Chapter 1.

INTRODUCTION AND REVIEW OF LITERATURE
1. INTRODUCTION

The industrial revolution has played a massive role in changing the socio-economic scenario of the modern world. Despite the large numbers of merits of industrial revolution, that have made the human life more easy and comfortable, it remains a fact that industrial revolution is one of the major causes for the environmental pollution as we see it today. Throughout the world there are various types of pollution that interfere with the quality of life for all living creatures and with the natural functioning of the Earth's ecological systems. Although some environmental pollution is a result of natural causes, such as methane emissions from cattle and toxic materials expelled from volcanoes, most pollution is caused by human activities.

Comprising over 70% of the Earth's surface, water is undoubtedly the most precious natural resource that exists on our planet. As society has become more technologically advanced, pollution has evolved from being primarily biohazards in our water to containing an ever-expanding mixture of dissolved manufactured chemicals. In addition to the increasing complexity of pollution, the sources of pollution are now evident throughout every region of the world. In response to the increases and complexity of both human and industrial pollution, methods for treating pollution have also evolved. Primarily because of cost, however, advanced treatment technologies have been implemented only selectively and non-point sources of pollution remain virtually uncontrolled. As a result, a wide range of pollutants from a variety of sources is being discharged into our water resources. Because of the biological and chemical diversity and complexity of today's population, the environment simply cannot assimilate all of these potentially harmful discharges. Furthermore, pollution regulations around the world allow chemical pollution of water resources as long as ambient water quality criteria are not exceeded thus; pollution of our water resources has become unavoidable. Since water pollution by industrial effluents has become one of the inevitable outcomes of expanding industrialization, the scientific community is focusing their attention towards developing efficient methods for the treatment of effluents. In the last few
decades much effort has been expended in developing suitable methods for protecting the ‘Mother Earth’ from the harmful effect of diverse pollutants. Hence the current phase of researches is two dimensional; one is aiming at developing new technologies for the welfare of humankind while the other is focusing on finding out solutions for minimizing any after effect that may be caused by the new innovations made. Thus, these two phases of researches have to be carried out in parallel to sustain appropriate development.

1.1. SOURCES OF WATER POLLUTION

There are many causes for water pollution; but two general categories exist: direct and indirect contaminant sources. Direct sources include effluent outfalls from factories, refineries, and waste treatment plants that emit fluids of varying quality directly into the water bodies. Indirect sources include contaminants that enter the water resources from soil/groundwater systems and from the atmosphere via rainwater. Whatever be the source of pollution, the magnitude of pollution is usually measured based on the nature of the compounds contaminating the water resources. Aromatic compounds with diverse structure and nature are one of the threatening sources that contribute to much of the chemical pollution in water. This group includes different phenolic, non-phenolic and substituted poly aromatic compounds of varying toxicity. Dyes and phenolic compounds are the two categories of chemical pollutants that the researchers are more concerned about, probably due to their high toxicity and abundance in the effluents.

The major sources of chemical pollution are the effluents from different industries. It is well known that textile, leather and pulp mills discharge highly colored industrial wastewater, which contains appreciable concentrations of dyes belonging to different categories. Synthetic dyes are extensively used for dyeing and printing in industries. Over 10,000 dyes with an annual production over $7 \times 10^5$ metric tones worldwide are commercially available and 5-10% of the dyestuffs are lost in the industrial effluents (Vaidya and Datye, 1982). Color is usually the first contaminant to be recognized in wastewater. A very small amount of dye in water
(10-50 mg/L) is highly visible and affects the aesthetic merit, water transparency and gas solubility of waterbodies. Many azo dyes, constituting the largest dye group, may be decomposed into potential carcinogenic amines under anaerobic conditions in the environment (Chung and Stevens, 1993). Color removal from wastewater is often more important than the removal of soluble colorless organic substances which usually contribute the major fraction of chemical or biochemical oxygen demand (Banat et al., 1996).

Phenols are the major organic constituents found in effluents of coal conversion processes, coke ovens, petroleum refineries, phenolic resin manufacturing, herbicide manufacturing, fiberglass manufacturing and petrochemicals. The terms "Phenols" or "Total Phenols" or "Phenolics" are used interchangeably either to denote simple phenol or a mixture of phenolic compounds in wastewater (Beszedits and Silbert, 1990). The concentration of phenols in effluents varies from 10 to 17 x 10³ mg/L. In general, COD contributed by phenolic compounds in the effluents ranges from 40% to 80% of the total COD. Phenols are toxic, carcinogenic, mutagenic and teratogenic (Autenrieth et al., 1991). A phenol concentration of 1 mg/L or greater affects aquatic life. Therefore, in most cases stringent effluent discharge limit of less than 0.5 mg/L is imposed (Chang et al., 1995; Tay et al., 2001).

1.2. TREATMENT OF INDUSTRIAL EFFLUENTS

In general, there are several methods for the treatment of industrial effluents that includes physical, chemical and biological methods. Physical methods aim at removing solid or liquid pollutants based on their density difference from water. They are essentially wastewater clarification methods and remove suspended or floating solids or liquids. Physical methods of effluent treatment are reverse osmosis, electrodialysis, filtration, foam separation, porous-bed filtration, adsorption etc. They help remove fine particles, and organic and inorganic dissolved materials, resulting in better water quality for re-use or disposal. The chemical method includes treatments like oxidative processes,
ozonation, photochemical and electrochemical destruction. Although different types of physico-chemical methods of effluent treatment are prevalent, they suffer from several drawbacks such as high cost, high sludge production, formation of toxic by-products etc. The disadvantages of physico-chemical methods necessitate the need for developing alternate methods of effluent treatment. It is at this particular scenario that the significance of biological methods of effluent treatment takes the course.

The biological methods of effluent treatment have gained much attention in the recent years. The biological method of purifying effluents is now becoming increasingly important because of the possibility of complete oxidation and decontamination of many impurities including toxic ones. The method also requires relatively low operating cost and simple equipments. This method makes use of the activity of microorganisms or their metabolites. In cases where the microorganisms are being used, they make use of the impurities as a nutritive substrate and form harmless oxidation products such as \( \text{H}_2\text{O} \) and \( \text{CO}_2 \). Different groups of bacteria, actinobacteria, fungi and protozoa are the commonly used biological agents. Another approach of biological treatment involves the use of microbial metabolites, which includes mainly the oxidative enzymes produced by microorganisms. The oxidative enzymes are those groups of enzymes that catalyze the oxidation-reduction reactions in a system and come under the major group called oxidoreductase. These enzymes are highly active against a wide range of compounds and are effective in the treatment of effluents from different sources especially those containing dyes, phenols and other related compounds.

### 1.3. RELEVANCE OF LIGNIN DEGRADING ORGANISMS AND ENZYMES IN POLLUTION ABATEMENT

The oxidative enzymes widely used for effluent treatment are lignin peroxidase, manganese peroxidase and laccases, coming under the general category called lignin degrading enzymes or ligninolytic enzymes. These enzymes are mainly produced by fungi and bacteria to achieve the task of degrading the highly
complex aromatic polymer, lignin. The treatment of industrial effluents has been carried out either with the help of lignin degrading organisms or their enzyme systems. Extensive studies have been carried out on the subject over last few decades. White-rot fungi (WRF) are among the few groups of microorganisms capable of completely degrading polymers of phenolic origin, including lignin. The random nature of the structure of lignin requires its degradation to function non-specifically; consequently other compounds of aromatic structure, including many xenobiotic compounds, are also susceptible to degradation by ligninolytic enzymes, and it is this property that confers their bioremediation potential on these organisms (Davis and Burns, 1990). The efficiency of white rot fungi in the decolourization of industrial effluents have been well accounted in the literature. Jaouani et al have reported the decolourization of olive oil mill waste waters by the white rot fungi Coriolopsis polyzona (Jaouani et al., 2006). Immobilized laccase from Lentinula edodes has been reported to be involved in the partial decolorization and decreased toxicity of an olive mill effluent (Annibale et al., 1999). Phenol is not readily biodegradable and has been reported to be toxic or growth inhibitory to most types of microorganisms, even to those species that have the metabolic capacity of using it as a growth substrate (Annachhatre and Gheewala, 1996). Phenolic removal from olive oil mill wastewater using loofah-immobilized Phanerochaete chrysosporium has also been reported. The system removed 90 % of total phenols and 50 % of COD (Ahmadi et al., 2006). Ryan et al (2007) have investigated the role of different parameters such as fungal growth, culture age and activity and enzyme (laccase) production on bioremediation of phenolic wastewaters by T. versicolor. They have proposed that the time of addition of the phenolic effluents to fungal cultures play an important role in the degradation of phenols by the strain.

Decolourization of dye containing effluents by different fungi has also been established. Decolourization of both synthetic and real textile waste water by Pleurotus flabellatus has been reported by Nilsson et al (2006). Similar studies have been reported from other white rots such as Thelephora sp and Trametes hirsuta (Selvam et al., 2003; Rodriguez Couto et al., 2006). Next to fungi, actinomycetes are the widely used organisms for effluent treatments. Dye
degradation by actinomycetes has been well documented. Works on degradation of recalcitrant azo dyes by *Streptomyces* sp were undertaken by Paszczynski et al. (1991) and the authors have reported that the linkage of a guaiacol molecule onto the azo dyes facilitated the degradation of recalcitrant dyes by five of the *Streptomyces* strains tested. The influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* sp has also been studied (Pasti-Grigsby et al., 1992). Decolorization of the industrial effluents containing reactive dyes, by actinomycetes strains were also proposed (Zhou and Zimmermann, 1993). The degradation of environmental pollutants by microorganisms is invariably effected with the aid of lignin degrading enzymes, either a single enzyme being involved in the process or a group of enzymes acting synergistically. The significance of lignin degrading enzymes in the treatment of industrial effluents is immense; which necessitates a thorough understanding of lignin, lignin degrading organisms and their enzyme systems.

1.3.1. Structure of Lignin

Lignin serves as the second major reservoir of fixed carbon sources in nature, next to cellulose, comprising 15% of the earth’s biomass (Hammel, 1992). True lignin is distributed widely but not universally throughout the Plant Kingdom. It is found in all vascular plants, where it is deposited in cell walls of supportive and water conductive tissues. The three primary polymers that make up plant cell walls consist of about 35 to 50% cellulose, 20 to 35% hemicellulose and 10 to 25% lignin. Lignin fills the spaces in the cell wall between different plant polysaccharides by covalent linking and thereby conferring mechanical strength to the cell wall and by extension the plant as a whole (Chabannes et al., 2001).

The chemical nature of lignin is known largely from studies of its biosynthesis, work pioneered by Freudenberg and his co-workers between about 1930 and 1965. Unlike other biopolymers, lignin contains no readily hydrolysable bond recurring at periodic intervals along a linear backbone. Instead, lignin is a three dimensional amorphous polymer containing many different stable C–C and
C–O–C linkages between phenyl propanoid monomeric units. It consists of an apparently random complex of phenolic and non-phenolic compounds (Crawford, 1981). The most common bond in lignin, the β–O–4, is a bond starting at the middle carbon atom (b) of the propyl sidechain on one repeat unit, linking through the oxygen of the next repeat unit to the number 4 carbon atom of the aromatic ring of that repeat unit (Meister, 2002).

The structural complexity of lignin makes it one of the most recalcitrant molecule and its breakdown involves multiple biochemical reactions, that has to take place more or less simultaneously; cleavage of inter monomeric linkages, demethylations, hydroxylations, side chain modifications and aromatic ring fission followed by dissimilation of the aliphatic metabolites produced. Activity against lignin has been demonstrated in a relatively limited range of microorganisms and an additional challenge for potential ligninolytic microorganisms is the need to gain access to the substrate by penetration of plant tissues. Fungi as well as lignocellulose degrading actinomycetes, accomplish this task by hyphal invasion of the various cell wall layers (Vicuna, 1988). The molecular mass (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson et al., 1990). Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes.

