CHAPTER-2

REVIEW OF LITERATURE

Lignin is the second most abundant renewable biopolymer in nature after cellulose. Though it comprises about 30% of plant material on average basis, but its distribution varies from one plant to another plant. For instance, distribution of lignin in plants i.e. angiosperms, gymnosperms and monocotyledons vary. Angiosperms contain 18-25% lignin on a dry weight basis, gymnosperms and monocotyledons possess 25-35% and 10-30% lignin, respectively (Grisebatch, 1981). It is found in cell walls of various types of supporting and conducting tissue, notably the tracheids and vessel elements of the xylem. It is an essential part of the plant cell wall, imparting rigidity. The mechanical rigidity of lignin strengthens stems and vascular tissue thereby allowing upward growth, permitting water and minerals to be conducted through the xylem under negative pressure without collapse of the tissue (Griesbatch, 1981). Because lignin is such a key component of water transport tissue, the ability to make lignin must have been one of the most important adaptations permitting primitive plants to colonize dry land. It is deposited chiefly in the thickened cell wall but can also occur in the primary wall and the middle lamella in the close contact with the cellulose and hemicellulose already present. The bulk of the lignin is in the thick secondary cell walls, but highest lignin concentrations are in
the middle lamellae (intercellular regions), where the lignin cements the plant cells together, thereby providing rigidity and strength to the plant.

2.1. Chemistry of lignin

Lignin is synthesized from phenyl propanoid precursors by polymerization in higher plants. The lignin precursors p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consist of an aromatic ring and a 3-carbon side chain (Fig.1). Lignin biogenesis involves free radical polymerization of the precursors, p-coumaryl, coniferyl, and sinapyl alcohols which give rise 3 types of subunits: p-hydroxyphenyl- (H-type), guaiacyl- (G-type) and syringyl subunits (S-type) in the lignin molecule. The relative proportions of these subunits in lignin vary depending on the type of plant and tissue. Lignins differ mainly in the proportion of the three subunits. Softwood lignins (gymnosperms, conifers such as spruce, cedar and hemlock) are made up of approximately 80% guaiacyl subunits, 14% p-hydroxyphenyl subunits and 6% syringyl subunits. In contrast, hardwood lignins (angiosperms such as poplar, willow, birch and alder) are composed of 56% guaiacyl subunits, 4% p-hydroxyphenyl subunits and 40% syringyl subunits. Grass (monocots) lignins are rich in p-hydroxyphenyl subunits. The elucidation of its chemical structure has been a formidable task that has taken almost 140 years of effort. Plant peroxidases catalyze the one-electron oxidation of these precursors to generate phenoxy radical intermediates, which diffuse away from the enzyme and couple with each other and the growing lignin polymer. This random coupling generates oligomeric quinone
methides susceptible to nucleophilic attack at the benzyl carbons by water, phenolic hydroxyls of other lignols, and also by the hydroxyls of hemicellulose to form lignin-carbohydrate complexes. Several structural models of lignin have been presented (Adler, 1977; Brunow, 2001). Lignin is a complex, three-dimensional, non-stereoregular polymer composed of phenylpropanoid units linked through several major types of carbon-carbon and ether bonds. The interunit bonds are characterized by the points of attachment: the standard nomenclature designates positions on the propyl side chains as α β γ (being proximal to the aromatic ring) and positions on the aromatic ring as 1-6 (1 indicating the point of attachment of the propyl side chain). Thus, the β-0-4 ether bond is quantitatively the most important interunit bond in spruce lignin (Fig.1).

**Fig.1.** Schematic structural formula for lignin, adapted from Adler (1977).
The structure illustrates major interunit linkages and other features described in the text; it is not a quantitatively accurate depiction of the various substructures. The three precursor alcohols are shown at the lower right; their polymerization, following one-electron oxidation, produces lignin.

A typical finding for the lignin polymer is that there is no single repeating bond between the subunits but a random distribution of at least 10 different types of bonds, the most common being the β-aryl ether (β-O-4) bond (Argyropoulos and Menachem 1997). Lignin synthesis, structure and chemistry have been reviewed by Harkin (1967), Adler (1977), Sarkanen & Ludwig (1971) and Higuchi (1990). Detailed reviews of lignin are found in Adler (1977), Eriksson et al., (1990), Argyropoulos & Menachem (1997) and Kuhad et al., (1997).

2.2. Microbial degradation of lignin

Cellulose and hemicellulose are composed of linear, repeating hydrolyzable interunit bonds and are relatively easily biodegradable whereas lignin has complicated structure with nonhydrolysable bonds and represents a formidable microbial substrate. Further, the lignin polymer encrusts the cellulosic microfibrils of plants and is chemically bonded to the hemicelluloses, making these polysaccharides less accessible to microbial decay. The molecular weight 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. The major challenges faced by a microorganism in its degradation are: Degradation...
system must be extracellular and must be oxidative rather than hydrolytic because the structure is comprised of interunit carbon-carbon and ether bonds. Ligninolytic agent must be less specific because lignin polymer is stereo irregular (Heinzkill & Messner, 1997).

The only organisms reported to degrade lignin efficiently are the white-rot fungi that under natural conditions mostly colonize dead or living wood (Eriksson et al., 1990). White rot fungi constitute a diverse eco-physiological group comprising mostly of basidiomycetes and litter decomposing fungi. Fungi can exploit marginal living conditions in large part because they produce unusual enzymes capable of performing chemically difficult reactions. These fungal enzymes can convert wood, plastic, paints and jet fuel among other materials into nutrients. Some of these enzymes have already been harnessed in pulp and paper processing (biopulping and biobleaching), and in synthesis of fine chemicals (bio-catalysis). The white rot basidiomycetes degrade lignin rapidly and extensively than any other microbial groups. They invade the lumens of wood cells, where they secrete enzymes that degrade lignin and other wood components. The electron microscopic studies showed that lignin is degraded at some distance from the hyphae and is removed progressively from the lumens towards the middle lamella. During its mineralization by white rot fungi, lignin undergoes oxidative changes including aromatic ring cleavage depolymerization with release of low-molecular weight fragments (Ward et al., 2004). They are selective degraders Selective lignin degraders are especially
interesting from the standpoint of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation. Among white rot fungi, *Phanerochaete chrysosporium* has been assessed for its lignolytic potential and well characterized in terms of participation of enzymes in lignin degradation (Farrell *et al*., 1989; Stewart & Cullen, 1999). For this reason *P. chrysosporium* is used as an experimental organism and a reference culture.

