Chapter – II

Review of Literature
A large number of microorganisms are involved in wood decomposition, however the dominant decomposers are fungi. Different processes are used to degrade wood because it is composed of differing complex components. Three types of filamentous fungi are able to utilize wood that have a preference for one or more of the wood polymers. Based upon the decay forms, color and texture of the resulting wood, the filamentous fungi were designated as white, brown and soft-rot. The soft-rot fungi include the Ascomycetes and Fungi Imperfecti, which decompose cellulose completely while lignin is partially degraded. Brown-rot fungi include some of the Basidiomycetes, and have a preference for hemicellulose and cellulose but will degrade lignin by demethylation. The third group is the white rot fungi (WRF) that include some of the Basidiomycetes which degrade the lignin more extensively and rapidly to carbon dioxide and water (Scklarz et al., 1989) than any other organisms.

Lignin-degrading fungi are a group of taxonomically heterogeneous higher fungi, which are widely distributed in a range of environments from tropical to temperate. Microorganisms in this group are mainly saprophytes that belong to the division Eumycota, subdivision Basidiomycotina (Burdsall, 1998). They are characterized by their ability to depolymerize and mineralize lignin efficiently using extracellular ligninolytic enzymes. Many classes of extracellular enzymes have been implicated in lignin degradation such as lignin peroxidases (LiPs), manganese peroxidases (MnP s) and laccases. A review of various WRF has demonstrated that the peroxidases and laccases are secreted by almost all ligninolytic fungi, however the enzymes may be differentially expressed in different species and even between various strains (de Jong et al., 1992; Esposito et al., 1991; Hatakka, 1994; Nerud et al., 1991; Orth et al., 1993; Palaez et al., 1995; Szklaez et al., 1989; Waldner et al., 1988).

One of the most intriguing questions is which, enzyme or enzyme combination is responsible for the most efficient lignin depolymerization and degradation. Two of the
best examined lignin-degrading fungi are *Phanerochaete chrysosporium* (*P. chrysosporium*) and *Trametes versicolor* (*T. versicolor*). *P. chrysosporium* is a ligninolytic basidiomycete, which has been found to be a good degrader of lignin. It has been used as a model organism to study physiological requirements for lignin biodegradation. It was found that only during secondary metabolism, triggered by nitrogen, carbon or sulphur limitation, do liquid cultures of *P. chrysosporium* produce ligninolytic enzymes (Erwin *et al.*, 1993). It was found to produce high levels of ligninolytic peroxidases, including lignin and manganese peroxidase, in response to nutrient depletion. For a long time it was believed that *P. chrysosporium* did not produce laccase, because its activity is repressed by glucose. However, laccase activity has been detected constitutively at a basal level in *P. chrysosporium* (Perez *et al.*, 1996; Srinivasan *et al.*, 1995). *T. versicolor* is another well studied WRF, which secretes substantial amounts of laccase. It is also known as *Coriolus versicolor* and *Polyporus versicolor*. Similarly to *P. chrysosporium*, *T. versicolor* secretes both LiP and MnP. Unlike *P. chrysosporium*, *T. versicolor* secretes substantial levels of laccase. Low concentrations of several laccases are produced constitutively during growth on wood, while higher concentrations are induced by the addition of aromatic compounds such as xylidine and ferulic acid. *T. versicolor* is also a well studied laccase producing fungus and most of the knowledge of laccase has been determined from these studies (Call and Mucke, 1997). *C. gallica* is another white rot fungus that secretes both MnP and laccase but not LiP. It produces high volumetric activity levels when grown by solid state fermentation on oat bran. The laccase activity has been correlated with decolorization of some industrial dyes (Rodriguez *et al.*, 1999).

**Wood Composition**

Wood and plant material are composed mainly of three structural polymers which are cellulose, hemicellulose and lignin. Cellulose determines the basic morphology of a wood cell wall and is being approximately 45% of the weight of wood. Glucose units in cellulose are linked by (β 1→4) glycosidic bonds in a linear polymer. Microfibrils are bundles of highly ordered cellulose molecules that are surrounded by lignin and
hemicellulose, which gives wood its mechanical strength and protection from microorganisms (Kirk and Cullen, 1998).

Hemicellulose, another biopolymer occupies 25% to 30% of the weight of wood. Similar to cellulose the backbone of hemicellulose is linear (β 1→4) linked monosaccharides, however the hemicellulose is shorter than cellulose polymers. Hemicellulose has three basic forms: 1,4-β-D-xylans, 1,3- and 1,4-β-D-galactans, and 1,4-β-D-mannans (Sarikaya et al., 1997). Presence of side groups such as sugars and acetyl esters impart a gel-like characteristic to hemicellulose (Kirk and Cullen, 1998).

