CHAPTER 1

Introduction
The increasing environmental pollution leads to progressive deterioration in our quality of life. These circumstances compel the world’s scientific community to look for effective means of environmental remediation with the purpose of human health and nature preservation. Biodegradation and biosynthesis of chemical matter in a natural ecosystem was well balanced before the advancement of industries. Humans have transformed the planet and population growth will accentuate human influence on the environment. The world population has reached seven billion and it continues to grow. To support this growth and maintain biosphere, our agriculture and industry must operate in sustainable manner. This requires the advanced environmental biology tactics. Environmental biology is broad; it involves cleaner manufacturing using biological methods for diminishing chemical inputs in agriculture and industry. Practices to develop eco-friendly and economical bioremediation processes to control environmental pollution. That reforms the balanced and sustainable development with quality life and contributing to wealth creation. The quality of life on earth is linked inextricably to the overall quality of the environment. In early times, we believed that we had unlimited abundance of land and resources. Today, however, the resources in the world show greater or lesser degree of our carelessness and negligence in using them.

Applications of chemical science have contributed significantly to the advancement of human civilization. With a growing understanding and ability to manipulate chemical molecules, the post-World War II, chemist was considered a societal problem solver. They synthesized crop-enhancing agricultural chemicals to ensure a constant and viable food supply. They played a significant role in the eradication of deadly diseases by developing life-saving pharmaceuticals and chemical
pesticides. Chemists also developed innovative plastics and synthetic fibres for use in both industrial and consumer products. As a result of industrial revolution and modern science, more and more chemicals are being synthesized to satisfy the human needs.

Since the industrial revolution, human activity has created a legacy of environmental contamination widespread through industrialized economies (Baveye et al. 1999). As modern economies move to post-industrial economic activity and heavy industry retreats, more sites affected with by-products from industrial processes are revealed. Now a days there is a general awareness about the detrimental effects of certain products and sub-products from industrial processes and that their release and disposal to the environment should be controlled or even prevented. Environmental protection, legislation, and monitoring become more effective, and also as ‘brown’ land is released for other used (Philip et al. 2005). However, there are large extensions of soils and sediments with high levels of pollution, as a heritage from activities in the past or as a result of current releases. In any case, pollutants pose a risk to all living forms and therefore must be removed.

A large number of manmade organic compounds have been found their way into our environment as a consequence of the activities of modern industry and agriculture. No organic compounds have infinite persistence built into its chemical structure. All terrestrial organic compounds in existence are thermodynamically unstable to varying degrees, all of them have been synthesized, at one time or another by a living organism, whether by a plant or an organic chemist and all of them to be oxidised to carbon-dioxide with the release of energy. However, in the kinetic sense, most of these compounds are perfectly stable and under physiological conditions in the absence of catalysts they will not be degraded or oxidised at significant rates. Such
catalysts are most commonly uncounted in soil as the enzymes of aerobic microorganism seeking to release and harness energy for growth.

Biodegradation is a process that has been going on in nature for years. It is a natural process, it poses less hazards to human health and the environment and is more acceptable to society than conventional treatment technology. Biodegradation has received little attention in pollution control as it is a slow process that occurs in nature over a long period of time. Biotechnological procedures, particularly biodegradation is being recognized as a valuable auxiliary or essential procedure to achieve the eco-friendly environment. Since, chemical or physical processes rarely bring about the change in structure of calcitrant molecule, in such instances, microorganisms capable of degrading specific or broad specificity chemicals may be instrumental in decomposing such chemical pollutants. The major trends in these studies include the isolation of specialized microbial strains exhibiting the required biodegradative capacities as regard to the specific organic compounds and elucidation of the intermediary metabolic pathway operating in the degradation of these compounds. The dramatic advancement of this fascinating field in its historical perspective may be best known by the numerous reports, reviews and monographs of several authors.

The study on microbial degradation of a number of aromatic compounds over past few decades has provided a wealth of knowledge on the metabolism of aromatic compounds. We shall outline the general background of aromatic metabolism relevant to the microbial degradation of aromatic compounds in the following paragraphs.
General Background of Aromatic Metabolism

The aromatic compounds during the microbial catabolism undergo intricate degradation pathways before entering into the central metabolic cycle that can yield energy or cellular constituents. The studies on aromatic compounds metabolism are mainly concerned with the isolation and identification of microbial strains that are capable of utilizing aromatic compounds and elucidation of the intermediary degradation pathways, which lead to the mineralization of these compounds. The principal methods employed to isolate the microbial strains is that of selective enrichment in which the compound to be metabolized is supplied as a growth limiting and usually the sole source of carbon and energy to the microorganisms. The study with the pure culture is preferred to understand the metabolic fate or catabolic sequence of inferred recalcitrant chemical. The microbial strains are normally identified on the basis of their morphological, physiological and biochemical characteristics by using various cultural and biochemical criteria.

Many experimental approaches have been employed to investigate the degradative mechanisms adopted by the microorganisms in the catabolic sequences of the aromatic substrate. These experiments are mainly concerned with the isolation and characterization of the intermediary metabolites and also the identification of the enzymes involved in the degradative process by employing different physico-chemical methods. A powerful tool that unravels the metabolic pathways is that of demonstration of the sequential induction of enzymes to oxidize a specific substrate and the intermediary metabolites (Stainer, 1947; Suda et al. 1950; Shamsuzzaman and Barnsley, 1974a, 1974b; Karegoudar and Pujar, 1984; Denome et al. 1993; Heider and Fuchs, 1997; Alexander, 1999; Kang et al. 2003; Seo, 2006; Seo et al. 2009; Pérez et
Further, an insight into the degradative pathways is also provided by assaying the probable key enzymes involved in various degradative pathways.