2.2.1. Lignolytic system

Lignin degradation by lignin-degrading microbes is an oxidative and non-specific process. The ligninolytic system evolved in white-rot fungi consists of extracellular peroxidases and oxidases that act non-specifically via the generation of lignin free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions. The main extracellular enzymes participating in lignin degradation are Cu-containing laccase (Lac) (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), lignin peroxidase (ligninase) (LiP) (Diarylpropane: oxygen, hydrogen-peroxide oxidoreductase; EC 1.11.1.14) and manganese peroxidase (MnP) [Mn (II): hydrogen peroxide oxidoreductase; EC 1.11.1.13] (Kirk & Farrell, 1987; Thakker *et al*., 1992; Hatakka, 2001). All three families of enzymes, implicated in the biodegradation of lignin, catalyze the one-electron oxidation of phenolic substrates to phenoxy radicals that can undergo certain degradation reactions of lignin (Leonowicz, 1999). These major lignin-degrading
enzymes have a great potential in industrial and bioremedial applications (Wesenberg et al., 2003; D'souza et al., 2006). Lignin-degrading enzymes are finding broad application base in industrial processes owing to the wide range of chemical reactions they can catalyze with relatively clean technology (Rodriguez Couto & Toca Herrera, 2006a). These lignin-degrading enzymes include both specific and non-specific enzymes. Feasible technologies with non-specific enzymes can be developed owing to their wide substrate range. Production of these enzymes from White-rot fungi has been well documented. The use of enzymes for the treatment or the removal of environmental and industrial pollutants has attracted increasing attention because of their high efficiency, high selectivity, and environmentally benign reactions. Extracellular fungal peroxidases, such as lignin peroxidase, manganese peroxidase and fungal laccases are the two major classes of enzymes that have been evaluated for the removal of toxic phenolic compounds from industrial wastewater and the degradation of recalcitrant xenobiotics (Christian et al., 2005). Focus is currently on the improvements of the production of these enzymes, such as discovery of new fungal strains, modification of growth conditions, use of inducers, and use of cheaper growth substrates such as agricultural and food wastes. The review of literature given below is therefore an account relating to laccases, lignin peroxidases and manganese peroxidases of their production, biochemical characterization and their applications.
2.2.1.1. Laccases (Benzenediol: oxygen oxidoreductase; EC 1.10.3.2)

Laccase (EC 1.10.3.2, p-diphenol oxidase) is one of old enzymes that have been studied since the nineteenth century. Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Levine, 1965; Thurston, 1994). In 1896 laccase was demonstrated to be a fungal enzyme for the first time by both Bertrand and Laborde (Levine, 1965; Thurston, 1994). Laccase is a member of the large blue copper proteins or blue copper oxidases, which comprise a small group of enzymes. Other members of this group include mammalian plasma protein - ceruloplasmin and ascorbate oxidase of plants. Laccases are either mono or multimeric copper-containing oxidases that catalyze the one-electron oxidation of a variety of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (Ducros et al., 1998). The ability of laccases to oxidize phenolic compounds as well as their ability to reduce molecular oxygen to water has led to intensive studies of these enzymes (Thurston, 1994; Xu, 1996).

Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones (Rodriguez Couto & Toca Herrera 2006; Mendonca et al., 2008). Laccases (EC 1.10.3.2) are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases, which oxidize diphenols and related substances, and use molecular oxygen as an electron acceptor (Thurston, 1994; Viswanath et al., 2008a). The biotechnological importance of these enzymes can also be attributed to their substantial retention of activity in organic solvents with applications in
organic synthesis. Laccases have widespread applications, ranging from effluent decolourisation and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents and washing powders, many of which have been patented (Yaver et al., 2001). The biotechnological application of laccase has been expanded by the introduction of laccase-mediator systems - 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) and 1-hydroxybenzotriazole, which are able to oxidize non-phenolic compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps (Bourbonnais & Paice, 1990; Eggert et al., 1996a). Enzymes are becoming an effective tool in industrial processes, from crude applications such as bioremediation to fine processes such as chirally selective biocatalysis (Pedroza et al., 2007; Cabana et al., 2007; Dritsa et al., 2007; Hu et al., 2007).

A novel application field for laccases is in cosmetics. For example, laccase-based hair dyes could be less irritant and easier to handle than current hair dyes (Viswanath et al., 2008). Laccases can generate a desired worn appearance in denim fabrics in textile industry (Viswanath et al., 2008a). Laccases have also potential applications in food industry - fruit juice, wine and beer stabilizing by removing the polyphenols that cause haze formation and discoloration (Minussi et al., 2002, Viswanath et al., 2008a).

One of the major environmental problems, faced by the world today, is the contamination of soil, water, and air by toxic chemicals. With industrialization and the extensive use of pesticides in agriculture, the pollution...
of the environment with mandate organic compounds has become a serious problem. Eighty billion pounds of hazardous organopollutants are produced annually in the United States and only 10% of these are safely disposed (Reddy & Mathew, 2001). Certain hazardous compounds, such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenols (PCP), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2, 2-bis (4-chlorophenyl) ethane (DDT), benzene, toluene, ethyl benzene, xylene (BTEX), and trinitrotoluene (TNT) are persistent in the environment and are known to have carcinogenic and/or mutagenic effects.