Lignin makes up 30% of the weight of wood. A typical lignin contains 10%-20% phenolic groups (Youn et al., 1995). Lignin is a rigid organic polymer which has evolved for preservation purposes, giving plants structural integrity and providing protection from microorganisms (Call and Mucke, 1997). Lignin physically protects cellulose and hemicellulose from enzymatic hydrolysis. Due to the heterogeneous nature of lignin, it is resistant to degradation. The structure of lignin (Figure 1) is different from that of cellulose and hemicellulose in being composed of branched substituted phenylpropane units joined by a random distribution of stable carbon-carbon and ether linkages (Youn et al., 1995, Kirk and Cullen, 1998). The polymer consists of aromatic compounds of three p-hydroxybenzyl alcohol including p-coumaryl, coniferyl and sinapyl alcohol (Youn et al., 1995).

Lignin is highly branched and heterogeneous, being made up of phenyl-propanoid units which are linked through a variety of different bonds of which the most abundant linkage is guaiacylglycerol-β-arylether.
Ligninolytic enzymes

Lignin molecules are too large to be transported into the cell. As a result the extracellular enzymes are involved in lignin biodegradation. The degradation mechanism is oxidative, as the inter unit carbon-carbon and ether bonds are present in the structure. Lignin is stereo irregular, so the mechanism must be less specific for typical degradative enzymes. These requirements are fulfilled by the extracellular peroxidases and oxidases of WRF that act nonspecifically by generating unstable free radicals that are able to undergo a variety of spontaneous cleavage reactions (Kirk and Cullen, 1998). Enzymes that play a role in ligninolysis are LiP, MnP and laccase, which catalyze single electron oxidations to produce a free radical that undergoes a variety of non-enzymatic coupling substitutions and fission reactions explaining the chemical changes in lignin during degradation by fungal cultures. These enzymes have been shown to utilize low molecular weight mediating substrates to perform lignin degradation. Not all WRF produce all of these enzymes, each fungus produces its own unique combination of enzymes and has a characteristic ability to degrade lignin (Kirk and Cullen, 1998).

White rot fungi degrade the complex natural polymer lignin and they are well established as primary degraders of lignin and aromatic compounds (Blanchette, 1984). This ability has been used in numerous strategies to offer new technologies and products and add value to wood and other lignocellulosic crops and by-products (Eriksson, 1990).

Lignin Peroxidase (E.C. 1.11.1.14)

The lignin peroxidase (LiP) was first discovered in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* (Glenn *et al.*, 1983). This hydrogen peroxide requiring extracellular enzyme, with an acidic pH optimum, catalyzes the oxidative breakdown of lignin and of model compounds. LiP catalyses the cleavage of the arylpropane side chains, ether bond cleavage, aromatic ring opening and hydroxylation. The LiP is capable of catalyzing one electron oxidation. The native enzyme contains a protoporphyrin prosthetic group with a high spin ferric iron, Fe (III). Oxidation of hydrogen peroxide removes a electrons and converts the prosthetic group of the enzyme
to an oxo-iron (IV) porphyrin radical cation, an enzyme form designated as compound I. Further, electron reduction by abstraction of an electron from a donor molecule produces an enzyme with an oxo-iron (IV) porphyrin, a form designated as compound II. A second one electron reduction completes the catalytic cycle by regenerating the native ferric enzyme. LiPs in *P. chrysosporium* are a relatively large family of isozymes with molecular weight ranging from 38,000 and 43,000 and pi values ranging from 3.3 to 4.7. After the discovery of LiP it was thought that this enzyme was the main lignolytic agent because it was able to attack the major (non-phenolic) lignin moiety. However, in recent years it has been demonstrated that laccase and manganese peroxidase are able to oxidize phenolic as well as non-phenolic substrate under certain conditions (Bourbannais and Paice, 1990; Jensen *et al.*, Eggert *et al.*, 1996).

**Manganese Peroxidase (E.C.1.11.1.13)**

Manganese peroxidase (MnP) is the most common lignin-modifying peroxidase produced by almost all wood colonizing Basidiomycetes. MnP preferentially oxidizes manganese II ions (Mn$^{2+}$), always present in wood and soils, into highly reactive Mn$^{3+}$, which is established by fungal chelators such as oxalic acid. Chelated Mn$^{3+}$ in turn acts as a low-molecular weight, diffusible redox-mediator that attacks phenolic lignin structures resulting in the formation of unstable free radicals that tend to disintegrate spontaneously (Boominathan and Reddy, 1992). MnP is capable of oxidizing and depolymerizing natural and synthetic lignins as well as entire lignocellulosics of wood and pulp in the cell-free system. In vitro depolymerization of lignin is enhanced in the presence of co-oxidants such as thiols (eg. glutathione) or unsaturated fatty acid and their derivatives (eg. Tween 80) (Hofrichter, 2002).