The studies on the biodegradation of a wide range of aromatic compounds adopting various methodologies over the past several years have led to a wealth of information on aromatic catabolism (Chapman, 1972; Dagley, 1981 and 1986; Chakrabarty, 1982; Taylor, 1983; Gibson and Subramanian, 1984; Zeyer et al. 1990; Young and Häggblom, 1991; Tierney and Young, 2010; Haddock, 2010; Teufel et al. 2010; Fuchs et al. 2011; Bugg et al. 2011).

A survey of the studies reveals the underlying unity as well as the diversity in microbial metabolism of the aromatic compounds. It may be observed that relatively a large class of compounds is metabolized through similar pathways by different microbial species and also that a small class of compounds is metabolized through different pathways by a single microbial species. We shall consider some of the common features involved in the biodegradation of aromatic compounds in the following pages:

**Common Aromatic Metabolic Mechanism**

Under the influence of microbial enzymes, the peripheral aromatic compounds by prior modification of the substituted group and/or by direct successive hydroxylation of the aromatic ring yield dihydroxy-substituted phenolic intermediates such as catechol, protocatechuate, gentisate etc. These terminal metabolites on subsequent fission of the aromatic ring produce smaller aliphatic fragments which through various degradative pathways depending upon the microbial strains ultimately enter into the Kreb’s tricarboxylic acid (TCA) cycle eventually resulting in mineralization (formation of CO₂ and H₂O).
Hydroxylation of the Benzene Nucleus

Hydroxylation of the benzene nucleus of an aromatic compound is essential step for the initiation and subsequent disintegration of the benzene nucleus. Hydroxylation of the benzene nucleus is accomplished through the insertion of an oxygen molecule by the influence of hydroxylase enzymes in the presence of co-factors and metal ions. The incorporation of two hydroxy groups is essential for the labialisation of the benzene nucleus (Dagley, 1971).

The peripheral aromatic substrates already possessing a hydroxy group are metabolized by the introduction of another hydroxy group most often at the ortho-position or sometimes at meta-position under the influence of flavin linked enzymes in the presence of either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H). The examples of monohydroxylation reactions are found in the reports of several investigators (Dagley, 1971; Groseclose and Ribbons, 1981; Spokes and Waker, 1974; Buswell and Clark, 1976; Chapman and Ribbons, 1976; Kachhy and Modi, 1976; Banat et al. 1992; Vannelli and Hooper, 1995; Butler and Mason, 1997; Gibson and Parales, 2000; Díaz et al. 2001; Chakraborty and Coates, 2005; Vaillancourt et al. 2006; Ullrich and Hofrichter, 2007; Cozzarelli et al. 2010; Arora et al. 2010; Vogt et al. 2011). The successive hydroxylation of the benzene yield dihydroxy compounds such as catechol, protocatechuate, gentisate, pyrocatechuate or resorcinol.

The unhydroxylated peripheral aromatic compounds are degraded by the introduction of two hydroxy groups most commonly through simultaneous hydroxylation under the influence of iron sulphur protein in the presence of FAD and NAD(P)H, or very rarely through successive hydroxylation in the presence of iron,
pteridine and NAD(P)H. The examples of double hydroxylation reactions are found in the reports of various investigators (Feist and Hegeman, 1969; Fewson, 1981; Johnson and Stanier, 1971; Channareddy et al. 1976; Gibson and Parales, 2000; Berkel et al. 2006; Pazmino et al. 2010). The unhydroxylated compounds such as benzoates on double hydroxylation yield dihydroxy compounds. The metabolism of benzoate by different microbial strains interestingly provides a typical example of the usual mode of simultaneous double hydroxylation and also the unusual mode of successive double hydroxylation. The simultaneous double hydroxylation of benzoic acid involves directly the formation of catechol, protocatechuate or gentisate via salicylate, m-hydroxybenzoate or p-hydroxybenzoate.

The hydroxylation pathway of aromatic compound discussed here are the most frequently encountered ones which invariably involve the formation of dihydroxy compounds as the terminal intermediates that are susceptible for the subsequent oxygenative cleavage of the benzene nucleus.

**Disintegration of the Benzene Nucleus into Smaller Fragments**

Dissimilation of the benzene nucleus is crucial step in the microbial catabolism of aromatic compounds. Benzene nucleus of a terminal aromatic metabolite is cleaved by the incorporation of both the atoms of molecular oxygen into the substrate under the influence of dioxygenase enzyme to yield aliphatic fragments. The disintegration of the benzene nucleus during the metabolism of aromatic substrates by different microorganisms is known to occur generally by two modes of ring cleavage namely ortho-cleavage and meta-cleavage which are also referred to as intradiol and extradiol fission respectively (Nozaki et al. 1970; Lipscomb, 2008; Krishnaswamy and Namasivayam, 2011).
1. **Ortho-cleavage:** In the case of *ortho*-cleavage the aromatic ring is cleaved by oxidative fission of the bond between the two consecutive carbon atoms bearing the hydroxyl groups.

2. **Meta-cleavage:** In the case of *meta*-cleavage the aromatic ring is cleaved by oxidative fission of the bond between the two consecutive carbon atoms, one bearing an hydroxyl group and another bearing a substituent group other than the hydroxyl group.