1.3.2. Lignin degrading organisms

1.3.2.1. White Rot Fungi (WRF)

Of all the ligninolytic groups of microorganisms, the white rot fungi (WRF) are the most efficient lignin degraders (Kirk and Farrell, 1987; Eriksson et al., 1990). In contrast to other fungi and bacteria, white rot fungi degrade lignin more extensively and rapidly than any other known group of organisms. The term white rot fungi were derived from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance of the substrate. In the case of WRF, lignin is degraded during secondary metabolism during their secondary metabolism since lignin oxidation provides no net energy to the fungus; synthesis and secretion
of these enzymes are often induced by limited nutrient levels; mostly carbon and nitrogen (Wesenberg et al., 2003). White rot fungi can cause selective or non-selective delignification of wood. In selective delignification, lignin is removed without any marked loss of cellulose and in non-selective delignification all the major cell wall components are degraded (Eriksson et al., 1990; Blanchette, 1995).

Among the white rot fungi, the most studied lignin degrading system is that of *P. chrysosporium*. Lignin degradation by *P. chrysosporium* is a classical secondary metabolic activity induced particularly by nitrogen starvation. This organism secretes LiP, MnP and laccase for lignin degradation (Tien and Kirk, 1983; Glenn and Gold, 1985; Srinivasan et al., 1995). Also there are reports about the production of dioxygenase, catalase, aromatic acid reductase (Leisola and Fietcher, 1985), aryl alcohol dehydrogenase, and vanillate hydroxylase and quinone oxidoreductase (Buswell and Eriksson, 1988) by *P. chrysosporium* under different conditions. An extracellular multicopper oxidase with ferroxidase activity was also discovered recently in *P. chrysosporium* but the role if any, this newly identified enzyme have in lignocellulose degradation was not studied by the authors in detail (Larrondo et al., 2003). *P. chrysosporium* has been considered as the model organism for studying lignin degradation and the enzymology and molecular biology of this organism has been studied in detail and reported by many authors (Reddy and D'Souza, 1994; Cameron et al., 2000; Macarena et al., 2005). The gene encoding lignin peroxidase of *P. chrysosporium* was first cloned by Tien and Tu in the year 1987 (Tien and Tu, 1987) and thereafter many workers have concentrated on exploring the molecular biology of *P. chrysosporium* and the different genes encoding LiP and MnP of this organism were characterized (Gold and Alic, 1993; Alic et al., 1997; Gaskell et al., 1994). Similar works were carried out with other white rot fungi like *Trametes versicolor* (Johnsson et al., 1994; Johansson and Nyman, 1996), *Phlebia radiate* (Saloheimo et al., 1989), *Bjerkandera adusta* (Kimura et al., 1991), *Pluerotus eryngii* (Ruiz-Duenas et al., 1999), *Dichomitus squalens* (Li et al., 1999) and *Ceriporiopsis subvermispora* (Lobos et al., 1998).

White rot fungi produce lignin-degrading enzymes in different combinations and based on this the fungi can be placed into different groups such as fungi
producing Lip and MnP, MnP and laccase, LiP and laccase (Hatakka, 1994). The lignin degradation capability of the fungi may differ greatly according to the group to which they belong. A recent study by Boer et al (2004) has suggested that manganese peroxidase was the main ligninolytic enzyme produced by *Lentinula edodes* although other ligninolytic enzymes have also been already reported in the species. Different species of white rot fungus *Trametes* (*T. Versicolor, T. hirsuta,* and *T. ochracea*) were reported to be producing LiP and MnP along with laccase (Tomsovsky and Homolka, 2003). The lignin degrading ability of the white rot fungus *Elfvingia applanata* has also been revealed recently and the organism was found to be producing Manganese peroxidase and laccases under ligninolytic conditions, but failed to produce LiP (Ohkuma et al., 2001). Production of lignin modifying enzymes by co-cultivated white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* has been reported by Koroleva et al (2002). Arora et al (2002) has studied the ligninolytic system of two *Phlebia* sp and found that they were capable of degrading lignin selectively and hence hold better prospects in various biotechnological applications than *P. chrysosporium*. Lignin degrading ability has also been attributed to the basidiomycete *Stropharia coronilla* that secretes MnP for benzopyrene degradation (Kapich et al., 2005). Reports on lignin degrading white rot fungi from the aquatic environment are sparse as compared to the land isolates. However qualitative screening of lignocellulose-degrading enzymes in selected marine fungi has been reported (Rohrmann and Molitoris, 1992). Lignin-Modifying Enzymes of the white rot fungus, *Flavodon flavus*, isolated from a coastal marine environment has been studied in detail by Raghukumar et al (1999). The same group has done considerable work on lignin degrading enzymes from marine and facultative marine fungi. Lignin modifying enzymes of *Coriolopsis polyzona* has also been established recently (Jaouani et al., 2006). Several isozymic forms of lignin degrading enzymes have been detected in *P. chrysosporium* cultures and a number of other white-rot fungi like *Trametes versicolor, Bjerkandera adusta, Phlebia radiata.*
1.3.2.2. Brown Rot Fungi

Brown rot fungi are usually defined as those wood-rotting fungi that decompose and remove wood carbohydrate, leaving a residue of modified lignin that is typically dark brown in colour and almost equal in weight to the lignin in the original wood (Kirk and Adler, 1970). Brown rot fungi are able to mineralize the methoxyl groups of lignin, but the mineralization of other parts is much lower (Buswell and Odier, 1987). They also introduce other chemical modifications into the lignin polymer such as the removal of R–O–CH3 side chains leaving phenols behind. The studies by Kirk (1975) have shown that there was probably a significant depletion of lignin during decay by the brown rot fungus *Gleophyllum trabeum*. Other species like *Wolfiporia cocos* and *Laetiporus sulphureus* were also reported to be capable of degrading *Eucalyptus grandis* wood to some extent (Machuca and Ferraz, 2001).

1.3.2.3. Soft Rot Fungi

Soft-rot fungi degrade wood in environments that are usually found to be unfavorable for white- or brown-rot fungi, generally in wet environments. Soft rot fungi attack moist wood, producing a characteristic softening of surfaces of the woody tissues. Among the soft rot fungi, *Aspergillus* species are the important lignin degraders. It has been reported that *A. fumigatus* was capable of liberating 38 % of the initial radioactivity labeled kraft lignins as $^{14}$CO$_2$ (Kadam and Drew, 1986). The lignocellulose degrading ability of the ascomycete, *Phaeosphaeria spartinicola* growing on decaying salt marsh grass *Spartina alterniflora* has also been reported (Newell et al., 1994). The soil fungi *Penicillium chrysogenum*, *Fusarium solani* and *Fusarium oxysporum* were found to be mineralizing 20–27 % of $^{14}$C-milled wood lignin from wheat straw in 28 days (Rodriguez et al., 1996). Degradation of phenolic compounds was established in other species of *Aspergillus* such as *A. niger* and *A. terreus* (Garcia et al., 1999). The production of lignin
peroxidase by *Aspergillus* sp isolated from mangrove region has been reported by
Shamla and Prema (Shamla and Prema, 2002). Kanayama et al (2002) have
isolated a strain of *Aspergillus terreus*, which produced LiP, MnP, and phenol
oxidase that were capable of lignin degradation.

1.3.2.4. Non-filamentous bacteria

Bacteria are known to display an ample metabolic versatility towards aromatic
substrates. This characteristic applies for naturally occurring compounds such as
lignin as well as for those of Xenobiotic origin. Bacteria of several genera,
including *Pseudomonas*, *Alcaligenes* and *Arthrobacter*, were reported to be readily
degrading the single-ring aromatic compounds that build up the lignin
macromolecule (Crawford, 1981). However the extent to which bacteria are able to
bring about the decay of the lignin polymer itself has not been properly assessed.

The ability of a Bacillus strain to convert $^{14}$C-[side chain]-lignin of spruce to
$^{14}$CO$_2$ was elucidated by Robinson and Crawford (Robinson and Crawford, 1978).
The authors hypothesized that the initial release of $^{14}$CO$_2$ from the side chain
labeled spruce lignins was due to the degradation of $^{14}$C that have been
incorporated into peripheral units of the lignin which were more susceptible to
attack than highly condensed lignins. Studies by Odier and Monties (1978) have
established that a *Xanthomonas* strain could decompose dioxane-lignin as a sole
carbon and energy source and they also observed 77 % degradation of lignin in
minimal medium after 15 days of growth. However, there was a view that it could
have been low molecular weight fractions of the extractive dioxane lignins that
were metabolized by *Xanthomonas* (Crawford, 1981). There are no available data
on degradation studies using $^{14}$C labeled lignins by this bacterial strain that
prevents direct comparison with that of fungi, which are reported to cause 75 %
mineralization of $^{14}$C – DHP. Synthetic lignin transformation has also been
observed in the marine bacterium *Sagitulla stellata*. The bacterium was found to be
releasing 3.5 % and 1 % $^{14}$CO$_2$ from [β-$^{14}$C] DHP and [ring-$^{14}$C] DHP respectively,
after 30 days of incubation (Gonzalez et al., 1997). It needs mentioning that
bacteria are able to degrade some of the structural components of lignin including side chains and methoxyl groups although the ability to decompose lignin is much less widely distributed among bacteria than fungi.

1.3.2.5. Filamentous bacteria (Actinomycetes)

Among bacteria, lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* species. Actinomycetes are a heterogeneous group of gram-positive bacteria, of which terrigenous saprophytes are the most common forms. The growth of most actinomycetes as branching hyphae is a trait shared by the filamentous fungi and their hyphal growth form is well suited to the colonization of plant biomass and they secrete a range of enzymes active against lignocellulose (McCarthy, 1987). Lignin degradation always accompanies growth in actinomycetes and is therefore presumed to be a primary metabolic activity. The primary degradative activity of actinomycetes is solubilization of lignin with low levels of mineralization to carbon dioxide (McCarthy, 1987). Haider et al (1978) have shown that *Nocardia* species were able to decompose lignin and to assimilate lignin degradation products as a carbon source. These strains were reported to be capable of releasing $^{14}\text{CO}_2$ significantly from the methoxyl group and transforming other carbons from the phenylpropane skeleton of lignin also into $^{14}\text{CO}_2$. Although lignin-degrading ability is observed among different genera of actinomycetes, the most promising lignin degraders are those coming under the genus *Streptomyces*. The list of actinomycetes involved in the production of lignin degrading enzymes is given in Table 1.1. The studies on lignin degradation by actinomycetes were initiated early in 1970s. Sutherland et al (1979) used scanning electron microscopy very successfully to show colonization of Douglas fir phloem by the actinomycete *Streptomyces flavovirens*. Microscopic observations indicated that the non-lignified walls of parenchyma cells were attacked first, followed by attack on the thick walled, heavily lignified scleroids. Lignin degradation studies by Phelan et al (1979) have revealed that six
*Streptomyces* strains were capable of degrading specifically $^{14}$C lignin labeled Douglas fir lignocelluloses.