**Versatile Peroxidase**

A new type of peroxidase, namely versatile peroxidase was described in *Pleurotus eryngii* (Martinez *et al.*, 1996), a fungus investigated for its ability to degrade lignin selectively (Martinez *et al.*, 1994), and in other *Pleurotus* species like *P. pulmonarius* (Camarero *et al.*, 1996) and *P. ostreatus* (Sarkar *et al.*, 1997) and *Bjerkandera adusta* (Heinfling *et al.*, 1998). Versatile peroxidase of *Pleurotus* and *Bjerkandera* is able to...
oxidize Mn\(^{2+}\) as well as non-phenolic aromatic compounds, phenols and dyes (Martinez, 2002). This new peroxidase oxidizes Mn\(^{2+}\) to Mn\(^{3+}\), degrades non-phenolic lignin model dimmer veratrylglycerol-β-guaiacyl ether yielding veratraldehyde, oxidizes veratryl alcohol and p-dimethoxybenezene to veratraldehyde and p-benzoquinone, respectively, as lignin peroxidase does, although with lower affinity (Caramelo et al., 1999).

Due to the low specificity and strong oxidizing abilities of the lignin degradation system, white rot fungi are also capable of degrading a broad spectrum of organic chemicals containing carbon skeletons similar to those found with the lignin polymer such PAH's, chlorinated phenols, PCB, dioxin, DDT, alkyl halides, nitro aromatics, chloroanilines and types (Field et al., 1993).

**Laccase (F.C.1.10.3.2.)**

Laccase is a type of copper-containing polyphenol oxidase that was discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883). Laccases are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize diphenols and related substances and use molecular oxygen as an electron acceptor. In contrast to most enzymes, which are generally very substrate specific, laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols, and even some inorganic compounds such as iodine (Xu 1996). When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical (Kersten et al., 1990; Thurston 1994). The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerisation (Thurston 1994).

**Distribution of laccases and their physiological roles**

Laccases are common enzymes in nature, and they are found widely in plants and fungi as well as in some bacteria and insects. The first laccase was reported in 1883 from *Rhus vernicifera*(R. vernicifera), the Japanese lacquer tree (review: Reinhammar 1984),
from which the designation laccase was derived. Laccases have subsequently been
discovered from numerous other plants, for example sycamore (Bligny and Douce 1983),
poplar (Ranocha et al., 1999) tobacco (De Marco and Roubelakis-Angelakis 1997) and
peach (Lehman et al., 1974). Plant laccases are found in the xylem, where they
presumably oxidize monolignols in the early stages of lignification (Bao et al., 1993; O'Malley et al., 1993; Mayer and Staples 2002; Gavnholt and Larsen 2002). In addition,
laccases have been shown to be involved in the first steps of healing in wounded leaves
(De Marco and Roubelakis-Angelakis 1997). 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), which is probably the
reason why detailed information about the biochemical properties of plant laccases is
limited. However, Rhus vernicifera laccase is an exception which has been extensively
studied, especially with regard to its spectroscopic properties (Malmstrom et al., 1970;
Woolery et al., 1984). R. vernicifera laccase has also widely been used in investigations
of the general reaction mechanism of laccases (Lee et al., 2002; Battistuzzi et al., 2003;
Johnson et al., 2003).

The majority of laccases characterized so far have been derived from fungi,
especially from white-rot basidiomycetes that are efficient lignin degraders. Well-known
laccase-producers include fungi such as Agaricus bisporus (Wood 1980). Botrytis cinerea
(Marbach et al., 1984), Chaetomium thermophilum (Chefetz et al., 1998), Coprinus
cinerus (Schneider et al., 1999), Neurospora crassa (Froehner and Eriksson 1974),
Phlebia radiata (Niku-Paavola et al., 1988). Pleurotus ostreatus (Sannia et al., 1986),
Pycnoporus cinnabarinus (Eggert et al., 1996b) and Trametes (Coriolus. Polyporus)
versicolor (Rogalski et al., 1991). The physiological roles of fungal laccases are various.
Laccases from white-rot fungi, such as T. versicolor and Pycnoporus cinnabarinus,
participate in lignin biodegradation, where they mainly oxidize the phenolic subunits of
lignin (Bourbonnais and Paice 1990; Eggert et al., 1996a; Eggert et al., 1996b; Thurston
1994; Hatakka 2001). In plant-pathogenic fungi, laccases are important virulence factors.
The grapevine grey mould, Botrytis cinerea, 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* (Rigling and van Alfen 1991; Choi et al., 1992; Mayer and Staples 2002) and
in the human pathogen *Cryptococcus neoformans* (Williamson 1994). In *Aspergillus
nidulans*, laccase activity is related to pigment production, and deletion of the laccase
gene yA abolishes the green color of conidial spores (Clutterbuck 1972; Aramayo and
Timberlake 1993; Adams et al., 1998). Laccases have also been proposed to participate in
fungal morphogenesis in *Armillaria* spp. (Worral et al., 1986), Lentinus edodes (Leatham
and Stahmann 1981) and *Volvariella volvacea* (Chen et al., 2004).