The fission products depending upon the mode of cleavage of the aromatic ring are further degraded through different pathways into smaller aliphatic fragments such as *cis, cis*-muconate, *β*-keto adipate, maleyl pyruvate or *α*-hydroxy muconic semialdehyde etc., by simple decarboxylation, hydrolysis and isomerization reaction under the influence of decarboxylase, hydroxylase or isomerase enzyme until the routes are finally led to the formation of succinate, fumarate, malate, citrate or oxaloacetate that are the constituents of Kreb’s cycle.

**Degradative Routes of Terminal Aromatic Metabolite**

From the preceding considerations, it is apparent that the microbial degradation of peripheral aromatic compounds essentially involves the formation of catechol, protocatechuate, gentisate or pyrocatechuate, etc., as the terminal aromatic metabolites which are further degraded to cellular components through *ortho*/or *meta*-cleavage pathways. The study of degradation of these terminal aromatic metabolites by several microorganisms has been made in detail by many investigators (Dagley et al. 1964; Ornston and Stanier, 1966; Dagley, 1971; Gibson, 1984). We shall consider briefly in following paragraphs the degradative pathways of some of these terminal aromatic metabolite relevant to the present study.
1.2.3.1. Degradative Pathways of Protocatechuic Acid

The degradation of protocatechuic acid follows both *ortho*-fission (Sleeper et al. 1950; Ornston and Stanier, 1966) and *meta*-fission (Dagley et al. 1986; Crawford, 1975). The nucleus of protocatechuic acid in the case of *ortho*-fission is cleaved between C₃ and C₄ positions by the action of protocatechuic acid 3, 4-dioxygenase to yield β-carboxy lactonizing enzyme, carboxy muconolactone decarboxylase and enol-lactone hydroxylase to β-ketoacidate which is eventually converted to succinate and acetate.

The nucleus of the protocatechuic acid in case of *meta*-fission is cleaved by both the proximal and the distal extradiol fissions. In the case of distal mode, the nucleus of protocatechuic acid is cleaved between C₄ and C₅ positions by the action of protocatechuic acid 4, 5-dioxygenase to yield α-hydroxyγ-carboxy-muconic semialdehyde. The semialdehyde is subsequently hydrolysed to 4-hydroxy-4-methyl-2-oxoglutarate which on aldol fission reaction forms pyruvate and acetoacetate. In the case of proximal mode, the nucleus of protocatechuic acid is cleaved between C₂ and C₃ position by the influence of protocatechuic acid 2, 3-dioxygenase to yield α-hydroxy muconic semialdehyde. The semialdehyde either on direct hydrolysis or on dehydrogenation followed by decarboxylation yields 4-hydroxy-2-keto valerate which eventually leads to the formation of pyruvate and acetaldehyde.

The degradative pathway of protocatechuic acid via *ortho*-cleavage in *Pseudomonas putida* (Ornston and Stanier, 1966) and via *meta*-cleavage through C₂-C₃ fission in *Bacillus macerons* (Crawford and Olson 1979) and through C₄–C₅ fission in *Pseudomonas testosterone* (Dennis et al. 1973) are illustrated in figure 1.1.
Figure 1.1. Degradative pathways of protocatechuic acid (A) *ortho*-cleavage and (B) *meta*-cleavage.
Review of Literature on Phenolic Compounds

Occurrence of Phenolic Compounds

Phenolic compounds may be defined as any compound with aromatic nucleus bearing a hydroxyl group directly linked to the aromatic nucleus. Phenolic compounds are environmentally important due to their extensive use in various industries, presence in wastewaters and their potential toxicity.

Lignin is the second most abundant carbon compound on the Earth and it is an aromatic polymer of phenyl propanoid units. Lignin is broken down through biological or abiotic means, the resulting phenolic compounds contribute a certain level of toxicity to the respective aquatic or soil system unless they are transformed or removed accordingly. Lignification, which is the metabolic process of sealing a plant cell wall by lignin deposition, occurs during the course of normal tissue development and is an important step during root growth. Lignin is one of the last products of phenylpropanoid metabolism in plants, and it plays a crucial role in a plant’s resistance to biotic and abiotic stresses (Vanholme et al. 2012; Chen et al. 2013). Lignin is resistant to reactions, industries that process plant material commonly remove the lignin fraction by acid or alkaline hydrolysis. The hydrolysis process forms a number of single-ring aromatic compounds that become contaminants of industrial effluent.

Monoaromatic compounds structurally similar to benzoic acid may also be produced by the breakdown of pesticides in the environment and can still be harmful substances to non-target organisms such as fish and algae [Sinclair et al. 2003]. Industrial effluents that contain a significant phenolic fraction include those from olive oil mills, wine distilleries, and paper-pulping mills (Andreozzi et al. 1995; Di Gioia et al. 2001; Estrada et al. 2001; Isidori et al. 2004). These industries can be also introduced
into the environment through degradation of natural substances (Davi and Gnudi, 1999) and industrial activities (e.g. dyes, plastics, pharmaceuticals and explosives) (Hoffsommer et al. 1980; Gutes et al. 2005). These lipophilic compounds have numerous industrial applications, which enhance the risk to the environment and to human health (Bradbury and Coats, 1989).