Enzymatic treatment is currently considered an alternative method for the removal of toxic xenobiotics from the environment (Dick & Tabatabai, 1993; Karam & Nicell, 1997; Gianfreda et al., 1999). Although laccases are known as diphenol oxidases, monophenols like 2, 6-dimethoxyphenol or guaiacol is used as better substrate than diphenol-catechol or hydroquinone. Laccases are of particular interest because of their capabilities to oxidize a wide range of environment ally dangerous substrates. Greater attention on laccase, an eco-friendly enzyme in recent past is generating information that appeared in a number of reviews (Baldrian, 2006; Riva, 2006; Singh & Chen, 2008) in the last couple of years. In the future laccases may also be of great interest as a green catalyst in synthetic chemistry, where they have been proposed to be applicable for oxidative deprotection and production of complex polymers and medical products.
Many lignolytic fungal species produce constitutively at least one laccase isoenzyme and lignolytic enzymes are dominant in soil environment. Laccases are of interest to researchers in the field of bioremediation as well as environmental pollutants such as endocrine disrupting chemicals, pesticides, herbicides, and certain explosives (Rodriguez Couto & Toca Herrera, 2006a). The capacity of laccases to function effectively in a number of detoxification schemes has been explored, including the degradation of xenobiotics (Christain et al., 2005), the decolorization of dyes (Nagai et al., 2002, 2004; Camarero et al., 2005), and pulp bleaching (Geng et al., 2004, Sigoillot et al., 2004). Laccases are among the main enzymes involved in delignification process of lignocellulosic mass by white-rot fungi for production of ethanol (Mayer & Staples, 2002) and to distinguish between morphine and codeine. Research in recent years has been intense, much of it elicited by the wide variety of laccases, their utility and their very interesting enzymology. Current status of knowledge in this regard is reviewed.

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi (Leontievsky et al., 1997) However, the occurrence of laccases in higher plants appears to be far more limited than in fungi (Mayer & Staples, 2002). Laccases have subsequently been discovered from numerous other plants (Bligny & Douce, 1983; De Marco & Roubelakis-Angelakis, 1997 and Ranocha et al., 1999) but their detection and purification of plant laccases is often difficult because
crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha et al., 1999), plant laccases are found in the xylem, where they presumably oxidize monolignols in the early stages of lignifications (Bao et al., 1993; O'Malley et al., 1993; Mayer & Staples, 2002; Gavnholt & Larsen, 2002) and also participate in the radical-based mechanisms of lignin polymer formation (Hoopes & Dean, 2004). In addition, laccases have been shown to be involved in the first steps of healing in wounded leaves (De Marco & Roubelakis-Angelakis, 1997). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965), as well as in bacteria (Diamantidis et al., 2000). The first bacterial Laccase was detected in the plant root-associated bacterium 'Azospirillium lipoferum' (Givaudan et al., 1993) where it was shown to be involved in melanin formation (Faure et al., 1994).

Among physiological groups of fungi, laccases are typical for the wood-rotting basidiomycetes and a related group of litter-decomposing saprotrophic fungi i.e., the species causing lignin degradation. Agaricus bisporus (Wood, 1980), Botrytis cinerea (Marbach et al., 1984), Coprinus cinereus (Schneider et al., 1999), Phlebia radiata (Niku-paavola et al., 1988; Campoy et al., 2008), Pleurotus ostreatus (Sannia et al., 1986), Stereum ostrea (Viswanath et al., 2008b) Fomitella fraxinea (Park and Park, 2008), Stereum hirsutum (Mouso et al., 2007), Lentinus tigrinus (Ferraroni et al., 2000), and Trametes versicolor (Rogalski et al., 1991) are some examples of basidiomycetes that produce Laccase.
Laccases have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi (Assavanig et al., 1992). In the fungi, Ascomycetes and Deuteromycetes have not been a focus for lignin degradation studies as much as the white-rot Basidiomycetes. Laccase from *Monocillium indicum* was the first laccase to be characterised from an ascomycete showing peroxidative activity (Thakker et al., 1992). Yeasts are a physiologically specific group of both ascomycetes and basidiomycetes. Until now, laccase was only purified from the human pathogen *Cryptococcus* (Filobasidiella) *neoformans*. This yeast produces true Laccase capable of oxidation of phenols and aminophenols and unable to oxidize tyrosine (Williamson, 1994). Additionally, these enzymes can protect fungal pathogens from toxic phytoalexins and tannins, thus they are important virulence factor in many fungal diseases (Mayer & Staples, 2002). In addition to plants, bacteria and fungi, laccases or laccase-like activities have been found in some insects, where they have been suggested to be active in cuticle sclerotization (Sidjanski et al., 1997; Dittmer et al., 2004).

In plants, laccase plays a role in lignification and in fungi, laccases have been implicated to be involved in many cellular processes, including, delignification, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston, 1994; Yaver et al., 2001). Only a few of these functions have been experimentally demonstrated (Eggert et al., 1996). Ligninolytic enzymes have mostly been reported to be extracellular but there is evidence in literature of the occurrence of intracellular laccases in white-rot.
fungi (Schlosser et al., 1997). Intracellular as well as extracellular laccases were identified for *Neurospora crassa* (Froehner & Eriksson, 1974), suggested that the intracellular laccase functioned as a precursor for extracellular laccase as there were no differences between the two laccases other than their occurrence.

Laccases also participate in formation of lignin by polymerization reactions in plants. Fungal laccases contribute to several processes such as lignin degradation, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston 1994, Mayer & Staples, 2002). Laccases are usually the first ligninolytic enzymes secreted to the surrounding media by the fungus. Almost all species of White-rot fungi were reported to produce laccases with different production levels (Hatakka 2001). The majority of laccases characterized so far have been derived especially from White-rot fungi because of their abundance (Gianfreda et al., 1999; Kiiskinen et al., 2004). Laccase plays a role in pigment formation in spores, detoxification of phenol compounds produced during lignin degradation and acts synergistically with other enzymes in the breakdown of lignin. These enzymes have been neglected in the past, probably because they were not commercially available. The search for new, efficient and environmentally benign processes for the textile and pulp and paper industries has increased interest in these enzymes that produce water as the only by-product, making them more generally available to the scientific community. Consequently, a significant number of reports have been published in the past decade that have focused on the biochemical properties of these proteins and/or
on their applications in technological and bioremediation processes in addition to their use in chemical reactions. In addition, many fungal enzymes are capable of breaking down a broad range of complex compounds, making them potentially useful for destruction of persistent pollutants (bioremediation and biodegradation). The typical molecular weight of fungal laccase is 60-80 kDa and the isoelectric point is between pI 3 and pI 4, but variations on these values occur.