Only a few bacterial laccases have been described. The first bacterial laccase was
detected in the plant root-associated bacterium *Azospirillum lipoferum* (Givaudan et al.,
1993), where it was shown to be involved in melanin formation (Faure et al. 1994). An
atypical laccase containing six putative copper-binding sites was discovered from
*Marinomonas mediterranea*, but no functional role has been assigned to this enzyme
(Solano et al., 1997; SanchezAmat et al., 2001). *Bacillus subtilis* produces a thermostable
CotA laccase which participates in pigment production in the endospore coat (Martins
et al., 2002). Laccases have recently also been found from *Streptomyces cyaneus* (Arias
et al., 2003) and *Streptomyces lavendulae* (Suzuki et al., 2003). In addition to plants, fungi
and bacteria, laccases or laccase-like activities have been found in some insects, where
they have been suggested to be active in cuticle sclerotization (Sugumaran et al., 1992;
Dittmer et al., 2004).

**Structure and catalytic mechanism of laccases**

The overall fold of laccases comprises three cupredoxin-like domains A, B and C,
that are about equal in size (Ducros et al., 1998; Bertrand et al., 2002; Piontek et al.,
2002; Enguita et al., 2003). The cupredoxin fold is common among copper-containing
proteins, and it has also been found in the simple copper proteins plant plastocyanin
(Guss and Freeman 1983; Inoue et al., 1999) and bacterial azurin (Norris et al. 1983), as
well as in the more complex multicopper oxidases ascorbate oxidase (Messerschmidt et
al., 1992) and ceruloplasmin (Zaitseva et al., 1996; Murphy et al., 1997). All three
domains are important for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains Band C, a mononuclear copper center is located in domain C, and a trinuclear copper center is located at the interface between domains A and C. The mononuclear copper center contains one type-1 (T1) copper atom that is trigonally coordinated to two histidines and a cysteine. The coordination bond between T1 and Scys is highly covalent, which causes a strong absorption around 600 nm and gives laccases their typical blue color (Solomon et al., 1996). T1 also has an distant axial ligand which is a leucine or phenyl alanine residue in fungal laccases (Ducros et al., 1998; Bertrand et al., 2002; III; Piontek et al., 2002) and a methionine residue in the bacterial Bacillus subtilis CotA laccase and in other multicopper oxidases (Enguita et al., 2003; Messerschmidt 1997). The trinuclear cluster contains one type-2 (T2) copper atom and a pair of type-3 (T3) coppers (Messerschmidt 1997). The T2 copper is coordinated by two and the T3 copper atoms by six conserved histidines (Bertrand et al., 2002; III; Piontek et al., 2002). The T1 and T2 coppers are paramagnetic and can be identified in electron paramagnetic resonance (EPR) spectrum. The T3 copper pair is antiferromagnetically coupled by a bridging hydroxide, which makes the T3 coppers EPR-silent (Solomon et al., 1996). However, they can be detected by their characteristic absorbance at 330 nm (Solomon et al., 1996).

Substrate oxidation by laccase is a one-electron reaction generating a free radical, which usually reacts further through non-enzymatic routes (Reinhammer and Malmstrom, 1981). Bourbannais and Paice (1990) have shown that the artificial laccase substrate ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) has the capacity to act as a mediator enabling the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own. Niku-Paavola and Viikari (2000) have reported that purified laccase of Trametes hirsute oxidizes alkenes, which are not natural substrate for this enzyme. The oxidation was the effect of a two-step process in which the enzyme first catalyzed the oxidation of primary substrate, alkenes. The main reaction products of alkenes were the corresponding ketones or aldehydes. The reduction of oxygen to water is accompanied by the oxidation, typically of a phenolic substrate. Laccases are remarkably non-specific as to their reducing substrate and the range of substrates
oxidized varies from one laccase to another (Wood, 1980; De Vries et al., 1986). Simple diphenols like hydroquinone and catechol are good substrates for most laccases, but methoxy substituted monophenols like guaiacol and 2,6-dimethoxy phenol are often better (Coll et al., 1993; Rigling and Van-Alfen, 1993 and Slomczynski et al., 1995), p-phenylenediamine (a diamine rather than a diphenol) (Wood, 1980 and Assavanig et al., 1992) widely used substrate and syringaldazine is considered to be uniquely a laccase substrate (Assavanig et al., 1992; Youn et al., 1995 and Chefetz et al., 1998).