Phenolics are a group of biologically active compounds with an extremely wide distribution and a well-known chemistry. Phenolic compounds are produced as waste products of many industrial activities and such, appears in industrial effluents that contaminate aquatic ecosystems. Phenolic compounds are known to enter water bodies via sewage waters from wood processing plants, industrial operations, petroleum refineries, coal producers and chemical plants. However, quite a variety of phenolic compounds are formed as a result of secondary pollution of natural aquatic ecosystem, i.e. in the process of vital activity of aquatic organisms, during microbiological degradation and transformation of organic compounds that are formed in the water column as well as in bottom sediments (Kondrat’eva, 2001).

Bioremediation continues to be the preferred method for household waste recycling and heavy metal, toxic chemical, and radioactive pollutant removal. There are three main processes in bioremediation. These include: (i) transformation or insignificant alteration of the molecule; (ii) fragmentation or degradation of the molecule to simpler compounds; and (iii) mineralization or conversion of the complex compound into simpler ones (H₂O, CO₂, H₂, NH₃, CH₄, etc.). Bioremediation processes mainly involve the use of microorganisms (bacteria, fungi, yeasts, and algae). For this reason, the evaluation of polluted areas prior to bioremediation often includes detection, quantification, and activity determination of the xenobiotic-degrading microorganisms.
The biodegradation activity of microorganisms has become particularly topical over the past decades with regard to the increased presence of resistant anthropogenic pollutants in the biosphere in extents exceeding the self-cleaning abilities of nature. Compounds that cannot be degraded naturally in the environment have been created, such as synthetic polymers, colorants, pesticides, pharmaceuticals, detergents, etc. These compounds, also known as xenobiotics, that are biologically active even as microimpurities. They are highly toxic and exhibit mutagenic, carcinogenic, allergenic, and teratogenic properties. Most of them are considerably stable, so decades are needed for their bioremediation. Mankind cannot renounce the use of such substances; therefore, the application of the biodegrading properties of microorganisms to the removal of anthropogenic pollutants from the environment is especially relevant.

**Impact of Phenolic Compounds on Environment**

Phenolics are defined as priority pollutants and a large number of organizations and documents regulate the safe norms for phenolic compounds in drinking water and the environment. In EU Directive 80/778/EEC, the admissible concentration for all phenolic compounds in drinking water is defined as 0.5 μgL⁻¹ (Commission of the European Communities, 1980). According to Japanese legislation, the permitted level of phenolic compounds is ten times higher (5 μgL⁻¹) (Ministry of Health and Welfare, 2000). The US Environmental Protection Agency defined a safe norm of 1 μgL⁻¹ for phenol and its nitro-, methyl-, and chloro-derivatives (US-EPA, 2007). Thus, the removal of monoaromatic compounds from wastewater and polluted soils is of primary significance. Therefore, various physical, chemical, and biological methods for wastewater treatment have been developed and applied (Mokrini et al. 1997; Chan and
European countries used to spread phenolics containing olive mill wastewaters on agricultural soil, though now that practice has been restricted due to the effluent’s toxicity (Isidori et al. 2004). Adverse effects have been observed for seed germination, crop yield, and general growth of plants, aquatic organisms, and bacteria (Di Gioia et al. 2004; Hartley and Whitehead, 1985; Isidori et al. 2004; Riffaldi et al. 1990). Toxicity of phenolic compounds for microorganisms is largely an issue of concentration, where large amounts of aromatics can accumulate in and disrupt cell membranes (Díaz et al. 2001). Nitrogen-fixing bacteria may be negatively affected by caffeic acid, coumaric acid, ferulic acid, or vanillic acid in amounts greater than 0.1mM (Hartley and Whitehead, 1985). Ruminal bacterial growth may be inhibited by concentrations above 1mM of coumaric or ferulic acids (Akin et al. 1993). Phenolic toxicity is additive and depends only on the total amount of phenolic acids, not on the concentration of one acid (Blum, 1998). Coconut husk retting is the basic process involved in the manufacture of coir. This small-scale industry practiced in the backwaters leads to deterioration of water quality (Jayasankar, 1985).

**Phenolic Compounds - Allelochemicals**

Phenolic compounds fall within the class of most important and common plant allelochemicals in the ecosystem. These compounds contain a range of compound types that include structures such as simple aromatic phenols, hydroxy and substituted benzoic acids and aldehydes, hydroxy and substituted cinnamic acids, coumarins, tannins, and perhaps a few of the flavonoids (Zeng et al. 2008). Many higher plants release allelochemicals into the environment through root excretion or exudation,
leaching, evaporation and decomposition of plant tissues/organs (Ding et al. 2007). These compounds can accumulate in the soil, influencing (positively or negatively) the growth and development of other species. This process is termed allelopathy and is broadly defined as any chemically-mediated interaction among plants. It also involves the contact of allelochemicals with the rhizosphere or bulk soil, and can be absorbed by receptor plants and exert its influence (Weir et al. 2004). Due to the multitude of potential molecular targets, the investigation of the mode of action of allelochemicals is a challenging endeavour (Reigosa and Pazos-Malvido, 2007).