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>Lignin source/substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nocardia</em></td>
<td>Corn stalks</td>
<td>Trojanowski et al., 1977</td>
</tr>
<tr>
<td><em>S. viridosporus</em></td>
<td>Milled wood lignin</td>
<td>Crawford., 1978</td>
</tr>
<tr>
<td><em>S. badius</em></td>
<td>Lignocellulose</td>
<td>Phelan et al., 1979</td>
</tr>
<tr>
<td><em>S. flavovirens</em></td>
<td>Douglas fir wood</td>
<td>Sutherland et al., 1979</td>
</tr>
<tr>
<td><em>S. setonii</em></td>
<td>Corn stover</td>
<td>Pometto and Crawford, 1986</td>
</tr>
<tr>
<td>Actinomadura spp</td>
<td>Ball milled straw</td>
<td>Mason et al., 1988</td>
</tr>
<tr>
<td><em>S. diastaticus</em></td>
<td>Corn stover</td>
<td>Pasti et al., 1990</td>
</tr>
<tr>
<td><em>S. rochei</em></td>
<td>Corn stover</td>
<td>Pasti et al., 1990</td>
</tr>
<tr>
<td>Thermomonospora mesophila</td>
<td>Ball milled straw</td>
<td>Godden et al., 1992</td>
</tr>
<tr>
<td><em>S. chromofuscus</em></td>
<td>Lignocellulose</td>
<td>Goszczynski et al., 1994</td>
</tr>
<tr>
<td><em>S. thermoviolaceus</em></td>
<td>Lignocellulose</td>
<td>Iqbal et al., 1994</td>
</tr>
<tr>
<td><em>S. cyaneus</em></td>
<td>Wheat straw</td>
<td>Berrocal et al., 1997</td>
</tr>
<tr>
<td><em>S. griseus</em></td>
<td>Lignocellulose</td>
<td>Endo et al, 2003</td>
</tr>
<tr>
<td><em>S. lavendulae</em></td>
<td>Lignocellulose</td>
<td>Suzuki et al, 2003</td>
</tr>
<tr>
<td><em>S. coelicolor</em></td>
<td>NA*</td>
<td>Machczynski et al, 2004</td>
</tr>
</tbody>
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NA* Information not available in the source material

Table 1.1. Lignin degrading actinomycetes
The results also showed that it was the aromatic ring components that were cleaved by the strains and a substantial percentage of the labeled ring carbons were released as $^{14}$CO$_2$ while the side chain components were attacked to only a limited degree. These observations suggest that the studied strains have the ability of ring opening within an intact lignin polymer. Studies by the same authors have established that a strain of *S. badius* could release about 13% of a $^{14}$C lignin labeled lignocellulose as $^{14}$CO$_2$. The degradation studies on lignin model compounds using six actinomycetes strains has been reported and the results have shown that all of the six strains used for the study could demethylate the substrates and oxidize Cα on the phenyl propane side chain while two strains; *Thermomonospora mesophila* and *S. badius* utilized the compounds and produced substantial amounts of monomeric products (Godden et al., 1992).

Although the above studies were capable of giving an insight into the lignin degradative ability of actinomycetes, valuable contribution in this area was given by Crawford (1978) who has done considerable works on the lignin degrading system of *Streptomyces viridosporus* T7A including the mechanisms involved. *S. viridosporus* T7A degraded lignin oxidatively and in the process released water soluble intermediates into the culture medium, which included single ring aromatic compounds and an oxidatively modified polymer – Acid Precipitable Polymeric Lignin (APPL) (Crawford et al., 1983; Pometto and Crawford, 1986). Studies by Crawford and Borgmeyer (1985) have also elucidated the differences between APPLs produced by *S. viridosporus* and another species, *S. badius* 252, which indicated that significantly differing mechanisms of lignin metabolism existed between these two ligninolytic species. *S. viridosporus* T7A produced APPLs by oxidative depolymerization while *S. badius* produced APPLs from the repolymerization of lower molecular weight intermediates of lignin degradation with the help of an extracellular phenol oxidase. The chemistry of the APPLs produced also differed. It was observed that the APPL of *S. badius* was less lignin like and increased substantially in average molecular weight over time while that of *S. viridosporus* was observed to be more lignin like and was slowly modified further over time.
1.3.3. Ligninolytic enzymes

The extremely complex nature of lignin requires an array of oxidative enzymes to be involved in its complete degradation. The characteristics of these enzymes differ widely with the microbial sources. The efficiency of an organism to produce one or more of these enzymes also varies greatly among different microbial groups. Although a wide range of enzymes have been reported to be involved in the tedious process of lignin degradation only a few enzymes like Lignin peroxidase, manganese peroxidase and laccases play major role and each of these enzymes exhibits specific mode of action.

1.3.3.1. Lignin Peroxidase

This enzyme commonly known as ligninase is one of the most important enzyme involved in the degradation of lignin. It was discovered in 1983 from the WRF, *P.chrysosporium*. Since then, this enzyme has been demonstrated in wide variety of organisms including brown rot fungi, soft rot fungi and filamentous bacteria. LiPs are oligomannose type glycoprotein with a molecular weight range of 38 KDa to 43 KDa (Schmidt et al., 1990). The crystal structure of lignin peroxidase (LiP) from the white rot fungus *Phanerochaete chrysosporium* was refined by Choinowski, et al (1999). They have used the program MOLSCRIPT (Kraulis, 1991) to depict the structure of LiP isozyme with an isoelectric point of 4.15 (LiP 415), and according to their study the final model comprised 343 amino acid residues, 370 water molecules, the heme, four carbohydrates, and two calcium ions. The authors also reported the evidence for a radical formation at Trp171 using spin trapping, which supported the concept of Trp171 being a redox active amino acid and being involved in the oxidation of veratryl alcohol.

LiP is having relatively high redox potential, so the compounds with high redox potentials that are not oxidized by other enzymes are also oxidized by LiP. It is this particular character of LiP that makes it an important part of ligninolytic
system. LiP can oxidize both phenolic and non-phenolic compounds. This enzyme employs free radical chemistry to cleave the propyl side chain of lignin substructures (Schoemaker et al., 1985) and have been shown to depolymerize lignin invivo (Hammel et al., 1993). LiPs have the unusual ability to cleave the recalcitrant nonphenolic units that comprise approximately 90% of lignin (Glenn et al., 1983; Tien and Kirk, 1983). The reactions catalysed by LiP include Cα-Cβ cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation and even aromatic ring cleavage of non-phenolic lignin model compounds (Tien and Kirk, 1984; Renganathan et al., 1985; Umezawa and Higuchi, 1987; Chun and Aust, 1995). This heme peroxidase has a classical peroxidase catalytic mechanism for which H₂O₂ is required. The native enzyme is oxidized by H₂O₂ and generates two-electron deficient compound I. Compound I can oxidize a compound and can be reduced to compound II, which is one electron deficient. A subsequent oxidation of another molecule by compound II returns the peroxidase to its native resting stage. When there is excess H₂O₂, it will combine with compound II of LiP, generating compound III, which is an inactive form of the enzyme. In many cases, the substrates are not directly accessible to heme of LiP and thus direct oxidation of substrate does not occur. In such cases involvement of redox mediator plays an important role. Veratryl alcohol is an excellent substrate for LiP and it acts as the redox mediator for indirect oxidation of other substrates. Veratryl alcohol stimulates oxidation by preventing enzyme inactivation (Valli et al., 1990) and it is oxidized by LiP to VA cation radical, which is a strong oxidant, and it acts as an electron transfer mediator in the catalytic reaction of LiP.

Lignin peroxidase is usually produced in combination with other lignin degrading enzymes like MnP and laccases. The most studied ligninolytic organism, Phanerochaete chrysosporium produced LiP along with MnP (Hatakka, 1994). Chrysosporium purinosum, considered to be a separate strain of \textit{P.chrysosporium}, similarly produced LiP and MnP activities (Waldner et al., 1998). Phlebia ochraceofulva produced higher LiP activities along with laccases.
but it failed to produce MnP even at elevated Mn (II) concentrations (Sarkanen et al., 1991). LiP is also produced by ascomycetes like *Aspergillus* sp (Shamla and Prema, 2002). There are also reports that several fungi which are efficient lignin degraders in nature and especially suitable for selective lignin degradation apparently did not produce LiP (Sarkanen et al., 1991). These include *Dichomitus squalens, Lentinus edodes, Rigidoporus lignosus* and *Sterreum hirsutum* (Paice et al., 1993). However there might be many reasons for difficulties in demonstrating LiP activity in a certain fungus, such as the use of unsuitable medium or cultivation conditions.

Lignin peroxidase has been well established among actinomycetes. The characterization of the extracellular peroxidase of *Streptomyces viridosporus* T7A was carried out by Ramachandra et al (1987) and they proposed that the lignin-oxidizing enzyme of *S. viridosporus* T7A, designated as actinomycete lignin peroxidase P3 (ALiP – P3) was a heme protein and the enzyme has been confirmed to be capable of catalyzing Cα – Cβ cleavage of lignin substructure model compounds. Pasti et al (1990) have isolated actinomycete strains from the gut of higher termites and found that the peroxidases of *S. Chromofuscus* A2 were superior to that of *S. viridosporus* and the rates of lignin solubilization in both organisms were correlated and subjected to glucose repression. However, it was not confirmed by these researchers whether the superior lignocellulolytic activity of *S. chromofuscus* A2 was due to the activities of other enzymes acting in concert with the peroxidases. The inducible nature of actinomycetes peroxidase has been studied and the authors have concluded that the peroxidase production by actinomycetes could be induced by growth on straw, indulin AJ, Syringic acid, 3, 4-dimethoxycinnamic acids and vanillic acid. One of the interesting works on peroxidases of actinomycetes was carried out by Mason et al (2001) who have investigated the appearance of the secreted pseudoperoxidase of the thermophilic actinomycete, *T. fusca* BD 25. The enzyme from *T. fusca* was found to be associated with the appearance of a heme like spectrum and the species responsible for this spectrum was found to be a metallo-porphyrin. Based on the above-mentioned spectral studies, they proposed that lignin degrading heme peroxidases
were not secreted by actinomycetes and the spectra recorded from culture media were due to zinc or copper containing porphyrins and not the heme. The same porphyrin was observed by this group of workers in the growth medium of the lignin solubilizing actinomycete *S. viridosporus* T7A. These results were contradictory to the reports on heme peroxidases of actinomycetes. The authors have reported that the earlier reports on heme peroxidases of actinomycetes were due to the incorrect assignment of optical spectra to heme groups rather than to non-iron containing porphyrins. Thus the studies implies that the exact nature of peroxidases from actinomycetes needs to be studied more to get a thorough knowledge on the same and in the present form the information on actinomycetes peroxidases remains incomplete with several missing links.

1.3.3.2. Manganese Peroxidase

Manganese peroxidase is another important enzyme produced by the lignin degraders. It is also a heme peroxidase and requires H$_2$O$_2$ for its activity. The redox potential of the MnP – Mn system is lower than that of LiP and normally it does not oxidize non-phenolic lignin models however Maltseva et al has reported that the MnP from *Panus tigrinus* is able to degrade nonphenolic lignin model compounds (Maltseva et al., 1991). MnP shows a strong preference for Mn (II) as its reducing substrate (Glenn and old, 1985). MnP oxidizes Mn$^{2+}$ to Mn$^{3+}$, which is stabilized by organic acid chelators viz; oxalate, malonate, glyoxylate etc and acts in turn as a low molecular mass, diffusible, redox mediator that attacks organic molecule and oxidizes various compounds nonspecifically via hydrogen and one electron abstraction. The organic acids also facilitate the release of Mn (III) from the active site of the enzyme. The crystal structure of manganese peroxidase (MnP) from the lignin- degrading basidiomycetous fungus *P. chrysosporium* showed that the enzyme has two structural calcium ions. MnP also has two N-acetylglucosamine residues N-linked to Asp131 that are readily visible in the electron density map. The active site, consisting of a proximal His ligand H-bonded to an Asp residue and a distal side peroxide-binding pocket consisting of a catalytic Histidine and Arginine. The 1.45 crystal structure of MnP complexed with
Mn (II) provided a more accurate view of the Mn-binding site including possible partial protonation of Glu 39 in the Mn-binding site and glycosylation at Ser 336 (Sundaramoorthy et al., 2005). The one electron oxidation of Mn (II) to Mn (III) in a multi step reaction cycle is as follows:

\[
\text{MnP + H}_2\text{O}_2 \rightarrow \text{MnP compound I + H}_2\text{O} \quad \text{(Reaction 1)}
\]
\[
\text{MnP compound I + Mn (II) \rightarrow MnP compound II + Mn (III)} \quad \text{(Reaction 2)}
\]
\[
\text{MnP compound II + Mn (II) \rightarrow MnP + Mn (III) + H}_2\text{O} \quad \text{(Reaction 3)}.
\]