Oxidation of lignin by fungal laccase has been studied intensively since the early 1970's. Oxidation of milled wood lignin, demethylation (Ishihara and Miyazaki, 1974) and formation of carboxylation were observed. A series of studies revealed that laccase could take part in many of the reactions required for lignolysis (Kersten et al., 1990; Bourbonnais and Paice, 1990, 1992; Regalado et al., 1999). In an in vitro system using pure enzymes from Rigidiporus lignosus, laccase and manganese peroxidase were found to act synergistically to degrade radiolabelled lignin (Galliano et al., 1991). Archibald and Roy (1992) have been shown that pure laccase from Coriolus versicolor can provide Mn (III) chelators from Mn (II) in the presence of phenolic accessory substrates.

Besides, playing a role in delignification, fungal laccases appear to be involved in numerous physiological functions including fruit body development, detoxification of phenolic compounds via oxidative coupling and polymerization (Thurston, 1994), pathogenesis, protection from plant defense compounds (Bar-Nun and Meyer, 1990), melanin production, pigment production and antimicrobial activity (Eggert et al., 1997).

The laccase enzyme is widely distributed among plants, where it is implicated in wound response and the synthesis of lignin, the complex polymer that constitute the main component of the plant cell wall (Lewis et al., 1998). But in fungi, particularly laccases of basidiomycetes are capable of mineralizing lignin which is apparently a unique feature in this group of organisms (Thurston, 1994).
In *Aspergillus sp.*, *Dadina concentria* and *Lentinus edodes*, the laccase activity is associated with pigment formation in structures that are more rigid than a simple mycelial aggregate. Formation of fruit body may involve phenol oxidases and the synthesis of pigments coupled to oxidative polymerization of cell wall components strengthening cell-to-cell adhesion (Bu'Lock, 1967; Leatham and Stahmann, 1981). Currently the potential of laccase is being investigated in detoxification of environmental pollutants.

**Culture conditions for laccase production**

Culture conditions and medium composition also play a major role on laccase expression. Physiological demands vary among white rot fungi and considerable research has been done on the influence of agitation, pH, temperature, carbon source, and nitrogen level. Buswell et al., (1995) reported that the production of lignin modifying enzymes is strongly affected by the nature and amount of nutrients especially nitrogen and trace elements in the growth medium. For example, repression of lignin degradation by high nitrogen levels and the occurrence of lignolytic activity only during secondary metabolism in *P.chrysosporium* and *T. versicolor* are well established traits (Kirk et al., 1978; Eriksson et al., 1990). However, the addition of nitrogen to certain fungal cultures increased the degradation of lignin related compounds (Eriksson et al., 1990). Such fungi may be found in nitrogen rich environments such as cattle dung, whereas, white rot fungi growing in wood encounter low nitrogen concentrations (Eriksson et al., 1990). Sethuraman et al., (1999) reported that the bird’s nest fungus *Cyathus stercores* produced higher levels of laccase and managanese peroxidase mineralization in the presence of 10 mM ammonium tartrate and 1% glucose but the rate of lignin mineralization was slow, however it was high at 1mM ammonium tartrate.

Earlier studies showed that the media composition and modification of media influenced the levels of lignin peroxidase and managanese peroxidase in *Bjerkandra sp* strain BOS55, strains of *T. versicolor* and *P. chrysosporium* (Kaal et al., 1993; Mester et al., 1996; Collins et al., 1997). Bolag and Leonowicz (1984) have described that laccase formation in a number of white rot fungi is known to be influenced by various physiological factors and has been studied extensively.
Both the nature and the quantity of available nitrogen sources exert a great influence on the extracellular lignolytic enzyme production of wood rotting Basidiomycetes. Although the concentration of nitrogen sources have an effect on lignolytic enzyme production. There is no strict relationship between these two factors. For several species, the lignolytic enzyme activity is suppressed rather than stimulated by high nitrogen concentrations (25-60 mM). The best known example of this type of regulation was observed in *P. chrysosporium* (Keyser et al., 1978) and *Pleurotus sajorcaju* (Zhao et al., 1996; Fu et al., 1997). *T. versicolor* (Zhao et al., 1996) and *P. brevispora* (Leatham and Kirk, 1993) have been show to produce more amount of laccase at low nitrogen concentration (2-3 mM). In contrast, high nitrogen concentrations stimulates the production of lignolytic enzyme in *Bjerkarea adusta* (Kaal et al., 1993; 1995), *Pleurotus ostreatus* (Kaal et al., 1995), *Coriolus subvermispora* (Ruttimann-Johnson et al., 1993), *Ganoderma lucidum* (D’Souza et al., 1999).