Many secondary metabolites have been referred to as allelochemicals. They are commonly found in soils at concentrations between 0.01 and 0.1 mM, and they affect plant growth at concentrations of up to 10 mM (Siqueira et al. 1991; Macias, 1995). Allelochemicals typically suppress seed germination, causing disorders of root growth and inhibiting plant growth. Moreover, they alter several physiological and biochemical processes such as water utilization, mineral uptake, photosynthesis, amino acid metabolism, protein synthesis, glycolysis, mitochondrial respiration and ATP synthesis and many more processes (Weir et al. 2004). One of these compounds is \textit{trans}-cinnamic acid, which is a well-known allelochemical that affects seed germination and root growth (Ding et al. 2007; Reigosa and Pazos-Malvido, 2007). In most studies, the effects of cinnamic acid have been related to its action on the plasma membrane and related processes, including the induction of oxidative stress (Ye et al. 2006), an increase in reactive oxygen species (ROS) levels (Ding et al. 2007), a disturbance in Ca\(^{2+}\) homeostasis (Yu et al. 2009), and a decrease in the net nitrate uptake and plasma membrane H\(^{+}\)-ATPase activity (Abenavoli et al. 2010). However, in higher plants, the cell wall is one of the first tissues affected by stress signals, which are then transmitted
to the cell interior and influence several processes (Komatsu et al. 2010). Most important factor
for crop decline in terms of growth, yield and quality is due to the presence of phenolic allelochemicals in soil, leading to tremendous agricultural losses (Chen et al. 2011a and b).

Plants introduce allelochemicals into the environment through foliar leaching, root exudation, residue decomposition, volatilisation and debris incorporation into soil (Inderjit and Duke 2003). Autotoxicity is a type of intraspecific allelopathy, where a plant species inhibits the growth of its own kind through releasing toxic chemicals into the environment (Singh et al. 1999). Root exudates and plant debris of several plant species have been shown to have autotoxicity potential (Yu et al. 2000). Phenolic acids (PAs), which are found in root exudates and plant debris, are a type of allelochemicals that could exert detrimental effects on plant growth and development under specific conditions, including autotoxicity. Therefore, PAs are considered to be autotoxins of these plants (Yu and Matsui 1994; Inderjit and Duke 2003).

In the soil microorganism plant system, soil microbial communities are critical to soil biological processes that are necessary for maintaining a healthy and fertile soil and suppressing plant diseases (Paul 2007). Changes in soil microbial communities may lead to changes in the functions performed (Acosta-Martínez et al. 2010). Artificially added root exudate components (quinic, lactic, maleic acids and sugars) were shown to increase soil dehydrogenase activity and bacterial taxon richness (Shi et al. 2011). Therefore, there is a possibility that PAs could influence plant growth through changing rhizosphere microbial communities (Zhou et al. 2012). Phenolic acids could inhibit or stimulate the development and growth of microorganisms invitro (Black and Dix 1976; Wu et al. 2008). However, much uncertainty remains on the effects of PAs on
rhizosphere soil microbial communities. Most studies investigating the effect of plant root exudates or certain PAs on soil microorganism communities report the responses of microbial populations using culture-dependent methods (Sparling et al. 1981; Blum and Shafer 1988; Shafer and Blum 1991; Qu and Wang 2008). Molecular fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) analysis of ribosomal RNA (rRNA) gene fragments and Real-Time polymerase chain reaction (RT-PCR) are appropriate molecular techniques for studying the response of soil microbial communities to PAs that cause autotoxicity in plants. Cucumber (*Cucumis sativus* L.) is a vegetable crop of high economic importance in many countries. Phenolic acids are thought to account for the autotoxicity of *C. sativus* (Yu et al. 2000). Vanillic acid (4-hydroxy-3-methoxybenzoic acid) (VA), a dihydroxybenzoic acid derivative, is one of the most common PAs detected in substrates used in *C. sativus* cultivation (Politycka et al. 1984), *C. sativus* root exudates (Pramanik et al. 2000) and soils under *C. sativus* (Zhou et al. 2012). Phenolic allelochemicals are utilized by many microorganisms as energy source, detoxify or mineralize these compounds, and catalyse their oxidation and polymerization reactions (Chen et al. 2011a; Zhang et al. 2010).

**Biodegradation of Phenolic Compounds**

Phenylpropanoids, such as *p*-coumaric acid, caffeic acid and ferulic acid, are aromatic compounds containing a phenyl ring with a C₃ side chain. These hydroxycinnamic acids are formed sequentially from cinnamic acid via the central phenylpropanoid pathway (Dixon and Paiva 1995). *p*-Coumaric acid is one of the most abundant constituents of plant cell wall found to be covalently linked to
saccharides and lignins by ester bonds and/or ether bonds (Hartley and Harris 1981; MacAdam and Grabber 2002). These hydroxycinnamic acids appear to be relatively inert as far as degradative processes in the plant are concerned; on the contrary, many species of microorganisms are able to transform or degrade plant aromatic compounds and to release vast amounts of carbon that otherwise would be locked away in plant secondary products such as lignin (Rosazza et al. 1995).

Because of the abundant availability of \( p \)-coumaric acid in nature, there is considerable interest in utilizing \( p \)-coumaric acid as a cheap source of natural substrate for microbial transformation aimed at producing value-added phenolic products such as caffeic acid (Nambudiri et al. 1969; Estrada Alvarado et al. 2003) and \( p \)-hydroxybenzoic acid (Estrada Alvarado et al. 2001). Hydroxylation at the meta position of \( p \)-coumaric acid resulted in the formation of caffeic acid (Douglas, 1996), while hydroxybenzoic acid derivatives \((C_6-C_1)\) were formed by cleavage of \( C_2 \) fragment from phenylpropanoids (Hertweck et al. 2001).

The use of microbial cells and their enzymes as catalysts in the manufacture of economically important substances is a rapidly developing field of biotechnology. For nearly every type of reaction known in organic chemistry (oxidative, reductive, hydrolytic or conjugative) there is a biocatalytic equivalent (Turner, 1995). The key advantage of biocatalysis is that enzymes and microorganisms catalyse reactions specifically under mild conditions, thereby saving energy (Faber 1992; Wong and Whitesides 1994). Moreover, bioprocesses are the only ones legally accepted in Europe and the US for the production of some food additives such as natural flavour.