This enzyme is mostly reported in WRF, where it is produced in combination with LiP or laccase. There are a number of efficient delignifying fungi that secrete MnP as the sole extracellular peroxidase, including *Lentinula edodes* (Leatham, 1986), *Bjerkandera adusta* (Wang et al., 2002), *Coprinus subvermispora* (Lobos et al., 1994), *P. sordida* (Ruttimann et al., 1994), *Dichomitus squalens* (Perie et al., 1996), *Pleurotus ostreatus* (Giardina et al., 2000) and *Rigidoporus lignosus* (Galliano et al., 1991). Some other ligninolytic fungi produce MnP in combination with lignin peroxidase. *P. chrysosporium*, *P. radiata*, *T. versicolor*, and *Nematoloma frowardii* are examples for LiP – MnP group (Hofrichter et al., 2001). A manganese peroxidase (MnP) from a wood-degrading fungus *Trichophyton rubrum* LSK was characterized earlier and the enzyme has the highest pI of 8.2 among MnPs reported so far and when compared with other MnPs, this MnP has been reported to be more stable in the presence of high concentrations of H\(_2\)O\(_2\) (Bermek et al., 2004). Manganese mediated lignin degradation by *Pleurotus pulmonarius* (Camarero et al., 1996) have also been studied and the results clearly indicated the stimulation of lignin mineralization by Mn\(_{2+}\). Two phylogenetically and structurally divergent manganese peroxidases (Pr- MnP 2 and Pr- MnP 3) from *Phlebia radiata* were recently described by Hilden et al (2005). The Pr-MnP2 with a long C-terminal extension has the highest structural similarity with the crystal structure of *P. chrysosporium* MnP1, whereas the shorter Pr-MnP3 protein was structurally more related to lignin peroxidase. Some differences between both Pr-MnPs were observed including the presence of helix G* (only in Pr-MnP2) and helix B* (only in Pr-MnP3). Both *P. radiata* MnPs have 12 helices, the position of one of the additional helices, depicted B** in the *P.*
radiata proteins was similar to those found in most fungal class II peroxidases. However, the second additional helix, helix G* in Pr-MnP2 and helix B* in Pr-MnP3, occupies very different positions. Both models also showed four-disulphide bridges (Hilden et al., 2005). Production of Manganese peroxidase by actinomycetes is not well established although there are citations on few species. The studies on biodegradation of pesticides by actinomycetes suggested the probable production of MnP by a Streptomyces strain CCT 4916 that was able to degrade the herbicide diuron by oxidative reactions (Esposito et al., 1998). The studies by Zou and Schrempf (2000) revealed for the first time that a bacterial catalase-peroxidase has a heme-independent manganese-peroxidase activity. The catalase-peroxidase CpeB reported by them was found to be catalyzing the peroxidation of Mn (II) to Mn (III), independent of the presence or absence of the heme inhibitor KCN.

1.3.3.3. Laccase

Laccase was first discovered in the sap of the Japanese lacquer tree Rhus vernicifera in 1883 (Reinhammar, 1984). It is a polyphenol oxidase, which belongs to the family of blue multicopper oxidases. These enzymes catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water. Laccases oxidize a broad range of substrates, preferably phenolic compounds. Laccases differ from LiP and MnP in that it does not require H₂O₂ to oxidize its substrates.

1.3.3.3.1. Classification of laccases

Laccase (EC 1.10.3.2) is a blue copper protein that falls within the broader description of polyphenol oxidases. Polyphenol oxidases are proteins with the common feature that they are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor (Mayer, 1987). Polyphenol oxidases are associated with three types of activities:
Catechol oxidase or o-diphenol: oxygen oxidoreductase (EC 1.10.3.1)
Laccase or p-diphenol: oxygen oxidoreductase (EC 1.10.3.2)
L-ascorbate oxidase (EC 1.10.3.3)

These different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion, 1980). There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase. Catechol oxidases and tyrosinases have o-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and para diphenol activity, usually with more affinity towards the p-diphenols. Only laccases have the ability to oxidize syringaldehyde (Thurston, 1994; Eggert et al., 1996).

13.3.3.2. Sources of laccases

Plant laccases

The first laccase to be reported was from a plant source, the Japanese lacquer tree *Rhus vernicifera*, belonging to the family Anacardiaceae. All the members of this family appear to contain laccase in the resin ducts and in the secreted resin (Huttermann et al., 2001). Laccases are encoded by multigene families in plants. However, the occurrence of laccases in higher plants appears to be far more limited than in fungi. The isolation and characterisation of laccases has been obtained mainly from dicot species, such as *Acer pseudoplatanus* (Sterjiades et al 1992), tobacco (Richardson and G.J. McDougall, 1997), *Arabidopsis thaliana* (McCaig et al, 2005) yellow-poplar (LaFayette et al, 1999) and cotton (Wang et al, 2004). *Pinus taeda* tissue has been shown to contain eight laccases, all expressed predominantly in xylem tissue (Sato et al., 2001). Other reports are those of Wosilait et al. (1954) on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been shown to be present in the xylem tissue of *Populus euramerican* (Ranocha et al., 1999). Laccases have also been characterized from a few monocots such as ryegrass and maize (Gavnholt et al 2002; Caparros-Ruiz et al 2006).
**Fungal laccases**

Laccase activity is widely distributed among different groups of fungi. Among fungi, different genera of white rot fungus are the leading laccase producers. Many workers have done extensive studies on the production of laccase from the fungi under differing conditions. Laccase production has been carried out by submerged as well as solid-state fermentation techniques (Tong et al., 2007; Janusz et al., 2007; Rodriguez Couto and Sanroman, 2005). Solid-state fermentation has been considered more suitable for laccase production by due to their filamentous nature. However, there are few designs available in the literature for bioreactors operating in solid-state conditions for laccase production. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture. SSF lacks the sophisticated control mechanisms that are usually associated with SmF. Control of the environment within the bioreactors is also difficult to achieve, particularly temperature and moisture.

The production of laccases under solid-state fermentation conditions by different fungal strains is summarized in Table 1.2. The enhanced formation of laccase by the white rot fungi *Trametes pubescens* in the presence of copper was studied by Galhaup et al (2001). The influence of copper on laccase production has been reported earlier in *Neurospora crassa* (Huber and Lerch, 1987). A blue laccase of litter decaying basidiomycete *Stropharia rugosoannulata* capable of oxidizing Mn$^{2+}$ in the presence of Mn$^{3+}$ chelators has also been suggested (Schlosser and Hofer, 2002). The important laccase producing white rot fungi are *Phlebia radiata* (Niku-Paavola et al., 1990), *Ganoderma lucidum* (Perumal, 1997), *Trametes versicolor* (Swamy and Ramsay, 1999), *Cyathus stercoreus* (Sethuraman et al., 1999), *Pycnoporus cinnabarinus* (Otterbein et al., 2000), *Pleurotus ostreatus* (Palmieri et al., 2000), *Pycnoporus sanguineus* (Pointing et al., 2000), *Coriolus hirsutus* and *C. sonatus* (Koroleva et al., 2001), *Trametes modesta*
<table>
<thead>
<tr>
<th>Support</th>
<th>Fungal strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagasse</td>
<td><em>T. versicolor</em>, <em>Pycnoporus cinnabarinus</em></td>
<td>Pal et al., 1995; Meza et al., 2005</td>
</tr>
<tr>
<td>Banana waste</td>
<td><em>P. ostreatus</em>, <em>P. sajor-caju</em></td>
<td>Reddy et al., 2003</td>
</tr>
<tr>
<td>Canola roots</td>
<td><em>Cyathus olla</em></td>
<td>Shinners-Carnelley et al., 2002</td>
</tr>
<tr>
<td>Corn</td>
<td><em>Lentinus edodes</em>, <em>P. pulmonarius</em></td>
<td>D'Annibale et al., 1996; Li et al., 2000</td>
</tr>
<tr>
<td>Cotton</td>
<td><em>P. ostreatus</em>, <em>P. chrysosporium</em></td>
<td>Jaszek et al., 1998; Sik and Unyayar, 1998</td>
</tr>
<tr>
<td>Sawdust</td>
<td><em>Coriolus hirsutus</em></td>
<td>Elisashvili et al., 2001</td>
</tr>
<tr>
<td>Wheat bran</td>
<td><em>P. pulmonarius</em>, <em>Ganoderma lucidum</em></td>
<td>Marques de Souza et al., 2002; Murugesan et al., 2007</td>
</tr>
<tr>
<td>Wheat straw</td>
<td><em>Phlebia radiata</em>, <em>P. ostreatus</em>, <em>P. pulmonarius</em></td>
<td>Vares et al., 1995; Baldrian et al., 2002; Marques de Souza et al., 2002</td>
</tr>
<tr>
<td>Wood</td>
<td><em>Ceriporiopsis subvermispora</em>, <em>P. ostreatus</em></td>
<td>Ferraz et al., 2003; Pradeep and Datta, 2002</td>
</tr>
<tr>
<td>Orange peelings</td>
<td><em>Trametes hirsuta</em></td>
<td>Rosales et al., 2007</td>
</tr>
<tr>
<td>Banana Skin</td>
<td><em>T. pubescens</em></td>
<td>Osma et al., 2007</td>
</tr>
<tr>
<td>Groundnut shell</td>
<td><em>P. ostreatus</em></td>
<td>Mishra and Kumar, 2007</td>
</tr>
<tr>
<td>Grape seeds</td>
<td><em>T. hirsuta</em></td>
<td>Rodriguez-Couto et al., 2006</td>
</tr>
<tr>
<td>Coconut flesh</td>
<td><em>T. hirsuta</em></td>
<td>Rodriguez-Couto and Sanroman, 2005</td>
</tr>
<tr>
<td>Chestnut shell</td>
<td><em>Coriolopsis rigida</em></td>
<td>Gomez et al., 2005</td>
</tr>
<tr>
<td>Barley bran</td>
<td><em>C. rigida</em></td>
<td>Gomez et al., 2005</td>
</tr>
<tr>
<td>Kiwi fruit waste</td>
<td><em>Trametes hirsuta</em></td>
<td>Rosales et al., 2005</td>
</tr>
</tbody>
</table>

Table 1.2. Production of laccase by different fungal strains under solid-state fermentation
(Nyanhongo et al., 2002), Bjerkandera adusta, Pleurotus sajor caju (Reddy et al., 2003) and Panus tigrinus (Zavarzina et al., 2004). The ascomycete fungi like Podospora anserine (Frese and Stahl, 1992), Trichophyton rubrum (Jung et al., 2002), Botryophphaeria sp (Alves-Da-Cunha et al., 2003), Physi sporinus rivulosus (Hakala et al., 2005) also exhibits laccase activity. Laccase activity has also been reported from Agaricus bisporus (Smith et al., 1998), the hyphomycete, Chalara paradoxa (Robles et al., 2000) and ascomycetes such as Mauginiella sp (Palonen et al., 2003), Trichoderma atroviride, Trichoderma harzianum (Holker et al., 2002) and Melanocarpus albomyces (Kiiskinen and Saloheimo, 2003). Gonzales et al (2002) have reported that the fungus Petriellidium fusoides produces laccase as the only enzyme involved in lignin metabolism and the mineralization of synthetic lignins by the laccase of this organism has also been discussed.