The extensively studied white rot fungus, *Phanerochaete chrysosporium* secretes LiP and MnP as the dominant Lignin modifying enzymes (LME) (Reddy and D’Souza, 1994), where both enzymes are inducible under specific conditions of low nitrogen, high oxygen tension, presence of the inducers veratryl alcohol and static cultivation. Laccase is produced in small titers under nitrogen sufficient conditions (Srinivasan et al., 1995) and secretes multiple laccase isoforms under high nitrogen (24 mM) levels with the presence high copper content in the culture medium (Dittmer et al., 1997). Production of LiP and MnP production by *P. chrysosporium* was observed at low nitrogen (2.4 mM) but it was completely suppressed at high nitrogen (24 mM) (Buswell and Odier, 1987; Boominathan and Reddy, 1992; Vander-Woude et al., 1993; Buswell et al., 1995). Laccase production by *P. chrysosporium* was not detected in low or high nitrogen medium with glucose as the carbon source but was produced when the organism was grown in low or high nitrogen medium with cellulose as carbon source (Srinivasan et al., 1995), whereas, in the white rot fungus *Ganoderma lucidum* higher levels of laccases were produced in high nitrogen medium with glucose as the carbon source (D’Souza et al., 1999).
Low concentration of several laccases are produced constitutively on wood and in submerged fungal cultures while, higher concentrations are induced by the addition of aromatic compounds such as xylidine and ferulic acid (Reinhammar, 1984). High concentrations of laccases have been observed in older non-induced cultures (Bourbonnais and Paice, 1992) suggesting that the influence of the age of culture.

**Copper in Laccase**

Copper is an essential micronutrient for most living organisms and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal, in the order of 1-10 μM (Cervantes and Gutierrez-Corona, 1994). Copper present in higher concentrations of its free, the form of cupric is extremely toxic to microbial cells. The binding and uptake of copper in fungi typically comprising two phases; metabolism-independent surface binding followed by an energy dependent metal, influx (Gadd, 1986). The stimulatory effect of Cu on laccase synthesis was also effective for several other Basidiomycetes and hence could be used as a simple method to boost the production of this enzyme (Galhaup and Haltrich, 2001). Increased production of laccase activity was obtained in the copper supplemented culture of *Pleurotus ostreatus* using Northern blot analysis (Palmieri et al., 2000).

Copper has been reported to be a strong laccase inducers in several species eg., *Neurospora crassa* (Huber and Lerch, 1987), *Trametes versicolor* (Collins and Dobson, 1997), *Phanerochaete chrysosporium* (Dittmer et al., 1997). *Pleurotus ostreatus* (Palmieri et al., 2000) *Pleurotus sajor-caju* (Soden and Dobson, 2001) and *Trametes trogii* (Levin et al., 2002).

Galhaup and Haltrich (2001) have been reported that CuSO₄ at 2.0 mM concentration exhibited high laccase activity (65 U/ml) in *Trametes pubescens*, and using Western blot analysis they reported that the synthesis of the laccase protein depend on the presence of copper ions in the culture medium. Also, they reported that both the
time and concentrations of copper supplementation were important for obtaining high levels of laccase.

In the Asomycete Podospora anserine, laccase mRNA is increased in response to copper and aromatic compounds and it was postulated that the laccase may involve in defense mechanisms against oxidative stress (Fernandez-Larrea and Stahl, 1996). This protective feature was partly attributed to the chelation of copper ions during the synthesis of laccase enzyme. Fungal laccases are believed to be involved in the formation of various pigments. In many fungi enhanced melanin accumulation was reported as a response to elevated copper concentrations in many fungi (Gadd and de Rome, 1988; Galhaup and Haltrich, 2001; Fogarthy and Tobin, 1996) Coll et al., (1993) reported that a metal responsive sequence is present in the promoter region of laccase gene of Basidiomycete PMI (CECT 2971), which is believed to be increased the laccase production. Recent work on the white rot fungus Trametes trogii revealed that addition of copper strongly stimulated lignolytic enzyme production and faster decolorization of polymeric dyes poly R-478. However, higher copper concentration (500 mM) inhibited the growth and notably decreased manganese peroxidase production although they did not affect secretion (Levin et al., 2002).