2.2.1.1. Reaction mechanism

Laccases catalyze four $1e^-$ oxidation of a reducing substrate with concomitant two $2e^-$ reduction of dioxygen to water (Riva, 2006). The stochiometry is four molecules of reducing substrate for each molecular oxygen, involving a total transfer of four electrons \[4RH+O_2 \rightarrow 4R+2H_2O\].

Laccases have been shown to contain four copper (II) atoms per molecule that are essential for its catalytic activity (Ragusa et al., 2002; Wong, 2008). These four copper (II) atoms can be classified into three groups, type-1, type-2, and type-3 pair (Gianfreda et al., 1999; Ragusa et al., 2002) and are defined in terms of their spectroscopic properties and their electronic potential as determined by and their electron paramagnetic resonance (EPR) pattern (Gianfreda et al., 1999; Ragusa et al., 2002). Type-1 and type-2 copper atoms display strong electronic absorption, and have well defined EPR spectra, while the type-3 pair of copper (II) atoms are strongly coupled and are EPR silent (Ragusa et al., 2002), which may be activated by strong anion binding (Gianfreda et al., 1999). Laccases
generally exhibit two absorption peaks when subjected to a UV-VIS wavelength scan, a strong absorbance is visible at 600 nm and is associated with the type-1 copper, while a shoulder at 330 nm is indicative of the type-3 pair of copper atoms. The occurrences of laccases that do not display this characteristic spectrum have been reported. A "white" laccase was said to be isolated from *Pleurotus ostreatus* (Palmieri *et al.*, 1997), while (Leontievsky *et al.*, 1997) reported the presence of "yellow" laccases. The loss of the absorption peak at 600 nm of the "white" laccase was attributed to the presence of only a single copper atom in the metal cluster, the other three atoms being replaced by two zinc and one iron atom (Palmieri *et al.*, 1997; Leontievsky *et al.*, 1997) attributed the loss of this peak in the case of "yellow" laccases to copper atoms being present in their reduced state.

The reaction mechanism may be described as the reduction of molecular oxygen, by various organic compounds, to water without the step of hydrogen peroxide formation (Yaropolov *et al.*, 1994). Laccases exhibit a high affinity for oxygen as their electron acceptor, however they display a low affinity for their reducing substrates (Fernández-Sánchez *et al.*, 2002). Laccases catalyze the oxidation of mono- and polyphenolic substrates and aromatic amines by the removal of a hydrogen atom from their hydroxyl group or removal of a single electron, to form a free radical. The radicals are susceptible to further oxidation by laccase or they may undergo non-enzymatic reactions such as hydration or polymerization (Yaropolov *et al.*, 1994). Although the exact mechanism of laccase
activity has not been elucidated (Burke and Cairney, 2002), it is believed to comprise three major steps;
(i) type-1 copper reduction by the reducing substrate,
(ii) internal electron transfer from type 1 copper to type 2 and type 3 copper trinuclear cluster,
(iii) molecular oxygen reduction to water at type-2 and type-3 copper atoms (Gianfreda et al., 1999).
The type 2 and 3 copper atoms are thought to be associated in a trinuclear cluster (Gianfreda et al., 1999), at the site where the electrons from the substrate (transferred to the type-1 copper) are transferred to the oxygen molecule (Yaropolov et al., 1994). The fundamental understanding of the catalytic mechanism of laccase has recently received considerable attention and several researchers are currently investigating the interaction of laccase with its substrates through crystallography and X-ray diffraction studies (Ducros et al., 1998; Antorini et al., 2002; Bertrand et al., 2002; Piontek et al., 2002). The four metal ions have been shown to be essential for optimal activity of laccase (Ducros et al., 1998).

2.2.1.2. Lignin peroxidases [1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase EC1.11.1.14]

Lignin peroxidases (LiP) are unusual oxidizing extracellular peroxidases produced by most of the lignolytic fungi that cause white-rot of wood. (Hammel et al., 1986; Kirk & Farrell, 1987; Buswell & Odier, 1987; Tien, 1987). In the
presence of H$_2$O$_2$ they catalyze the one-electron oxidation of a wide variety of aromatics to yield, as initial products, aryl cation radicals that subsequently undergo substituent-dependent reactions of both radical and ionic nature (Kirk & Farrell, 1987; Buswell & Odier, 1987). Lignin peroxidases [1, 2 bis (3, 4-dimethoxyphenyl) propane-1, 3-diol: hydrogen-peroxide -oxidoreductase EC1.11.1.14] catalyze the H$_2$O$_2$-dependent oxidative depolymerization of lignin (Tien & Kirt 1983; Hammel et al., 1993). LiP is relatively nonspecific to its substrates and has been known to oxidize phenolic aromatic substrates and also a variety of non-phenolic lignin model compounds as well as a range of organic compounds with a redox potential up to 1.4 V in the presence of H$_2$O$_2$ (Valli et al., 1990).

Lignin peroxidases play a central role in the initial degradation of the complex aromatic polymer lignin by *P. chrysosporium*. The glycosylated enzyme was identified and described independently by two American teams and a Japanese group (Shimada & Higuchi, 1983) in supernatant of nitrogen and carbon limited cultures of the corticoid basidiomycete *P. chrysosporium* that were flushed with pure oxygen. Lignin peroxidases were first designated as "ligninase". Discovery of lignin peroxidase was based on the H$_2$O$_2$-dependent Cα- Cβ cleavage of lignin model compounds and subsequently shown to catalyze the partial depolymerization of methylated lignin in vitro (Glenn et al. 1983; Tien & Kirk, 1983; Tien & Kirk, 1984). Since the first report of lignin peroxidase, several isozymic forms have been detected with *P. chrysosporium* and
a number of other white-rot fungi. The enzymology of lignin biodegradation by *P. chrysosporium* under lignolytic conditions has been clearly unraveled (Cullen & Kersten 1996).