**Bacterial laccases**

There is increasing evidence for the existence of typical multi-copper laccases in prokaryotes (Alexandre and Zhulin, 2000; Claus, 2003). Recent rapid progress in the whole genome analysis suggests that laccases are widespread in bacteria (Sharma et al., 2007). Corresponding genes have been found in gram-negative and gram-positive bacteria, including species living in extreme habitats, e.g. in Oceanobacillus iheyensis or Aquifex aeolicus (Deckert et al., 1998), Thermus thermophilus (Miyazaki, 2005) and in the archaeabacterium Pyrobaculum aerophilum (Fitz-Gibbon et al., 2002). Laccases have been identified also in Pseudomonas stutzeri (Kumar et al. 2005). The first convincing data for a prokaryotic laccase activity was presented for Azos rillum lipoferum (Givaudan et al., 1993). It is a multimeric enzyme, composed of a catalytic subunit and one or two larger chains. Marinomonas mediterranea is a melanogenic marine bacterium expressing both an SDS-activated tyrosinase and a laccase (Sanchez-Amat and Solano, 1997). The laccase, which was heterologously expressed in Escherichia coli, revealed the typical copper-binding domains of laccases and two additional potential copper-binding sites near the N-terminus (Sanchez-Amat et al., 2001).
laccase-like enzyme activity has been detected in spores of a *Bacillus sphaericus* strain (Claus and Filip, 1997). Recently the spore protein CotA of *Bacillus subtilis* has been recognized to be a laccase (Hullo et al., 2001, Jones and Henriques, 2002; (Martins et al., 2002). Laccase-like activity has also been found in other bacteria, e.g., CopA protein from *Pseudomonas syringae* (Mellano and Cooksey, 1988) and PcoA protein from *Escherichia coli* (Brown et al., 1995). Recently, a protein encoded by ORF bh2082 of *Bacillus halodurans* C-125 has been identified as a potential bacterial laccase by genome mining. The enzyme showed an alkaline pH optimum with syringaldazine as the substrate (Ruijssenaars and Hartmans 2004). Dalfard et al (2006) have demonstrated laccase activity in a newly isolated *Bacillus* strain HR03 which exhibited laccase activity along with cresolase and cathecolase activities.

**Actinomycete laccases**

Although some specific phenol oxidases (Yoshimoto et al., 1985) (tyrosinases) has been described in actinomycetes, the presence of phenol oxidases, possibly involved in the lignin degradation have been reported only in a few *Streptomyces* species. Extracellular phenol oxidase produced by *S. badius* has been implicated in lignin metabolism, which involved the production of low molecular weight intermediates of lignin degradation and the repolymerization of the intermediates to produce APPL (Borgmeyer and Crawford, 1985). The phenol oxidase secreted by *S. cyaneus* CECT has been reported as capable of solubilizing and mineralizing the lignin fraction of lignocellulose at the rate of 44.96 % and 3.41 % respectively after 21 days of incubation and it has been cited as the first report on laccase activity produced by a *Streptomyces* strain during growth in SSF (Berrocal et al., 1997). The presence of a laccase like phenol oxidase (Epo A) has been reported in *S. griseus* (Endo et al., 2003) and the enzyme shared many of the characters of typical laccase but failed to oxidize compounds like guaiacol and syringaldazine that were known model laccase substrates. A recent work by Arias et al (2003) elucidated the potential of extracellular laccases from *Streptomyces* for use in combination with
mediators for biobleaching of Kraft pulps. Suzuki et al (2003) have identified a thermo stable laccase from *S. lavendulae*. The structural details of actinomycete laccases were also unveiled in the recent years. Machczynski et al (2004) have identified a new family of laccases – the two domain laccases in *S. coelicolor* that lacked the second domain at the substrate binding cleft and it has also been claimed to be the first known paramagnetic NMR spectrum for the trinuclear copper cluster of laccase. The results of the above study are of great significance as it revealed the occurrence of a new laccase family in *Streptomyces*, which could instigate the search for different types of laccases from actinomycetes.

**Insect laccases**

Laccases have been detected in the cuticles of several dipterans like *D. virilis* (Yamazaki, 1969) and *L. cuprina* (Barrett, 1987), as well as in the lepidopteran *B. mori* (Yamazaki, 1972). In insects, several forms of laccases have been identified. “Laccase-1” and “laccase-2” have been identified in *Manduca sexta* and *Tribolium castaneum* (Arakane et al. 2005, Dittmer et al. 2004). Suderman et al., (2006) have investigated the role of laccase in insect cuticle sclerotization. Different isoforms of laccase have also been documented in *Drosophila melanogaster* and *Anopheles gambiae* (Arakane et al. 2005). In *Anopheles gambiae*, 5 different forms of laccases have been identified (Dittmer et al. 2004). A feature that is unique to insect laccases (relative to fungal and plant laccases) is a longer aminoterminal sequence characterized by a region with conserved cysteine, aromatic and charged residues (Dittmer et al. 2004). Hattori et al. (2005) have detected laccase in the salivary glands of the green rice leafhopper, *Nephotettix cincticeps*.

**1.3.3.3.3. Functions of laccases**

Laccases are distributed widely among fungi, bacteria and also in higher plants. Hence the properties of different laccases show a great deal of divergence
according to the source of its origin. It has been proposed that plant laccases could be implicated in the polymerization step of the lignification process. The possibility that laccases are involved in the lignification process in higher plants was first raised by Freudenberg (1958) and confirmed by Sterjiades et al (1992). This group did suggest, however, that laccase was involved only in the early stages of lignification, while peroxidases were involved later. Bao et al. (1993) showed that laccase activity was correlated with lignification of xylem in *P. taeda*. In the cases when laccase activity has been correlated with lignification, it has been located in or near the cell walls of lignifying cells. In other tissues, such as leaf or stem tissue, neither the cellular nor the sub-cellular location has been determined except in the case of the resin ducts of the Anarcardiaceae. Hence there is a lack of information about some of the basic factors relating to the exact function of laccases in plants (Mayer and Staples, 2002).

Information on the role of fungal laccases is more abundant in the literature. Fungal laccases play important role in physiological processes related to pathogenesis, morphogenesis, i.e. fruitbody development, pigmentation and to cell detoxification. One of the laccases of the edible mushroom *Lentinula edodes* has been assigned a role in fungal morphogenesis (Zhao and Kwan, 1999), and laccase has been reported to be specifically expressed in the green-spored conidia of *A. nidulans* (Aramayo and Timberlake, 1990; Clutterbuck, 1972). *Aspergillus fumigatus*, a filamentous fungus producing bluish-green conidia, is an important opportunistic pathogen that primarily affects immuno-compromised patients. Conidial pigmentation of *A. fumigatus* significantly influences its virulence. The conidial pigmentation of this fungus has been reported as being regulated by a cluster of genes such as abr1 and abr2. The abr1 gene (aspergillus brown 1) possessed two signatures of multicopper oxidases while the abr2 gene product showed homology to the laccase of *Aspergillus nidulans* (O’Hara and Timberlake,
1989). Similarly, laccase has been identified as responsible for the virulence of the encapsulated fungus *Cryptococcus neoformans*, which is a human pathogen (Williamson, 1997). In *C. neoformans*, laccase is present as a tightly associated cell wall enzyme that is readily accessible for interactions with host immune cells (Zhu et al., 2001). The grapevine mould, *Botrytis cineria* produces a laccase that is necessary for pathogenesis, and the role of the laccase is presumably related to detoxification of toxic defence metabolites produced by the plant (Bar-Nun et al., 1988). Laccases have also been shown to be important for pathogenesis in the chestnut blight fungus *Cryphonectria parasitica* (Choi et al., 1992; Mayer and Staples 2002). Laccases have also been proposed to participate in fungal morphogenesis in *Armillaria* sp, *Lentinus edodes* and *Volvariella volvacea* (Worrall et al., 1986; Leatham and Stahmann, 1981; Chen et al., 2004).

The bacterial laccases have been assigned specific roles in relation to their location in the cell. The cot A laccase of *Bacillus subtilis*, which is a component of the endospore coat was found to have a role in the appearance of a brown pigment characteristic of colonies in the late stages of sporulation (Donovan et al., 1987; Rogolsky, 1968) which appears to protect spores against UV light (Hullo et al., 2001). The CopA protein from *Pseudomonas syringae* and PcoA protein from *Escherichia coli* have been shown to be important for bacterial copper resistance (Mellano and Cooksey 1988; Brown et al. 1995). EpoA from *Streptomyces griseus* appears to have a role in morphogenesis in *Streptomyces* sp (Endo et al., 2002).

Laccase is hypothesized to play an important role in insect cuticle sclerotization by oxidizing catechols in the cuticle to their corresponding quinones, which then catalyze protein cross-linking reactions (Dittmer et al, 2004). Salivary laccase from the green rice leafhopper has been reported to be involved in the rapid oxidization of toxic monolignols, resulting in the formation of nontoxic polymers that allows the insect to feed successfully (Hattori et al. 2005).
1.3.3.3.4. Structure of laccases

The overall structure of laccases comprises three cupredoxin-like domains; A, B and C, that are about equal in size. (Ducros et al., 1998; Piontek et al., 2002). All three domains are important for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains B and C, a mononuclear copper centre is located in domain C, and a trinuclear copper centre is located at the interface between domains A and C. The crystal structure of laccases from different fungi has been elucidated. Ducros et al (1998; 2001) reported the crystal structure of a laccase from the fungus *Coprinus cinereus*. This was found to be a copper type-2-depleted form in which the putative T2 copper was completely absent and therefore was in a catalytically incompetent state. The difficulties in successfully crystallizing the active form of laccase have been unanimously attributed to the occurrence of extensive microheterogeneity, presumably caused by variable glycosylation of the enzyme. Unfortunately, deglycosylation to obtain high quality diffracting crystals of the *C. cinereus* laccase (CcL) resulted in the loss of copper. Antorini et al (2002) have carried out the purification and crystallization of laccase isozymes from two white rot fungi, *Trametes versicolor* and *Pycnoporus cinnabarinus*. *T. versicolor* laccase was crystallized in two crystal forms, both with the orthorhombic space group P212121, which diffracted to 1.9 and 2.95 Å resolution, respectively. The crystals of *P. cinnabarinus* laccase belonged to the monoclinic space group C2 and diffracted to at least 2.2 Å resolution. All the laccase crystals were suitable for X-ray structure determination and contained full complement of copper ions. (Antorini et al., 2002). The laccase isozymes from *T. versicolor* had a sequence identity of about 70% to the laccase from *C. cinereus*, but the value dropped to about 55% for *P. cinnabarinus* laccase isozymes with the *C. cinereus* laccase (Antorini et al., 2002).
Fig. 1.1. Ribbon diagram of laccase from *Trametes versicolor* (TvL)

The arrangement of the domain structure is depicted in different color coding (D1–D3). Copper ions are drawn as blue spheres. Carbohydrates and disulfide bonds are included as stick models (Piontek et al., 2002)

Fig. 1.2. Ribbon diagram of laccase from *Escherichia coli* (CueO)

The domain 3 of CueO is shown in blue and red (the antiparallel connections of the first and second β-strands of domain 3 are highlighted in green). Red, blue, green, and orange spheres indicate the labile (regulatory) Cu (rCu), type I Cu, type II Cu and type III Cu ions respectively
The crystal structure of a *T. versicolor* laccase (TvL) in its oxidized, copper-complete state has been described by Piontek et al (2002). This structure has given insight into the coordination of all the four copper centers in the fully active enzyme. The geometry of the trinuclear copper cluster in TvL was similar to that found in the ascorbate oxidase and that of mammalian ceruloplasmin structures, suggested a common reaction mechanism for the copper oxidation and the O2 reduction (Piontek et al., 2002). The TvL structure was a monomer organized in three sequentially arranged domains and had dimensions of about 65 x 55 x 45 Å3. Each of the three domains was of a similar β-barrel type architecture. The trinuclear copper cluster (T2/T3) was embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. The third domain had the highest helical content with one 3_{10}-helix and two α-helices located in the connecting regions between the strands of the different β-sheets. Finally, at the C-terminal end of domain 3, three sequentially arranged α-helices complete the fold. A 13-aminoacid- long α-helix at the C-terminal portion was stabilized by a disulfide bridge to domain 1 (Cys-85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connected domains 1 and 2. Both N-terminal and C-terminal amino acids benefited from hydrogen bonding networks to the rest of the protein, which provided sufficient rigidity so that excellent electron density was observed for these regions in the crystal structure.

