biochemical and physico-chemical techniques.

The second aspect of our study concentrated on the biotransformation of dimethylterephthalate by *Sclerotium rolfsii*. It was shown to catalyse hydrolytic conversion of dimethylterephthalate to free terephthalic acid through monomethylterephthalate. Many of the phthalate esters tested were hydrolysed to phthalate and respective alcohols. We further observed that the free acids did not support the growth of the fungi. Transformed products were isolated and identified by physico-chemical methods.

The thesis comprises five chapters, of which the present chapter provides a brief introduction to the general background of the studies on the microbial degradation of phthalates and phthalate esters including aromatic metabolism and fungal metabolism of aromatic compounds. The second chapter consists of the descriptions of the materials and methods used in our studies. The methods of maintenance of microbial cultures and the various analytical tools such as chromatographic, spectroscopic, manometric and enzymatic techniques
employed to trace the metabolic pathways are described in this chapter. The third chapter deals with the isolation of a microorganism, namely *Bacillus* sp. metabolising dimethylterephthalate by using the enrichment culture technique. Various tests for the identification, characterisation and growth behaviour of the microorganism are described in this chapter. The fourth chapter embodies the results and discussion on the degradation of dimethylterephthalate by *Bacillus* sp. The metabolic pathway adapted by this microorganism in the degradation of dimethylterephthalate is described in this chapter. The fifth chapter contains the results and discussion on the transformation of dimethylterephthalate by *Sclerotium rolfsii*. 