Lignin peroxidases have also been shown to oxidize various polycyclic aromatic hydrocarbons and related structures, including pyrene, anthracene, benzo[a]pyrene, dibenzo[p]dioxin, and thianthrene (Hammel *et al.*, 1986; Haemmerli *et al.*, 1986; Schreiner, 1988). Certain halogenated aromatics are also oxidized, including 2-chlorodibenzo[p]dioxin (Hammel *et al.*, 1986).

Various isoenzyme forms have been purified and characterised (Gold, 1984; Paszczynski *et al.*, 1986; Farrell *et al.*, 1989). The major studied lignin peroxidase has a pI of 3.5 and has been referred to as H8 (Kirk *et al.*, 1986). A nomenclature scheme has been proposed recently, in which H8 is referred to as LiP1 (Farrell *et al.*, 1989). Lignin peroxidase has also been isolated from the lignin-degrading basidiomycetes *Trametes versicolor* (Jonsson *et al.*, 1987) and *Phlebia radiata* (Kantelinen *et al.*, 1988). The information on lignin peroxidase has been reviewed (Kirk & Farrell, 1987; Wong, 2008). The principal criteria for identifying specific lignin peroxidase isozymes are their isoelectric points (pI) and their order of elution from a Mono Q anion exchange column (Renganathan *et al.*, 1985; Kirk *et al.*, 1986a; Leisola *et al.*, 1987). Ten peroxidases are separated by Mono Q chromatography and are designated H1 through H10. Six of these, H1 (pI 4.7), H2 (pI 4.4), H6 (pI 3.7), H7 (pI 3.6), H8 (pI 3.5), and H10 (pI 3.3) have veratryl alcohol oxidation activity.
characteristic of lignin peroxidase (Farrell et al., 1989). Analytical isoelectric focusing has resolved 15 proteins with lignin peroxidase activity (Leisola et al., 1987). Growth conditions (e.g., N vs. C starved), purification methods, and storage can affect relative isozymic levels.

Lignin peroxidase comprises of heme in the active site called heme-containing peroxidases and its molecular mass varies between 38 and 47 kDa. It catalyses several oxidations in the alkyl side chains of lignin related compounds (Tien & Kirk, 1988), C-C cleavages in the side chains of lignin subunit, oxidation of veratryl alcohols and related substrates to aldehydes or ketones, intradiol cleavage of phenylglycol structures and hydroxylation of benzylic methylene groups. It is also active against highly methylated lignin (Kent et al., 1987). Lignin peroxidase is also capable of cleaving aromatic rings via one electron substation and subsequent incorporation of oxygen. The optimum activity of lignin peroxidases is extremely low between pH 2.5 and 3.0.

2.2.1.2.1. Reaction mechanism

Enzyme intermediates in the catalytic cycle of lignin peroxidase are analogous to other peroxidases (e.g., horse radish peroxidase); steady state and transient-state kinetics have been studied in detail (Renganathan & Gold, 1986; Andrawis et al., 1988; Marquez et al., 1988; Harvey et al., 1989). H$_2$O$_2$ is the preferred co-substrate and oxidant in the lignin peroxidase-catalyzed reactions. The general mechanism of LiP catalyzed reactions consists of two steps - oxidation and reduction.
The native LiP reacts with $\text{H}_2\text{O}_2$ and generates two electron LiP-I with a second-order rate constant of $5.4 \times 10^5 \text{ M}^{-1} \text{ S}^{-1}$. $\text{H}_2\text{O}_2$ is the preferred substrate. The reaction is independent of pH from 2 to 7.5 (Andrawis & Marquez, 1988). The reason for the lack of pH effect is not clear.

Compound II- The reaction of LiP-I with a reducing substrate to form LiP-II is pH-dependent with the rate decreased with increasing pH (Marquez et al., 1988; Tien et al., 1986), and reduces to one electron deficient LiP II. The subsequent reaction of LiP II with a second molecule of reducing substrate to yield the native enzyme is also pH-dependent (Fig. 2). It is clear that the pH-dependence of the reduction of LiP-I and LiP-II rather than the formation of LiP-I dictates the unusual low pH optimum for the enzyme. LiP is having high redox potential (Kersten et al., 1990).

Fig. 2. Mechanism of direct oxidation of lignin peroxidase
In many cases, chemicals are not directly accessible to heme of LiP and thus direct oxidation does not occur. In such cases involvement of redox mediator plays an important role. Veratry alcohol (VA) produced by white rot fungi is an excellent substrate for LiP, VA serves as an electron mediator to facilitate oxidation of pollutants. VA is oxidized by LiP to VA cation radical (VA^+) which is a strong oxidant responsible for indirect oxidation of lignin and pollutants (Fig. 3).

Fig. 3. Lignin peroxidase catalyzed indirect oxidation

EDTA was found to be indirectly decarboxylated by LiP H2. The apparent inhibition of veratryl alcohol oxidase activity of LiP H2 by EDTA is suggestive of the reduction of (VA^+)back to VA during oxidation of EDTA (Shah et al., 1992).
Chemicals that have been found to be indirectly oxidized by LiP include herbicide aminotriazol (Tuisel et al., 1992), pentachlorophenol (Chung & Aust, 1995) and phenol (Chung & Aust, 1995).

LiP catalyzes reduction of various chemicals in the presence of VA (Fig. 4). The role of VA in lignin degradation has been the subject of several proposals. One suggestion considers VA\(^+\) as a redox mediator in the oxidation of lignin (Harvey et al., 1986). VA\(^+\) is capable of mediating oxidation of secondary substrates typically not oxidized by LiP (Harvey et al., 1986; Koduri, 1995; Goodwin et al., 1995; Tien & Ma, 1997; Christian et al., 2005). VA\(^+\) generated in LiP reaction oxidizes carboxylic acids to respective acid-derived anion radicals, which, in turn, serve as reductant. Such radicals effectively reduce cytochrome C nitro blue tetrazolium, ferric ion and molecular oxygen and are also involved in the reduction of carbon tetrachloride to the trichloromethyl radical which is neither a substrate for enzyme nor a good reductant (Shah et al., 1992).