The attention on the catabolism of low-molecular-weight aromatic compounds, in particular ferulic acid and \( p \)-coumaric acid, which are the main phenolic monomers
released from graminaceous cell wall (Hartley and Ford, 1989) and are very important in repressing lignocellulose utilization in the rumen, besides being toxic *invitro* to rumen bacteria, protozoa, and fungi (Borneman et al. 1986; Chesson et al. 1982). These two lignin-related aromatic compounds are extremely abundant and widely distributed in higher plants, and thus they could be of interest as a renewable resource for the production of useful and value-added chemicals such as guaiacol derivatives through biotransformation (Huang, 1993).

The bacterium *Bacillus pumilus* PS213 isolated from the bovine rumen has been shown to convert ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4-vinylphenol, respectively, by a nonoxidative decarboxylation, while its ability to degrade the two products further is still unknown. Decarboxylation of both classes of phenolic monomers, substituted cinnamic and benzoic acids, has already been described for several microorganisms (Crawford, and Perkins, 1978; Huang et al. 1993a and 1993b; Nazareth and Mavinkurve, 1986), including *Bacillus* species (Indahl and Scheline, 1968) such as *B. pumilus* (Arfmann and Abraham, 1989).

Biodegradation of aromatic compounds by soil bacteria (*Pseudomonas, Cellulomonas, Achromobacter*) was studied earlier, by measuring the consumption of oxygen by oxidation of the added aromatic compounds manometrically (Kunc, 1971a and 1971b; Kunc, 1974). Vaughan and Butt, (1970) analysed bioconversion of *p*-coumaric acid, resulting in the formation of caffeic acid. The hydroxylase enzyme responsible for the conversion of cinnamic to *p*-coumaric acid was extensively studied by Hill and Rhodes (1975); Nimura et al. (2010) and Katsuragi et al. (2010) and ferulic acid to 5-hydroxyferulic acid by Knockaert et al. (2011).
The shortening of the side chain of cinnamic acid by elimination of the acetic acid molecule (so-called /3-oxidation of cinnamic acids) and decarboxylation was described by Kindl (1971) and Hilton and Cain (1990), p-coumaric acid by Alvarado et al. (2001); Sachan et al. (2006) and ferulic acid by Baqueir-Peña et al. (2010) and Yuan et al. (2013).

Environmental pollution due to the release of natural phenolic compounds from agro-industrial operations has become widespread in the world. The structure of the compounds present is similar in many industrial effluents and residues like those produced in wine-distillery, olive oil extraction, green olive debittering, cork preparation, wood debarking and coffee production (Field and Lettinga, 1991; Borja et al. 1993; Brand et al. 2000; Lesage-Meessen et al. 2001; Minhalma and de Pinho, 2001; Aggelis et al. 2002). Chemically, agro-industrial wastes contain sugars, tannins, polyphenols, polyalcohols, pectins, lipids and a wide variety of simple aromatic compounds. (Chamkha et al. 2001). The major phenolic compounds in agro-industrial waste include p-coumaric, caffeic, ferulic, cinnamic, protocatechuic, syringic, vanillic, veratric, p-hydroxyphenylacetic acids, tyrosol, hydroxytyrosol, (Balice and Cera, 1984; Hamdi, 1993; Labat et al. 2000).

Cinnamic acid is the first metabolite of the phenylpropanoid pathway and is consequently a precursor for lignin and flavonoid biosynthesis (Boerjan et al. 2003; Kovácik et al. 2007; Lee et al. 2011). Substituted cinnamic acids, such as ferulic acid and p-coumaric acid are plant secondary products and are widely distributed in the cell walls of gramineous plants (up to 4.2g kg⁻¹ dry matter), extensively ester-linked to lignin or polysaccharides (Pan et al. 1998) are found in soil as breakdown products of lignin and accumulated in soil (Boudet et al. 2003). Olive mill wastewaters contain 250
mg kg\(^{-1}\) dry weight of phenolic compounds causing a major environmental problem in the Mediterranean area (Lesage-Meessen et al. 2001). High accumulation of phenolic acids in soil inhibit the growth of neighboring plants (Chou and Patrick 1976; Kuiters and Sarink, 1986; Zeng and Mallik 2006), causes the partition and loss of cell membrane of the fermenting organisms reducing cell growth and sugar assimilation (Philip et al. 2009), above 1 mM accumulation of phenolic acids in soil inhibits the growth of many species of ruminal bacteria (Borneman et al. 1986) and phenolic acids also delay the metabolism of sugars and citric acid by wine lactic acid bacteria (LAB) (Campos et al. 2009; Rozès et al. 2003). The presence of caffeic, coumaric, ferulic, or vanillic acids at concentration of above 0.1 mM affects nitrogen-fixing bacteria (Hartley and Whitehead 1985; Akin et al. 1993). Phenolic compounds have considerable inhibitory effect and are more toxic than furfural and Hydroxymethylfurfural (Philip et al. 2009).