The structure of *Rigidoporus lignosus* laccase (R1L) containing a full complement of copper ions has been elucidated by Garavaglia et al (2004). Structural comparison between R1L and TvL revealed only subtle differences. R1L folded into three sequentially arranged domains, each of them with a β-barrel type topology. As observed in all laccases, the type-1 copper was located in domain 1 whereas the trinuclear copper cluster (T2/T3) was embedded between domains 1 and 3. The structure was stabilized by two disulfide bridges: Cys85-Cys487 located between domain I and III; and Cys117-Cys210 connecting domains 1 and 2. The crystal structure of CueO (Fig. 2) from *Escherichia coli* has also been determined (Roberts et al., 2002; Roberts et al., 2003). Differing from other MCOs, the substrate-binding site of CueO is deeply buried under a methionine-rich helical
region including α-helices 5, 6, and 7 that interfere with the access of organic substrates (Kataoka et al., 2007). The asymmetric unit contained two Δα 5–7 CueO molecules (molecules A and B), which were related by a pseudo-2-fold symmetry. The overall structure of Δα5–7 CueO (molecule A) comprised three domains as other MCOs. Molecules A and B have essentially the same fold, although the C-terminal 6xHis-tag region of molecule B was disordered (Kataoka et al., 2007).

The crystal structure of another bacterial laccase which exists as a component of the spore coat of Bacillus subtilis has been disclosed by Enguita et al (2003). The overall CotA fold comprises three cupredoxin-like domains. The cupredoxin fold is mainly formed by an eight-stranded β-barrel, comprising two β-sheets composed by four strands, arranged in a sandwich conformation. The first domain comprises eight strands organized in a β-barrel form, starting with a coiled section which is absent in plant and fungal multicopper oxidases such the laccase from C. cinereus and ascorbate oxidase. However, a similar coiled section is present in the E. coli CueO protein (Roberts et al., 2002). Domain 2 of CotA acts as a bridge between domains 1 and 3, but a short α-helical fragment, encompassing residues 177–182, makes the connection between domains 1 and 2, whereas a large loop segment including residues 341–368 links domains 2 and 3. In both the structures of CotA and CueO, this region represents an external connection between domains 2 and 3, whereas in plant and fungal multicopper oxidases the corresponding link is made through an internal connection. Therefore, this feature may be a characteristic of the prokaryotic variants of these enzymes. Domain 3 of CotA not only contains the mononuclear copper center, but also contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between domains 1 and 3. Moreover, domain 3 includes the putative substrate binding site, located at the surface of the protein, close to the type I mononuclear copper center. A protruding section formed by a loop and a short α-helix, comprising amino acids from 434 to 454, forms a lid-like structure over the substrate binding site. No similar element has been found in the previously analyzed multicopper oxidases with known three-dimensional structure. Therefore, this structural elemental has been reported as a distinctive feature of CotA (Enguita et al., 2003).
1.3.3.3.5. Active site of laccases

The blue multicopper laccases typically employ four copper ions at their active site (Fig. 1.3). Type I copper confers the typical blue colour to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper-cysteine bond. Due to its high redox potential of ca. 790 mV, type 1 copper is the site where substrate oxidation takes place. Type 2 copper shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. The type 3 copper center is also the common feature of another protein superfamily including the tyrosinases and haemocyanins (Decker and Terwilliger, 2000). The typical coordination of type-1 coppers in blue multi copper oxidases and in the small copper enzymes consists of two histidines, one cysteine, and one axial methionine and is therefore 4-fold. Axial coordination has been considered to be one factor affecting the redox potential of copper enzymes. Mutational studies on azurin showed that the substitution of methionine by a leucine resulted in an increase of the E\text{0} by about 0.1 V (40). In CcL, which had a redox potential of 550 mV, the axial position was occupied by a leucine, whereas in TvL, with a redox potential of 800 mV, there was a phenylalanine in the corresponding position. Thus, it has been speculated that a phenylalanine in the axial position was responsible for the very high E\text{0} of TvL (Piontek et al., 2002). Type 2 and type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place. Type 2 copper is coordinated by two and type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the two type 3 copper atoms, is maintained by a hydroxyl bridge.
Fig. 1.3. Active site of laccase. Model of the catalytic cluster of the laccase from *Trametes versicolor* made of four copper atoms

(Piontek et al., 2002)

Type I (T1) copper confers the typical blue colour to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place.

Multiple sequence alignments of more than 100 laccases resulted in identification of four ungapped sequence regions, L1–L4, as the overall signature of laccases, distinguishing them within the broader class of multi-copper oxidases (Kumar et al., 2003). The amino acid ligands of the trinuclear cluster are the eight histines, which occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the T1 copper while each of the histidines is bound to one of the two type 3 copper. Intra-protein homologies between signatures L1 and L3 and between L2 and L4 suggested the occurrence of duplication events (Claus, 2004). In the structure of RIL, the mononuclear Cu1 center was trigonally planar coordinated by two ND atoms from His457 and His396 and by a SG atom of Cys452, with coordination distances similar to those observed in other fungal laccases (Garavaglia et al., 2004). Type-I copper center
usually has an additional axial ligand provided by Leu or Phe. In RIL, Leu462 occupies such a position with its CD2 atom at 3.76 Å from Cu1, which confirmed that no additional axial ligand for type-1 copper was present in laccase. Cu1 lies therefore within the plane formed by the two nitrogen atoms and the sulfur ligands. The trinuclear copper center of RIL lies between domains 1 and 3 at about 13 Å from the protein surface. The distance between the two T3 copper ions observed in RIL, appears to be longer than usually observed in other blue multi-copper oxidases. In RIL the two T3 copper ions are 5.1 Å apart, about 1 Å more than in Tvl (Piontek et al., 2002) and AO (Messerschmidt et al., 1992). RIL was a high E° enzyme, showing a redox potential of 730 mV (Bonomo et al., 1998). When comparing the coordination distances for the type-1 copper center among different laccases, it was noted that RIL showed the longest value for the Cu1–His distance (2.20 Å) (Garavaglia et al., 2004). Hence the authors concluded that the distance between the type-1 copper and the coordinating nitrogen atoms is a key factor for the modulation of the redox potential in laccases.

Recently, Hakulinen et al. (2006) have investigated the effect of X-ray radiation upon the crystal structure of a recombinant laccase from Melanocarpus albomyces through the use of crystallography and crystal absorption spectroscopy. It has been reported that the trinuclear site has an elongated electron density amidst coppers, suggesting dioxygen binding. Two crystal structures of rMaL were refined at 2.0 Å resolution from data sets collected rapidly (1.5 h) and slowly (8 h) with synchrotron radiation. At the trinuclear site, several differences between the low, and the high-dose structures were observed. The distance of type-3 coppers from each other was 4.7 and 4.8–4.9 Å for the low, and the high-dose structures, respectively. In addition, the distance of the opposite chloride ligand of the T2 copper was 2.5 Å in the low-dose structure but clearly longer in the high-dose structure, approximately 2.9 Å. The authors have concluded that X-ray radiation could alter the active site of laccase from M. albomyces. The spectral studies showed no band at 590 nm due to the reduction of T1 copper during the long X-ray measurements and the colour of the crystal changed from blue to colourless. Structural comparison between CueO from E. coli and three typical fungal laccases
revealed an obvious difference in the substrate binding pocket. In fungal laccases, the substrate binding pockets are fully open and type I copper is exposed to solvent. However in CueO, an additional α-helix from Leu351 to Gly378 is located over type I copper and makes the substrate binding pocket smaller. The in vitro laccase substrates such as ABTS might not be preferred by CueO due to this extra α-helix (Li et al., 2007). Several different types of recombinant CueO (rCueO) have also been expressed. The active site of engineered CueO of *E. coli* (Δα5–7 CueO) has been reported to include a bridging group between type III Cu atoms. In the case of Δα5–7 CueO, an oxygen atom has been satisfactorily refined as a bridging species with an acceptable temperature factor at the trinuclear Cu center (Kataoka et al., 2007). On the other hand, a Cl− ion bridged between type III Cu atoms in recombinant CueO (rCueO), although the OH−-bridged form of rCueO has been reported very recently (Li et al., 2007).

1.3.3.3.6. Mode of action

*Direct oxidation*

Substrate oxidation by laccase is a one-electron reaction generating a free radical. As one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen, the reaction mechanism cannot be straightforward (Thurston, 1994). The Cu atoms arranged at three different sites play an essential role in the catalytic mechanism of laccase. There are three major steps in laccase catalysis. The first step of the catalytic cycle involves the formation of a fully reduced laccase in which all four coppers are in a reduced state. Molecular oxygen then oxidizes the fully reduced laccase, presumably via a peroxy intermediate, and is reduced to water. (Shin et al., 1996; Solomon et al., 1996; Lee et al., 2002). In a typical oxidation of a substrate by laccase, there is a one-electron reaction that generates a free radical which is typically unstable. The free radical has several options for further reaction. A second enzyme-catalyzed oxidation may take place which converts a phenol to a quinone or it may undergo a non-enzymatic reaction such as hydration, disproportionation or polymerization to produce amorphous insoluble
melanin-like products (Thurston, 1994). Molecular oxygen acts as the electron acceptor to remove protons from the phenolic hydroxyl groups. This reaction gives rise to phenoxy radicals that can spontaneously rearrange, which can lead to fission of carbon-carbon or carbon-oxygen bonds of the alkyl side chains, or to cleavage of aromatic rings (Marzulla et al., 1995; Salas et al., 1995).

![Diagram of a laccase catalytic cycle](image)

**Fig. 1.4. Schematic representation of a laccase catalytic cycle**

(Riva, 2006)

In the reaction two molecules of water from the reduction of one molecule of molecular oxygen and the concomitant oxidation (at the T1 copper site) of four substrate molecules to the corresponding radicals.
The suitability of a chemical compound as a laccase substrate depends on two factors. Firstly, the substrate must dock at the T1 copper site, which is mainly determined by the nature and position of substituents on the phenolic ring of the substrate, especially those with bulky side chains (Xu 1996; Bertrand et al., 2002). Secondly, the redox potential (\(E^0\)) of the substrate must be low enough, because the rate of a laccase catalyzed reaction has been shown to depend on the difference between the redox potentials of the enzyme and the substrate (Xu et al. 2000; Xu et al., 2001). The redox potential of the substrate is determined by its chemical structure and nature of the substituents. Different substituents have different impact on \(E^0\) depending on their ability to withdraw or donate electrons. Methoxy substituents are electron donating and increase the electron density at the phenoxy group, thus making it more readily oxidized (Xu 1996, Garzillo et al., 1998).