The mediation model provides a mechanism by which LiP oxidizes the lignin substrate indirectly, with VA\(^+\) diffusing from the enzyme to act as direct oxidant on lignin. Measurement of the life time of VA\(^+\) suggests that it is able to act as a diffusible oxidant (Khindaria et al., 1996; Baciocchi et al., 1998; Baciocchi et al., 2002). Other studies however, suggests that VA\(^+\) is unstable and hence unlikely to effect long-range oxidation in LiP-catalyzed reactions (Joshi et al., 1996).
VA$^+$ oxidizes EDTA as well as oxalate to their corresponding anion radicals. These carboxylate anion radicals, in the absence of another electron acceptor, reduce molecular oxygen to $\text{O}_2^-$ which will reduce ferric iron to ferrous iron (and has been shown to reduce some chemicals). $\text{H}_2\text{O}_2$ then readily reacts with chelated ferrous iron to produce $\cdot\text{OH}$ (Barr et al., 1992) Fig. 4. $\cdot\text{OH}$ is having incredible oxidizing ability and makes up a potential non-enzymatic biological system known as Fenton reagent. Fenton's reaction has been widely used for degradation of xenobiotic compounds including PCBs, herbicides and dyes (Pratap & Lemley, 1998; Nerud et al., 2001), oxidizes EDTA as well as oxalate to their corresponding anion radicals.

![Fig. 4. Lignin peroxidase catalyzed reaction and generation of radicals](image_url)
2.2.1.3. Manganese peroxidases [Mn (II): hydrogen peroxide oxidoreductase; EC 1.11.1.13]

Manganese peroxidases (MnP) (EC 1.11.1.13) are heme-containing peroxidases. The principle function of the enzyme is to oxidize Mn$^{2+}$ to Mn$^{3+}$, using H$_2$O$_2$ as oxidant. Mn (III) is chelated by organic acids (e.g., oxalate or malate in nature). Chelated Mn (III) oxidizes phenolic lignin compounds to phenoxy radicals that degrade spontaneously (Hofrichter, 2002).

Another heme peroxidase was found in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* and required Mn (II) and H$_2$O$_2$ for activity (Kuwahara et al., 1984; Paszczynski et al., 1985). This enzyme was designated as manganese peroxidase. Activity of the enzyme was stimulated by simple organic acids, which stabilized the Mn$^{3+}$, thus producing diffusible oxidizing chelates (Glenn and Gold 1985; Glenn et al., 1986). Recently, physiological levels of oxalate in *P. chrysosporium* cultures have been shown to stimulate manganese peroxidase activity (Kuan & Tien, 1993). The MnPs are distributed in certain other lignin-degrading fungi. In view of nonspecific nature of its action, focus on biochemical properties of MnP has been paid. The molecular weight of extracellular fungal MnPs falls with in a range of 40-50 KDa and the isoelectric point (pI) is usually acidic (pI 3-4), but neutral MnPs have also been found.
MnP oxidizes a wide range of compounds from lignin to polycyclic aromatic hydrocarbons (PAHs) (Steffen, 2003). During recent years there has been a great interest in potential application of MnPs in biopulping and bio bleaching as well as in bioremediation processes (Harazono et al., 1996; Hofrichter et al., 1998; Breen & Singleton, 1999). MnPs is an extracellular heme-containing glycoprotein produced only by lignolytic (wood rotting and litter-degrading) basidiomycetes, especially during the secondary metabolism (Gold et al., 2000). This enzyme catalyzes the \( \text{H}_2\text{O}_2 \) - dependent oxidation of \( \text{Mn}^{2+} \) to a highly reactive \( \text{Mn}^{3+} \). The latter, stabilized by chelating dicarboxylic acids, is a low molecular-mass diffusible mediator, which non-specifically oxidizes a variety of phenolic and non-phenolic substances, including lignin and toxic pollutants (Perez & Jeffries, 1992; Moreira et al., 1997a). The aromatic structures are depolymerized via formation of phenoxy or aryl cation radicals, which finally result in the breakdown of the molecule (Hatakka, 1994).

Due to the important degradative potential of Mn peroxidase, there is general interest in producing the enzyme biotechnologically. An intensive research program was embarked upon to define nutritional, physiological and environmental factors related to the activation of the MnPs system and development of an efficient production system for large-scale operations. Production of MnPs by white-rot fungi is highly regulated by nutrients. In the most-investigated white-rot fungus- \textit{Phanerochaete chrysosporium}, MnPs are
synthesized in response to nitrogen, carbon or sulfur limitation. Particularly, manganese and nutrient nitrogen have been shown to produce strong regulating effects (Jeffries et al., 1981; Bonnarme & Jeffries, 1990; Gold & Alie, 1993).

Additionally environmental factors such as nutrient feed rate, agitation rate, use of oxygen versus air, and fungus immobilization have also been proven to promote MnP synthesis (Moreria et al., 1997b). Immobilization of fungal cells is an attractive technique for accomplishing high cell densities in order to achieve a rapid extra cellular enzyme production. Entrapment is a commonly used immobilization procedure, but surface adsorption has two advantages - namely its simplicity and better physiological functions.

Nematoloma frowardii – the South American agaricus white-rot fungus has been intensively investigated in Germany as a producer of manganese peroxidase and degrader of lignin and humic substances (Hofrichter & Fritsche, 1997; Hofrichter et al., 1999). MnP from this fungus is also capable of mineralizing different aromatic directly, including xenobiotics and polycyclic hydrocarbons (Sack et al., 1997; Hofrichter et al., 1998). These features suggest this organism to be a good candidate for further applications at a larger scale and make it an interesting objective to scale-up lignolytic enzyme production.