Bacterial phenolic acid decarboxylases (PADs), decarboxylates some 4-hydroxy cinnamic acids namely ferulic acid, \(p\)-coumaric acid and caffeic acid into their corresponding 4-vinyl derivatives: 4-vinylguaiacol, 4-vinylphenol, and 4-vinylcatechol, respectively. These are responsible for the detoxification of these 4-hydroxyl cinnamic acids and the vinyl derivatives which are value added in the food industries used as flavoring agents (Huang et al. 1994; Degrassi et al. 1995; Cavin et al. 1997 and 1998; Salgado et al. 2012). The biodegradation of phenylpropanoids is important for the global carbon cycle from an environmental point of view, since these compounds are released from plant wastes as breakdown products from lignin (Peng et al. 2003).
Industrial effluents that contain a significant phenolic fraction include those from olive oil mills, wine distilleries, and paper-pulping mills (Andreozzi et al. 1995; Di Gioia et al. 2001; Isidori et al. 2004). Many European countries used to spread phenolic-containing olive mill wastewaters on agricultural soil, though now this practice has been restricted due to the effluent’s toxicity (Isidori et al. 2004). Olive oil extraction process releases wastewater which is a liquid effluent formed by olive cell wall degradation during oil extraction. This effluent contains high concentrations of proteins, polyphenols, polyalcohols, sugars, lipids, tannins, pectins, and other simple aromatic compounds. The aromatic compounds include tyrosol, hydroxytyrosol, cinnamic, syringic, \( p \)-hydroxyphenylacetic, vanillic, protocatechuic, veratric, caffeic and \( p \)-coumaric acids (Balice and Cera 1984; Hamdi 1993; Labat et al. 2000).

Several reports have described the biodegradation of the hydroxycinnamic acids viz, \( p \)-coumaric acid, ferulic acid and caffeic acid by microorganisms. Biodegradation is the ecofriendly and cost effective to reduce the toxic level of these phenolic acids in contaminated soil and biotransformation gives rise to value added products. These hydroxy cinnamic acids are converted to hydroxy benzoic acids. \( p \)-Hydroxybenzoic acid and its derivatives find important applications as dietary antioxidant (Tomas-Barberan and Clifford, 2000), natural flavours (Walton et al. 2003), preservatives, medicines and also as monomers for liquid crystal polymers currently used in various electronic devices (Mcqualter et al. 2005). Ferulic acid can be used as a starting material to produce vanillin (4-hydroxy-3-methoxybenzaldehyde), an important flavour compound used in beverages and other food industries, such as in bread, cake, ice cream, chocolate and confectionery products, as well as fragrances. (Tanruean et al. 2013). Due to the abundance of these natural aromatic products, there
Table 1.1. Molecular structures of various phenolic compounds used in the present study.

<table>
<thead>
<tr>
<th>Name of the compound</th>
<th>Molecular structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p</em>-Coumaric acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td><em>p</em>-Hydroxybenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td><em>p</em>-Hydroxybenzoic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Vanillin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Vanillic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>
is scientific interest in utilizing them as substrates in biotechnological processes for the production of flavouring compounds labelled as “natural” (Casey and Dobb 1992; Krings and Berger 1998).

**Bacterial Consortium – An Approach for Effective Degradation**

Biological processes play a major role in the removal of contaminants and take advantage of the astonishing catabolic versatility of microorganisms to degrade such compounds. New methodological breakthroughs in sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information. Microbes found in natural water and soils have a broad ability to utilize (catabolize) many xenobiotic compounds as their sources of carbon and energy, thus recycling the fixed organic carbon back into harmless biomass and CO₂. Identification of such microorganisms and development of a suitable consortium system with favourable biological, chemical and environmental parameters, can effectively biodegrade or biotransform the toxic pollutants generally found in wastewater plants. Microbial mediated environmental protection and restoration processes involve process cultures comprising multiple microbial consortia and it is the consortium performance, rather than individual strain performance, that is critical as far as both process efficiency and economics are concerned. Moreover, a mixed bacterial consortium comprises of different kinds of bacteria with varied metabolic activities. Hence, a microbial consortium is a better candidate to be studied with respect to biodegradation and biotreatment purposes. Different industries manufacture different kind of products, thus leading to emittance of a wide variety of pollutants in the environment. Fertilizer, pesticide and chemical industries are prevalent on a large scale in India. These industries discharge their treated effluents directly into the water bodies. Generally,
these industries, experience difficulties in complying with the Pollution Control Board norms for discharge of industrial effluents into water bodies, as their effluents contain a large number of not easily biodegradable pollutants. These pollutants cannot be treated effectively in the common effluent treatment plants (CETPs), since they require a special bacterial seed having the potential to degrade them. If amenable to biological treatment, it affords a cost-effective option of reducing the carbon to below permissible limit. Industrial effluents having high COD are environmentally hazardous.

Phenolic compounds are present in the effluents of various industries including petrochemicals, coal coking, coal gasification, tanneries etc. They are difficult to be degraded by common soil microflora and persist for a long time in nature causing environmental pollution (Chakraborty et al. 2015). Biodegradation of phenolics by aerobic, anaerobic bacteria and fungi have been reported that evolved their metabolic capacities to degrade hydrocarbons. A mixed culture has been reported to result in complete degradation of phenolics with a high efficiency in degradation process than a pure culture (APHA, 1995; Kharoune et al. 2002; Chakraborty et al. 2013; Veena and Vasudevan, 2015). Most of the previous studies on biodegradation of phenolics have used sewage water from various industries as source of microorganisms (Saravanan et al. 2008). Very few works in this regard have used soil as the source of microorganisms for bioremediation (Chakraborty et al. 2015).