**Laccase mediator system**

There are instances in which the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called ‘chemical mediators’, which are suitable compounds that act as intermediate substrates for the laccase, (Fig. 1.5) whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate targets (Riva, 2006). Approximately 100 different potential mediator compounds have been described for the LMS, but ABTS and HBT (1-Hydroxybenzotriazole) remain the most commonly used (Bourbonnais et al., 1995; Bourbonnais et al., 1997; Johannes and Majcherczyk, 2000). Synthetic mediating substrates are heterocyclic compounds belonging to the general classes of phenoxazinones, phenothiazines or phenoxybenzothiazoles (Eggert et al., 1996). Natural mediators include phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol. The use of natural mediators proved to be as efficient as the commonly used ABTS and HBT (Johannes and Majcherczyk, 2000).
Activity of laccase-mediating substrate systems towards compounds depends on a combination of two main factors: the redox potential of the enzyme and the stability and reactivity of the radical generated by oxidation of the mediating substrate (Bourbonnais et al., 1998). Several hypotheses have been proposed for the mechanism of mediating substrate systems. The first hypothesis is that the mediating substrate can act as a redox mediating substrate, i.e., reversible. It is thought that laccase oxidizes the mediating substrate and this oxidized form of the mediating substrate can oxidize the substrate, and is consequently reduced back to its non-oxidized form: the species responsible for the oxidation of the substrate would be the oxidized mediating substrate (Li et al., 1998). Another hypothesis is that active intermediates are generated during the oxidation of the mediating substrate by laccase. These intermediates can abstract hydrogen. In such a reaction the mediating substrate would be continuously consumed (Li et al., 1998) and not be reversible. According to the results of the study performed by Li et al. (1998) the first hypothesis is more probable.

Fig. 1.5. Schematic representation of role of mediators in laccase catalyzed reactions (Fabbrini et al., 2002)

One of the first reports of a mediated system was by Bourbonnais and Paice (1990) using the laccase from *T. versicolor*. It was shown that laccase was able to cleave a nonphenolic dimer in the presence of ABTS. The presence of the mediating substrate, ABTS, prevented and reversed the polymerization of kraft lignin by *T. versicolor* laccase (Bourbonnais et al., 1995). The oxidation of ABTS
by laccase produces the stable dark green cation radical, ABTS+ and then the
dication (ABTS2+) is formed. Both oxidized species were relatively stable and the
reactions are highly reversible. It was observed that the dication was the
intermediate responsible for the oxidation of non-phenolic compounds such as
veratryl alcohol. The cation radical was shown to react only with the phenolic
structures. *T. versicolor* laccase has a redox potential of 585 mV. This is 300 mV
below the redox potential of ABTS+/ABTS2+ couple. Laccase can slowly oxidize
ABTS to ABTS2+ provided that the reaction is driven forward by the subsequent
reaction of the dication with veratryl alcohol or some other compound
(Bourbonnais et al., 1998). It is clear that the enzyme must produce ABTS2+ for the
oxidation of nonphenolic compounds to occur but oxidation by ABTS+ is limited to
phenolic compounds.

HBT is oxidised by laccase to form a nitroxide cation radical (Call and
Mucke, 1994; Bourbonnais et al., 1997). The nitroxy radical intermediate was not
stable and decayed rapidly but was shown to catalyze the oxidation of veratryl
alcohol to veratraldehyde. The nitroxy radical formed when HBT is oxidized by
laccase is a potent electrophile that easily abstracts hydrogen. So it is clear that
mediating substrates work to increase the substrate range of laccase by increasing
the difference in redox potential. The laccase/HBT system has given good results in
trials done for the bleaching of pulp and has the ability to oxidise the nonphenolic
β-O-4- linked subunits that are predominant in lignin as well as β-1 linked dimers
(Bourbonnais et al., 1997, Srebotnik and Hammel, 2000; Xu et al., 1997; Ander
and Messner, 1998). This mediator however has been reported as unable of acting
as a recyclable mediator (Li et al., 1998). Delignification by the laccase/HBT
system is not fully understood but as in the case of ABTS, HBT is small enough to
access lignin.
1.3.3.3.7. Properties of laccases

**pH and temperature**

The catalytic performance of laccases is greatly influenced by their activity and stability at different pH and temperature conditions. The pH activity profiles of laccases are often bell shaped, with optima around 4-6, when measured with phenolic substrates (Palmieri et al., 1993; Eggert et al., 1996, Xu 1997; Chefetz et al., 1998; Garzillo et al., 2001). The variation in pH optima may be due to changes to the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997). The decrease in laccase activity in neutral and alkaline pH values is affected by increasing hydroxide anion inhibition, because as a small anion, hydroxide ion is also a laccase inhibitor (Xu, 1997). The hydroxide anion (OH\(^-\)) binds to the T2/T3 coppers and results in the inhibition of the laccase activity due to the disruption of the internal electron transfer between the T1 and T2/T3 centres. On the other hand, the increasing pH decreases the redox potential of the phenolic substrate, which makes the substrate more susceptible to oxidation by laccase (Xu, 1997). The bell-shaped pH profile is thus the result of two opposite effects: increasing ΔE_o [laccase-substrate] and inhibition by hydroxide anion. In contrast to their activity, the stability of laccases is generally highest at pH values around 8-9 (Nishizawa et al., 1995; Xu et al., 1996; Chefetz et al., 1998). Saito et al (2003) has reported that laccase of a fungus belonging to the family Chaetomiaceae was stable at pH 4.5-9.

The optimum temperature of laccases usually ranges between 30-60 °C. Laccase from *T. versicolor* has been reported to have a temperature optima at 60 °C (Call and Mucke, 1997) while the laccase from the actinomycete strain, *S. cyaneus* has a temperature optima of 70 °C. Temperature stabilities of laccases vary considerably, depending on the source organism. In general, laccases are stable at 30-50 °C and rapidly lose activity at temperatures above 60 °C (Xu et al., 1996, Heinzkill et al., 1998; Schneider et al., 1999, Palonen et al., 2003). The most thermostable laccases have been isolated from bacteria; the half-life of *Streptomyces lavendulae* laccase was 100 min at 70 °C (Suzuki et al., 2003) and
that of *Bacillus subtilis* CotA was 112 min at 80 °C (Martins et al., 2002). The laccases isolated from a strain of *Marasmius quercophilus* (Farnet et al., 2000) were found to be stable for 1 h at 60 °C.

**Substrate specificity**

The low substrate specificity is one of the most important characters that have made laccase useful in varying industrial applications. Laccase is an important oxidant for aromatic rings substituted with electron donating groups, such as phenolics and aromatic amines. These are the preferred electron rich substrates of laccase. There is some difficulty in defining laccase by its reducing substrate. Laccase has an overlapping substrate range with tyrosinase, another type of Cu-containing oxidase, but laccase does not oxidize tyrosine itself (Thurston, 1994). Thus laccases are non-specific regarding to their reducing substrate, and the range of substrates oxidized varies from one laccase to another (Thurston, 1994).

The reducing substrate spectrum for laccase is diverse as long as the redox potentials are not too high (>1V) (Gianfreda et al., 1999). While laccase has low specificity for its reducing substrates, it has a strong preference for its oxidizing substrate, O2 (Gianfreda et al., 1999). Thus laccase can oxidize o- and p-diphenols, aminophenols, methoxyphenols, polyphenols, polyamines, lignin, some organic ions, aryl diamines and a considerable range of other compounds (Thurston, 1994; Call and Mucke, 1997; Gianfreda et al., 1999). In general, laccases show more affinity towards p-diphenols than the o-diphenols and very less reactivity has been observed generally with the meta-substituted phenols (Jolivalt et al., 1999).

**Carbohydrate content**

All laccases characterized to date are glycoproteins and this characteristic imparts resilient properties to laccases. The growth conditions of the organism can have a marked effect on the amount of glycosylation, as seen in a study of chloroperoxidase (Pickard and Hashimoto, 1988). The total carbohydrate content of
<table>
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<td>6.5</td>
<td>50</td>
<td>3.0</td>
<td>55</td>
<td>7.4</td>
<td>Guaiacol</td>
<td>0.18</td>
<td>Okamoto et al., 2000</td>
</tr>
<tr>
<td>S. cyaneus</td>
<td>4.5</td>
<td>70</td>
<td>5.6</td>
<td>75</td>
<td>NA</td>
<td>ABTS</td>
<td>0.38</td>
<td>Arias et al., 2003</td>
</tr>
<tr>
<td>S. griseus</td>
<td>6.5</td>
<td>40</td>
<td>5.3</td>
<td>114</td>
<td>NA</td>
<td>DMP</td>
<td>0.42</td>
<td>Endo et al., 2003</td>
</tr>
<tr>
<td>S. lavendulae</td>
<td>4.5</td>
<td>50</td>
<td>NA</td>
<td>73</td>
<td>NA</td>
<td>Catechol</td>
<td>0.04</td>
<td>Suzuki et al., 2003</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>3.0</td>
<td>75</td>
<td>7.7</td>
<td>65</td>
<td>NA</td>
<td>ABTS</td>
<td>0.10</td>
<td>Martins et al., 2002</td>
</tr>
</tbody>
</table>

NA – Information not available

Table 1.3. Properties of laccases from different organisms
Laccases usually vary from 1% to 15% depending on the source of the enzyme. *P. eryngii* has two isoenzymes with carbohydrate content of 1% and 7% (Munoz et al., 1997). *C. subvermispora* laccases have an approximate sugar content of 15% and 10% for L1 and L2 isoenzymes respectively (Fukushima and Kirk, 1995). *C. zonatus* laccase has been reported to have 10% carbohydrate per mole of protein (Koroljova et al., 1999).

Relative glycosylation levels can impart different properties to enzymes. It has been demonstrated that the carbohydrate moiety of the laccase of *T. versicolor* imparts resistance to proteolytic attack and elevated temperatures (Yoshitake et al., 1993). The resistance to proteolytic attack can be attributed to the specificity of proteases as it will specifically cleave the peptide bonds in the primary structure but the carbohydrate can obscure the peptide bonds, thereby conferring resistance. The carbohydrate can also confer thermophilic stability by additional hydrogen bonding between the carbohydrate portion and the protein. Some of the important properties of laccases from different organisms are summarized in Table 1.3.

### 1.3.3.8. Applications of laccases

Laccases have received much attention from researchers in last decades due to their ability to oxidise both phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes (Couto and Herrera, 2006). The important applications of laccases include detoxification of dye containing industrial effluents from textile and leather industries (Wong and Yu, 1999; Couto 2007), pulp biobleaching (Arias et al., 2003), use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics (Couto and Herrera, 2006). Laccases also find application in food industry that includes beverage (wine, fruit juice and beer).
processing, sugar beet pectin gelation, baking etc (Minussi et al., 2002). The major sectors where laccases are being used are as follows:

**Dye decolourization**

The application of laccases in dye decolourization has increased in recent years and many studies have been demonstrated for dye decolourization using both crude and purified forms of laccase. Some of the chemicals serving as redox mediators facilitate the dye degrading activity of laccase and enhance its specificity to wide range of dyes. Laccases have been used for the decolourization of dyes belonging to different categories such as azo, anthraquinone, heterocyclic, triphenylmethane dyes etc. Laccases have been extensively studied for their degradation of azo dyes (Zille ct al., 2005). Chivukula and Renganathan (1995) have reported the oxidation of phenolic azo dyes by the laccase from *Pyricularia oryzae*. Laccase from *Thelephora* sp. has been reported to decolourize azo dyes at different rates. The enzyme decolourized 19% of orange G, 12.0% of congo red and 15.0 % of amido black 10B (Selvam et al., 2003). Purified laccase from *Trametes hirsute* has been reported as capable of degrading triarylmethane, indigoid, azo, and anthraquinonic dyes. Anthraquinonic dyes and indigo carmine (Acid Blue 74) were degraded more than twofold faster than the azo dyes by the purified laccase (Abadulla et al., 2000). Nyanhongo ct al (Nyanhongo et al., 2002) have screened the ability of laccase from different *Trametes* sp to decolorize eight synthetic dyes (anthraquinone, azo, indigo and triarylmethane). All the tested dyes were decolorized by the laccase from *T. modesta* most efficiently under acid conditions (pH 3–6) but the optimum pH for decolorization of the individual dye varied. The four crude fungal laccases differed remarkably in their decolorization efficiency of eight different synthetic textile dyes. Specificity of *T. hirsuta* laccase towards different dye structures has been investigated by Couto et al., 2006. The enzyme decolourized dyes at different rates and 83 % decolourization of the azo dye methyl orange was attained with the enzyme. In another study, Cameselle et al., 2003 have observed that the azo dye methyl orange was easily degraded than the triphenyl methane dye bromophenol
blue. This behaviour was explained in terms of the structure and size of each dye, the first dyes to degrade are the ones with a low number of aromatic rings. Laccases immobilized on different supports have also been implemented in dye decolourization. Zamora et al (2003) have reported the decolourization of different reactive dyes by immobilized laccase. Laccase from *Sclerotium rolfsii* immobilized on alumina pellets has been employed for the decolourization of wool azo dye Diamond Black PV 200 (Ryan et al., 2003). The decolourization of the commonly used anthroquinone dye RBBR by laccases has also been cited (Hou et al., 2004).