Laccase is a copper containing protein. The enzymatic activity of laccase is due to four copper (II) ions, which are arranged in three different sites, each characterized by unique spectroscopic properties (Solomon et al., 1996). The T₁ or blue copper is responsible of the intense change in transfer absorption band around 600 nm; the T₂ site has no absorption feature and the T₃ site contains two copper ions which are antiferromagnetically coupled, and has a characteristic shoulder at 330 nm. Electrons abstracted from a substrate by T₁ copper, which then delivers them to T₂ and T₃ sites. In fact, these latter sites (T₂ and T₃) are arranged in a unique trinuclear cluster that is capable of binding oxygen which is the final electron acceptor.

The measured copper content of the purified fungal laccase varied between four and two atoms per enzyme molecules. The UV-visible spectrum of the purified laccase of many white rot fungi showed a peak of absorption at 614 nm typical for type one
copper that is responsible for deep blue colour of the enzyme and a shoulder at 325 nm suggests the presence of the type three binuclear copper pair (Fukushima and Kirk, 1995; Youn et al., 1995; Eggert et al., 1996; Schliphake et al., 2000)

The electron spin resonance spectrum of purified laccase of *Pycnoporus cinnabarinus* has two superimpose signal of type one and type two copper centres (Eggert et al., 1996; Schliphake et al., 2000). The same result has been reported in the laccase of *Pleurotus osteratus* (Youn et al., 1995) and *Marasmius queroophilus* (Dedeyan et al., 2000).

**Manganese in Laccase**

Manganese is found in lignin, the natural substrate for white trot fungi and its ability to induce manganese peroxidase (MnP) transcription is well established (Gold and Alic, 1993). Archibald and Roy (1992) have shown that the laccase from *T. versicolor* can produce Mn III in chelates from Mn II in the presence of phenolic accessory. They suggested a dual role for both laccase and MnP in lignin degradation. Similarly recent works on the white rot fungi *Clitocybula dusenii, Nematoloma frowardii* (Scheel et al., 2000) and *Pleurotus sajor-caju* (Soden and Dobson, 2001) showed increased laccase mRNA levels due to manganese supplementation in cultures.

Low molecular weight aromatic compounds have shown to significantly influence the growth and activity at lignocelluloses degrading microorganism (Varadi, 1972; Bomeman et al., 1986; Martin and Akin, 1988; Orth et al., 1993). Several compounds with a methylated p-phenolic group are the products of ferulic acid metabolism in *P.chryosporium* (Gupta et al., 1981). Aromatic Vertryl alcohol (3,4-dimethoxybenzyl alcohol), a secondary metabolite synthesized de-novo by several fungi (De Jong et al., 1994), encodes the transcription of genes for laccases (Mansur et al., 1998, Scheel et al., 2000) and manganese peroxidase (Scheel et al., 2000) but not lignin peroxidase (Cancel et al., 1993) in certain Basidiomycetes and act as an inducer of laccases in the ascomycete *Botryophaeria* sp., (Barbosa et al., 1996; Vasconceles et al., 2000; Dekker and Barbosa, 2001). But veratryl alcohol failed to stimulate laccase
production in *Pycnoporous sanguineus* (Pointing et al., 2000; Sethuraman et al., 1999) reported 3,4-dimethoxy-cinnamic acid, at 500 µM concentration, was the most effective inducer of laccase in the white rot fungus *Cyathus stercoreus*.

**Solid State Fermentation**

The large-scale production of lignolytic enzymes is of great importance because of their potential biotechnological applications in bioremediation. Solid-state fermentation (SSF) holds tremendous potential for the production of enzymes. The main advantage of SSF systems is that they stimulate the fermentation reactions occurring in nature. The increasing interest around SSF in recent years has focused on its applications in food fermentation and enzyme production. It can be of special interest in that process where the crude fermented product may be used directly as the enzyme source (Tengerdy, 1998). SSF is now gaining importance in enzyme production because of its favorable energetics, lower capital and operating expenses as compared to liquid culture. This system offers numerous advantages over submerged fermentation (SmF) system including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments (Pandey, 1994; Doelle et al., 1992).

Agro-industrial residues are generally considered the substrates for the SSF process and use of solid substrates for the production of enzymes is if no exception to that. A number of such substrates has been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran, maize bran, rice straw, rice husk, sago hampas, saw dust, coir pith, etc. (Pandey, 1994; Mitra et al., 1994; Selvakumar et al., 1994). In SSF process the solid substrate not only supplies the nutrients but also serves as an anchorage for the cells. Laccase production was stimulated most by wheat husk and bran; when wheat bran was formulated into pellets with additional of yeast extract, laccase activity was enhanced further (Ullah et al., 2000). Hatvani and Mecs
(2001) reported that *Lentinus edodes* produced high amount of laccase in the solid state fermentation using malt containing industrial by products.