2.2.1.3.1. Reaction mechanism

Mn-dependent Peroxidases are unique in utilizing Mn (II) as the reducing substrate (Glenn et al., 1985; Glenn et al., 1986; Faszcynski et al., 1986). MnP oxidizes Mn (II) to Mn (III), which in turn oxidizes a variety of monomeric
phenols including dyes as well as phenolic lignin model compounds. Mn (III) is stabilized by organic acid chelators and acts in turn as a low molecular mass, diffusible redox mediator that attacks organic molecules and oxidizes various chemicals non-specifically via hydrogen and one electron abstraction (Glenn & Gold, 1985). Fig. 5 the catalytic cycle thus entails the oxidation of Mn (II) by compound I (MnP-I) and compound II (MnP-II) to yield Mn (III).

\[
\text{MnP} + \text{H}_2\text{O}_2 \rightarrow \text{MnP-I} + \text{H}_2\text{O}_2 \\
\text{MnP-I} + \text{Mn}^{2+} \rightarrow \text{MnP-II} + \text{Mn}^{3+} \\
\text{MnP-II} + \text{Mn}^{2+} \rightarrow \text{MnP} + \text{Mn}^{3+} + \text{H}_2\text{O}
\]

Mn (III) in turn mediates the oxidation of organic substrates.

\[
\text{Mn}^{3+} + \text{RH} \rightarrow \text{Mn}^{2+} + \text{R}^- + \text{H}^+
\]

The characteristics of the cycle are very similar to that of LiP. Addition of one equivalent of H\textsubscript{2}O\textsubscript{2} to the native enzyme yields MnP-I, which is a Fe (IV)-oxo-porphyrin radical cation [Fe (IV) =O\textsuperscript{*}]. The peroxide bond of H\textsubscript{2}O\textsubscript{2} is cleaved subsequent to a 2e\textsuperscript{-} transfer from the enzyme heme-porphyrin. The formation of MnP-I is pH independent, with a second-order rate constant of 2.0\times10\textsuperscript{6} M\textsuperscript{-}1 S\textsuperscript{-}1 (Wariishi et al., 1989a). Addition of one equivalent Mn (II) rapidly reduces compound I to compound II. The conversion of MnP-I to MnP-II can also be achieved by the addition of other electron donors, such as ferro cyanide and a variety of phenolic compounds (Wariishi et al., 1988). In the reduction of compound II to generate the native enzyme, however, Mn (II) is an obligatory redox coupler for the enzyme to complete its catalytic cycle.
The Mn (III) formed is dissociated from the enzyme and stabilized by forming complexes with α-hydroxy acids at a high redox potential of 0.8–0.9 V. Oxalate and malonate are optimal chelators that are secreted by the fungus in significant amounts (Wariishi et al., 1992; Kishi et al., 1994). It has also been shown that MnP reacts with oxalate–Mn (II) instead of free Mn (II) as the true substrate with the chelator involved in the redox reaction of the metal (Kuan et al., 1993), it also causes one-electron oxidation of various substrates chelates of Mn (III)+ and carboxylic acids can react with each other and are converted to alkyl radicals. Oxidation of oxalic acid by Mn III+ produces a formate radical HCO2− that reacts with dioxygen to form superoxide O2− and subsequently, H2O2 (Khindaria et al., 1994; Urzua et al., 1998). Versatile peroxidases produced
by some white-rot fungi possess the ability to oxidize, in addition to Mn II\(^+\) also phenolic (phenol red) and non phenolic (veratryl alcohol) aromatic compounds (Martinez et al., 1996).

MnP catalyzes reduction reactions in the presence of hydroquinones and MnII\(^+\) (Chung et al., 1993). Mn III\(^+\) oxidizes hydroquinones to corresponding semiquinone radicals, which has been shown to reduce more oxidized chemicals. The quinine formed by this process is reduced back to hydroquinone by quinone reductases. Thus, highly oxidized pollutants, are indirectly reduced by MnP and LiP facilitating further metabolism (Chung et al., 1993) (Fig. 6).

![Fig. 6. Manganese peroxidase catalyzed reduction](image)

Fig. 6. Manganese peroxidase catalyzed reduction
Table 1. Summary of the ligninolytic enzymes and their substrates and reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Co-factor</th>
<th>Substrate, Mediator</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase</td>
<td>Lac</td>
<td>O₂</td>
<td>Phenols, e.g., ABTS</td>
<td>Phenols are oxidized to phenoxy radicals; Other reactions in the presence of mediators</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td>LiP</td>
<td>H₂O₂</td>
<td>Veratryl alcohol</td>
<td>Aromatic ring oxidized to cation radical</td>
</tr>
<tr>
<td>Manganese peroxidase</td>
<td>MnP</td>
<td>H₂O₂</td>
<td>Mn</td>
<td>Mn (II) oxidized to Mn (III); chelated Mn (III) oxidizes phenolic compounds To phenoxy radicals; other reactions in the presence of additional compounds</td>
</tr>
</tbody>
</table>

2.3. Industrial and biotechnological applications of lignolytic enzymes

The industrial development over the last five decades has resulted in an exponential increase in the production and consumption of chemicals. Production, use and disposal of numerous chemicals cause widespread contamination of soils as well as ground waters and surface waters. Indiscriminate applications, high persistence, unknown environmental pathway and pollutant’s potential to bioaccumulate have resulted in severe repercussions,
The recognition that environmental pollution is a worldwide threat to public health has given rise to a new industry for environmental restoration. Physical and chemical treatment processes (i) typically remove organic pollutants at low level, (ii) are highly selective in terms of the range of pollutants removed and (iii) prohibitively expensive for the treatment of wastes. Clean up of environmental pollution also presents a serious economic burden and therefore cost effective yet efficient and environment-friendly methods of decontamination are vital in solving the hazardous waste problems. The use of indigenous or introduced microorganisms provides a very attractive, ecofriendly and economic solution to many of our hazardous pollution problems. For both economic and ecological reasons, biological degradation (bioremediation) has become an increasingly popular alternative for the treatment of hazardous wastes. One such method of bioremediation involves the white rot fungi; a group of basidiomycetes characterized by their ability is unique among these fungi and has made them an important link in the global carbon cycle.