Aerobic degradation of phenolics with pure cultures has been studied extensively, for example *Pseudomonas putida* has been widely used for biodegradation of phenolics (Wang and Loh, 1999; Banerjee et al. 2001; Abuhamed et al. 2004; Kumar et al. 2005; Kulkarni and Chaudhari, 2006; Rodriguez et al. 2006). However, a mixed community of microbes is needed for complete mineralisation, although many reports
on phenolics degradation using pure species of microorganism are available. But reports on the same due to mixed culture of microorganism are scant (Abuhamed et al. 2004; Kumar et al. 2005; Rodriguez et al. 2006; Stoilova et al. 2006).

Utilization of natural phenolic compounds by microorganisms is predominant for the global carbon cycle from an ecological perspective (Peng et al. 2003). Phenolic compounds such as cinnamic, \( p \)-coumaric, ferulic, caffeic, syringic, vanillic, protocatechuic and \( p \)-hydroxyphenyl acetic acids are released from agro-industrial operations in free and mixed form (Hamdi 1993; Labat et al. 2000). Limited studies have been documented on the utilization of mixed phenolic acids by both pure and mixed cultures (Di Gioia et al. 2000; Mendonça et al. 2003). The treatment systems composed of mixed microbial populations possess higher degree of biodegradation and mineralization due to synergistic metabolic activities of microbial community and offers considerable advantages over the use of pure cultures in the degradation of compounds. In the microbial consortium, the individual strains may attack the molecule at different positions or may utilize metabolites produced by the co-existing strains for further decomposition. The degradation capability of individual strains, not necessarily contributes to the total degradation capability of the microbial consortium forming the association. The current study focuses on development of bacterial consortium and enhanced utilization of single and mixture of phenolic acids by individual and mixed strains.
Objectives of the present work:

➢ Isolation, characterization and identification of potential bacterial strains capable of utilizing phenolic compounds as sole source of carbon and energy.
➢ Studies on the utilization of single (individual) and mixed phenolic compounds in different combinations by the isolated bacterial strains.
➢ Elucidation of catabolic pathways of different phenolic compounds based on characterization of metabolic intermediates and by assay of different enzymes.
➢ Enhanced utilization of single and mixed phenolic compounds by bacterial consortium.

1.4. Organization of the Thesis

In the present study, we have focused on the utilization of phenolic compounds like p-coumaric acid, ferulic acid, caffeic acid and cinnamic acid. The study concerns with the investigations on the degradation of p-coumaric acid, ferulic acid and caffeic acid by *Pseudomonas* sp. TRMK1 and degradation of cinnamic acid by *Stenotrophomonas* sp. TRMK2. We have isolated phenolic compounds utilizing bacterial strains by using selective enrichment culture technique and identified the microorganisms by applying various physiological, cultural, morphological, biochemical tests and 16S rDNA sequencing. Catabolic pathways for p-coumaric acid, ferulic acid and caffeic acid in *Pseudomonas* sp. TRMK1 and degradation of cinnamic acid in *Stenotrophomonas* sp. TRMK2 has been elucidated by various biochemical methods. We have also studied the utilization of various phenolic compounds by all the three strains. As well, the bacterial consortium was prepared by using these strains and demonstrated the utilization capability on single and mixed phenolic compounds.
The thesis reports the results of our investigations carried on these observations. The thesis comprises of five chapters of which the present chapter evidently provides a brief introduction to the general background of the environmental pollution, the quality of life on earth, aromatic metabolism and survey of the previous studies on the microbial degradation of phenolic compounds.

The second chapter consists of the description of materials and methods employed in our studies. The use of different types of media, maintenance of bacterial culture, various biochemical techniques such as chromatographic, spectroscopic, and enzymatic studies that are employed to study the biodegradation of different individual phenolic compounds and in different combinations. This chapter also deals with the materials and methods employed in elucidation of catabolic pathway of p-coumaric, ferulic, caffeic and cinnamic acid. Further, method employed for preparation of bacterial consortium and utilization of individual and mixed phenolic compounds.

Third chapter deals with the isolation and identification of microorganisms namely *Pseudomonas* sp. TRMK1, *Stenotrophomonas* sp. TRMK2 and *Xanthomonas* sp. TRMK3 capable of utilizing phenolic compounds. Microorganisms were isolated by selective enrichment culture technique using phenolic acid as sole source of carbon and energy. The isolated microorganisms were identified by various cultural, biochemical tests and 16S rDNA sequencing. All the three strains were gram negative, aerobic and motile. The 16S rDNA sequence of strains TRMK1, TRMK2 and TRMK3 were deposited and are accessible in NCBI GenBank database with accession numbers KT717679, KU522144 and KU522145 respectively.

Fourth chapter describes the utilization of different phenolic compounds by bacterial strains and elucidation of catabolic pathways. This chapter is divided into two sections; (i) Utilization of p-coumaric, ferulic and caffeic acid by *Pseudomonas* sp.
TRMK1 and elucidation of catabolic pathways and (ii) Degradation of cinnamic acid by *Stenotrophomonas* sp. TRMK2 and elucidation of its catabolic pathway. This chapter includes studies on the identification of the catabolic pathway of *p*-coumaric, ferulic, caffeic and cinnamic acid by employing metabolite characterization and enzymatic investigations. Further, the ability of the strain TRMK1 to utilize mixture of phenolic acids was checked by preparing the synthetic effluent containing the mixture of phenolic acids.

Fifth chapter focuses on the development of bacterial consortium and enhanced utilization of single and mixture of phenolic acids by individual and mixed strains.

At the end, the thesis presents a comprehensive summary of our studies as a conclusion followed by the list of references.