**Pulp biobleaching**

The major problem encountered in the pulping process is the characteristic brown colour of the pulp due to the presence of residual lignin. The chemical processes are usually employed in the industries for the removal of lignin and hemicellulose from the pulp thereby the brightness of the pulp is achieved. Although the chemical treatments are effective method to achieve this task, the high cost and related pollution harms make them unattractive. Hence, there arises the necessity for alternate methods of pulp bleaching. Biobleaching can be defined as an ecofriendly treatment of pulp that involves either microorganisms or their enzymes. This process helps in the selective removal of lignin and hemicellulose components without degrading cellulose. Xylanases are the most widely used enzyme for biobleaching however, the role of laccases in the process has also been elucidated in the recent years.

The use of laccase mediator system along with chemical treatments has also been suggested for biobleaching. Sealey and Ragauskas (1998) have demonstrated that the laccase/n-hydroxybenzotriazole bleaching system can very effectively remove lignin from kraft pulps via a series of oxidative degradation reactions. Balakshin et al (2001) have studied the biobleaching of pulp with dioxygen in laccase-mediator system. The authors have concluded that rate of pulp delignification in the laccase mediated system is determined by the oxidation of a mediator with laccase or/and by the reaction of the oxidized mediator with the
residual lignin in pulp. Laccases from three different white rot fungi; *P. cinnabarinus*, *T. versicolor* and *Pleurotus eryngii* have been reported in the efficient bleaching of high-quality flax pulp in a totally-chlorine-free (TCF) sequence that involved laccase-mediation system. The treatment resulted in high brightness and low lignin content (Camarero et al., 2004).

The laccase from *S. cyaneus* has been employed for the biobleaching of eucalyptus kraft pulp in the presence of ABTS and it has been reported by the authors that the process has increased the brightness of the pulp by 2.2% (Arias et al., 2003). The potential of thermostable laccase from the fungus *P. cinnabarinus* along with the chemical mediator (1-hydroxybenzotriazole) to improve totally chlorine-free (TCF) bleaching of *Eucalyptus globulus* kraft pulps has been investigated by Ibarra et al (2006). The authors have claimed that the new TCF sequence including the laccase stage permitted to improve eucalypt pulp delignification to values around kappa 5 compared to kappa 7 using only TCF chemical reagents. In a similar way, the final brightness obtained, over 91 % ISO, was 3–4 points higher than that obtained in the chemical sequences. The studies by different groups prove that laccase-mediator system is an efficient alternative for chemical methods involved in pulp bleaching. A recent study by Camarero et al (2007) has established the potent role of natural laccase mediators in pulp biobleaching. The authors have claimed that the natural mediators such as acetosyringone and syringaldehyde enabled over 15% increase of final brightness and a decrease of final kappa number similar to that obtained by synthetic laccase mediators.

**Food industry**

In the food industry laccase finds application in different processes like fruit juice processing, wine stabilization, sugar beet pectin gelation, baking and in improving food sensory parameters (Minussi et al., 2002). Wine stabilization is one of the main applications of laccase in the food industry (Minussi et al., 1999). The color and taste of the wines depend particularly on the phenolic compounds present in
different types of wines (Brenna & Bianchi, 1994). The polyphenols present in musts and wines should be selectively removed to prevent any alterations in taste and colour imparted mainly by the oxidation of polyphenols. Different methods have been used in order to prevent the decolorization and flavor alteration in wines, such as the removal of phenolic groups with polyvinylpolypyrrolidone (PVPP), and the use of sulfur dioxide to block oxidizers, among others (Minussi et al., 2002). An alternative for the physicalchemical adsorbents could be the use of enzymes that selectively target specific polyphenols during the madeirization process. These polyphenolic substances would be oxidized by the enzyme, polymerized and then removed by clarification (Zamorani, 1989). One enzyme studied for this purpose is laccase (Cantarelli, 1986). There are several studies in the literature which states that laccase treatment promotes wine stabilization (Cantarelli & Giovanelli, 1990; Plank and Zent, 1993; Servili et al., 2000). According to Cantarelli (1986), mutant laccase from *Polyporus versicolor* (optimum pH 2.7) eliminated up to 70% catechin and 90% of anthocyanidins in a model solution in 3 h of treatment. The studies by Maier et al (1990) have shown that wines made by laccase treatment were the best, suggesting that a stable and high quality wine can be made with little or no added SO₂. The feasibility of using laccase for phenol removal from white must have also been suggested (Minussi et al., 2007).

It is well known that browning, both enzymatic and chemical, is one of the major faults in beverages (Giovanelli and Ravasini, 1993). Various pre- and post treatments are available to avoid post-turbidity and discoloration of fruit juices. Various enzymatic treatments have been proposed for fruit juice stabilization, including the use of laccase (Piacquadio et al., 1998). Stutz (1993) proved that is possible to produce clear and stable juices/ concentrates with a light colour by means of ultrafiltration and laccase, without any large additional investment. Ritter and Dietrich (1996) and Piacquadio et al (1998) have reported that the use of laccase improves stability in apple juice. There is a considerable interest in the food industry for finding new functional ingredients. Sugar beet pectin is a food ingredient with specific functional properties. It may form gels by an oxidative cross-linking of ferulic acid (Norsker et al., 2000). Micard and Thibault (1999)
showed that it is possible to crosslink the beet pectin through the oxidative coupling of the feruloyl groups using laccase. In the baking industry, the use of laccase results in an increased volume, an improved crumb structure and softness of the baked product, as well as increased strength, stability and reduced stickiness and thereby improved machinability of the dough. The effect on the dough has been found to be particularly good when poor quality flour has been used (Minussi et al., 2002).

**Phenol removal**

Removal of phenols from industrial aqueous effluents is an important practical problem, because virtually all phenols are toxic and their presence in a number of industrial waste waters is a health hazard. The use of free laccase, tyrosinase and peroxidase, which catalyse the oxidative coupling of phenol compounds resulting in the formation of water insoluble oligomeric and polymeric products which are then removed by sedimentation or filtration, has been proposed (Shuttleworth and Bollag, 1986). The use of enzymes has been suggested as an alternative method for other physico-chemical methods that suffer from serious drawbacks such as high cost and formation of hazardous by-products (Atlow et al., 1984).

The efficiency of laccases in the degradation of individual phenolic compounds as well as phenolic mixtures has been proved (Lante et al., 2000; Krastanov, 2000). The decolourization of phenolic effluents from different industrial sources has been reported (Davis and Burns, 1990). The effect of reactive co-substrates such as guaiacol and 2, 6-dimethoxyphenol on the removal of chlorinated phenols by horseradish peroxidase (HRP) and a laccase from the fungus *Trametes versicolor* has been investigated by Roper et al. (1995). They found that the addition of co-substrates will enhance the precipitation of phenols by laccase. Annibale et al. (2000) have reported the efficiency of an immobilized laccase from *Lentinula edodes* in removing the phenolics from olive mill waste water. The authors have reported that the laccase immobilized on Eupergit C was able to remove the phenols efficiently.
Aromatic compound degradation

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that consist of fused aromatic rings and do not contain heteroatoms or carry substituents. These compounds can be point source pollutants (e.g. oil spill) or non-point source (e.g. atmospheric deposition) and are one of the most widespread organic pollutants. Some of them are known or suspected carcinogens, and are linked to other health problems. The degradation of PAHs are of great significance in maintaining a proper ecosystem for human life. The laccase–mediator systems have been applied for the degradation of environmental xenobiotics such as polycyclic aromatic hydrocarbons (PAHs). Oxidation of anthracene and benzo[a]pyrene by laccase from *Trametes versicolor* has been reported by Collins et al (1996). The oxidation of five polycyclic aromatic hydrocarbons; anthracene, benzo(a)pyrene, fluoranthene, phenanthrene and pyrene by laccase from *Coriolus hirsutus* in the presence of the redox mediators has been suggested by Cho et al (2002). The authors have reported 40 % degradation of the least oxidizable PAH, pyrene by laccase within 1 h. The ability of laccase from *Pleurotus ostreatus* to degrade different PAHs has been investigated by Pozdnyakova et al (2006). The PAH degradation by the enzyme differed with the type of the aromatic compound as well as with the mediator used in the study (Pozdnyakova et al., 2006).

Denim washing

Denim washing includes one of the new areas of application for laccases. Cellulase enzymes are usually used in denim garment processing to get stone wash look on to the denim garments. Laccases are new generation enzymes for the finishing of denim jeans. Laccase can be used for bleaching delicate denim fabrics as well as for making fashionable effects without compromising the strength of the fabric. Campos et al. (2001) reported the degradation of indigo both in effluents and on fabrics using purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii* in combination with redox-mediators and reported that bleaching of fabrics by the
laccases correlated with the release of indigo degradation products. Pazarlogliu et al (2005) showed that a phenol-induced laccase from *Trametes versicolor* was an effective agent for stonewashing effects of denim fabric without using a mediator. Moreover, they found that *T. versicolor* laccase without a mediator was more effective than commercial laccase.

**Cosmetics industry**

Laccases find application even in production of cosmetics. Laccase-based hair dyes are less irritant and easier to handle than current hair dyes, since laccases replace H₂O₂ as an oxidizing agent in the dye formulation (Roure et al., 1992; Aaslyng et al., 1996; Lang and Cotteret, 1999). More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (Golz-Berner et al., 2004).

**Biosensors**

A biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component. The function of a biosensor depends on the biochemical specificity of the biologically active material. Enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. A number of biosensors containing laccase have been developed for different purposes such as immunoassays (Bauer et al., 1999), glucose determination (Wollenberger et al., 1986), aromatic amines (Simkus and Laurinavicius, 1995) and phenolic compound determinations (Freire et al., 2002; Vianello et al., 2004; Jegan Roy et al., 2005).
1.4. CONCLUSION

The growing concern about the increasing environmental pollution has led the scientific community to reorient their research attitudes. The term 'eco-friendly' has received much attention in the recent years and strict regulations have been made to assure that the new technologies should be environmental friendly. It is high time to consider effective and harmless methods for pollution abatement too. The use of biological methods is a promising alternate to the harsh physico-chemical methods usually employed for many of the industrial purposes, including pollution abatement. The use of microorganisms and their enzymes; especially the oxidative enzymes like laccases are nowadays widely used for these purposes and the success of these processes keeps the researches on characterization of enzymes from newly identified organisms at a great pace.

1.5. OBJECTIVES AND SCOPE OF THE PRESENT STUDY

Scope of the present study was confined to the following objectives

- Isolation and screening of actinomycete cultures for the production of lignin degrading enzymes
- Identification of the selected strain
- Optimization of cultural and nutitional parameters for laccase production in submerged fermentation (conventional and statistical methods)
- Optimization of process parameters for laccase production under solid-state fermentation (SSF)
- Enhanced laccase production in SSF using inducers and packed bed bioreactors
- Purification and characterization of laccase
- Application studies on dye degradation and phenol degradation using the selected strain and laccase
- Use of cell immobilization and enzyme immobilization technologies for application studies.