The white rot fungal technology is very different from other better-established methods of bioremediation. The mechanisms used by the fungi provide them with several advantages for pollutant degradation. The lignin-degrading system, being extracellular, has evolved to degrade insoluble chemicals such as lignin and many of hazardous environmental pollutants at including the loss of food sources, mutagenic and carcinogenic effects to the man kind.
considerably higher concentrations. The intracellular machinery of bacteria is poorly accessible to the pollutants whereas, bacterial enzymes are highly specific and a consortium may be required to degrade successfully and completely such chemicals.

The lignin-degrading system is expressed in response to nutrient (C, N or S) limitations and therefore organism does not require preconditioning with the pollutant to be degraded. Enzymes are not repressed even when the pollutant concentration is reduced to ineffective levels for enzyme induction. Bacteria fail to degrade the pollutants when the concentration is reduced below threshold value and ineffective for enzyme induction. White rot fungi can effectively degrade very low concentrations of pollutants to non-detectable levels. White rot fungi can be cultivated on inexpensive growth substrates like wheat straw, corncobs, wood chips or other crop residues and also on liquid media as well as in soil that promote the use of white rot fungi for bioremediation. In addition to being able to grow under nutrient limitation, the fungi also produce oxygen radicals such as OH⁺, which is capable of oxidizing biomolecules, such as proteins and DNA that could result in the death of other microbes. Using the plasma membrane - dependent redox system, the fungus is able to adjust the pH of its surrounding environment. Thus microbes with pH optimum that differ from that of the fungus might not grow well after the fungus has been introduced (Christian et al., 2005).
Dyes are widely used within the food, pharmaceutical, cosmetic, textile and leather industries. During industrial processing, up to 40% of the used dyestuff are released into the process water (Vaidya & Datye, 1982), producing highly colored wastewaters that affect aesthetics, water transparency, and gas solubility in water bodies. Moreover and most importantly, there is a general concern regarding toxicity of some of these dyes. Because of both the high discharged volumes and the effluent composition, wastewaters from the textile industry can be considered as the most polluting among all industrial sectors, thus greatly requiring appropriate treatment technologies (O’Neill et al., 1999). All the dyes used in the textile industry are designed to resist fading even upon exposure to many chemicals including oxidizing agents. Although some a-biotic methods for the reduction of several dyes exist, these require highly expensive catalysts and reagents (Robinson et al., 2001b). Biotechnological approaches were proven to be potentially effective in treatment of this pollution source in an eco-efficient manner (Willmott et al., 1998; Beydilli et al., 1998; McMullan et al., 2001; Zissi & Lyberatos, 2001; Robinson et al., 2001b). Dyes containing effluents represent highly problematic wastewaters because of their high COD and BOD. Suspended solids and the content of toxic compounds, but also because of the color, which makes them easily, recognized and pose esthetic problems. When dyes are released in the environment, they often exhibit toxic effects on different organisms and are a threat to ecosystems by reducing sunlight penetration, which in turn reduces photosynthetic activity and dissolved oxygen.
concentration (Banat et al., 1996). The white rot fungi are, so far, the microorganisms most efficient in degrading synthetic dyes, with basidiomycetous fungi that are able to depolymerized and mineralize lignin. This white rot fungi property is due to the production of extracellular lignin-modifying enzymes which, because of their low substrate specificity, are also able of degrading a wide range of xenobiotics compounds (Barr & Aust, 1994; Scheibner et al., 1997; Pointing, 2001) including dyes (Glenn & Gold, 1983; Pasti-Grigsby et al., 1992; Paszczynski et al., 1992; Spadaro et al., 1992). The main lignin modifying enzymes are manganese peroxidases (MnP), (Glenn et al., 1986), lignin peroxidases (LiP), and laccases (Lac) (Edens et al., 1999). LiP, MnP, and Lac play significant roles in dye metabolism by white rot fungi (McMullan et al., 2001) due to the structural similarity of the most commercially relevant dyes to lignin (sub) structures amenable to be transformed by lignin modifying enzymes. However, the profiles of lignin-modifying enzyme production during dye decolourization by cultures of Phlebia tremellosa (Robinson et al., 2001a) whereas, MnP, with or without LiP cooperation, was reported as the main enzyme involved in dye decolourization by Phanerochaete chrysosporium (Chagas & Durrant, 2001), and LiP was considered as the principal decolorizing enzyme of Bjerkandera adusta (Robinson et al., 2001b).

It is clear from the foregone survey of literature that the enzymes implicated in lignin degradation are: lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, manganese-dependant...
peroxidase and laccase, which oxidize phenolic compounds to give phenoxy radicals and quinones; glucose oxidase and glyoxal oxidase for H₂O₂ production and cellobiose-quinone oxidoreductase for quinone reduction. The different degrees of degradation of lignin with respect to other wood components depend on the environmental conditions and the fungal species involved. It has been demonstrated that there is no unique mechanism to achieve the process of lignin degradation and that the enzymatic machinery of the various microorganisms differ. Moreover microorganisms exhibit diversity in metabolic patterns. For instance, *Pleurotus ostreatus* belongs to a subclass of lignin-degrading microorganisms that produce laccase, manganese peroxidase and veratryl alcohol oxidase but no lignin peroxidase (Palmieri *et al.*, 1997). *Pycnoporus cinnabarinus* has been shown to produce laccase as the only ligninolytic enzyme (Eggert *et al.*, 1996), and *P. sanguineus* produces laccase as the sole phenol oxidase (Pointing & Vrijmoed, 2000). Lignolytic activity of organisms such as *P. chrysosporium, P. ostreatus, T. versicolor* has been clearly evaluated in terms of participatory enzymes. However information on enzymes involved in biodegradation of lignin by *Stereum ostrea* is fragmentary.