**Heterologous production of laccases**

Laccase genes are often expressed at very low levels in the native hosts. In order to improve laccase production, fungal laccases have been expressed heterologously in *Saccharomyces cerevisiae* (Kojima et al., 1990), *Trichoderma reesei* (Saloheimo and Niku-Paavola 1991), *Aspergillus oryzae* (Yaver et al., 1996; Wahleithner et al., 1996; Berka et al., 1997b; Yaver et al., 1999; Sigoillot et al., 2004), *Pichia pastoris* (Jonsson et al., 1997; Otterbein et al., 2000; Brown et al., 2002; Soden et al., 2002; Liu et al., 2003), *Aspergillus sojae* (Hatamoto et al., 1999), *Aspergillus niger* (Record et al., 2002; Larrondo et al., 2003a), *Aspergillus nidulans* (Larrondo et al., 2003a), tobacco (LaFayette et al., 1999) and maize (Bailey et al., 2004). In addition, heterologous yeast expression systems have been developed to facilitate protein engineering of laccases (Gelo-Pujic et al., 1999; Bulter et al., 2003) or to improve the resistance of yeast to phenolic growth inhibitors (Cassland and Jonsson 1999). Bacterial laccases from *Bacillus subtilis* and *Streptomyces lavendulae* have been expressed in *Escherichia coli* (Martins et al., 2002; Suzuki et al., 2003) but successful expression of fungallaccases in *E. coli* has not been reported.

Laccases have been expressed in *Aspergillus spp.* under the control of the strong constitutive TAKA-amylase (amyA) (Yaver et al., 1996; Wahleithner et al., 1996; Berka et al., 1997b; Yaver et al., 1999 and Larrondo et al., 2003a) or glyceraldehyde3-phosphate dehydrogenase (gpdA) promoters (Record et al., 2002). In *Trichoderma reesei*, the promoter region of the major cellulase gene cbhl has been used (Saloheimo and Niku-Paavola 1991). Secretion of heterologous laccases has generally been directed by using native laccase signal sequences in the expression constructs. However, it may be possible to increase the production levels by using signal sequences derived from host genes. For example, *Pycnoporus cinnabarinus* Lac 1,was produced 80 times more efficiently in *Aspergillus niger* when the laccase signal sequence was replaced by the prepro sequence of the A. niger glucoamy lase gene glaA (Record et al., 2002). The effect of signal sequences on heterologous laccase production has also been studied in *Pichia pastoris*.
strains expressing various laccases, but in these experiments the native laccase signal sequences have performed better than the commonly used N-terminal signal peptide from the *Saccharomyces cerevisiae* mating-type factor MFa gene (Jonsson et al., 1997; Brown et al., 2002; Soden et al., 2002 and Liu et al., 2003).

Laccase production levels have often been improved significantly by expression in heterologous hosts, but the reported levels have still been rather low for industrial applications. The highest yields have been obtained in filamentous fungi, especially in *Aspergillus spp.* that are widely used in the production of industrial enzymes. Improved laccase production levels have also been achieved by expression in *Pichia pastoris*, whereas expression in *S. cerevisiae* has generally resulted in very low activity levels (Larsson et al., 2001 and Bulter et al., 2003). The highest reported laccase production levels thus far have been obtained in homologous production systems in a shake flask cultivation of *Pycnoporus cinnabarinus*, which yielded 1000-1500 mg l\(^{-1}\) laccase (Lomascolo et al., 2003), and a fermentor cultivation of *Trametes pubescens*, which yielded 700 mg l\(^{-1}\) laccase (Galhaup et al. 2002b). The production of two bacterial laccases from *Bacillus subtilis* and *Streptomyces lavendulae* in *E. coli* resulted in extensive intracellular aggregation of laccases (Martins et al., 2002; Suzuki et al., 2003). *Bacillus subtilis* laccase could not be recovered from the inclusion bodies, and only the soluble fraction representing about 10% of the heterologous laccase was purified (Martins et al., 2002). *Streptomyces lavendulae* laccase was refolded to an active form after treatment with urea and 2-mercaptoethanol (Suzuki et al., 2003).

Production of heterologous laccase has often been improved by varying the cultivation conditions. For example, better production of heterologous laccase has been achieved in yeast systems by controlling the pH of the culture medium and by lowering cultivation temperatures (Jonsson et al., 1997; Cassland and Jonsson 1999; Larsson et al., 2001; O'Callaghan et al., 2002; Soden et al., 2002; Liu et al., 2003). Buffering of the culture medium to maintain the pH above 4 has been proposed to be important for stability of secreted laccases and inactivation of acidic proteases (Jonsson et al., 1997; Larsson et al., 2001; Soden et al., 2002), whereas lowered cultivation temperatures may
result in better production due to improved folding of heterologous proteins (Cassland and Jonsson 1999). In addition, overexpression of Sso2p, a membrane protein involved in the protein secretion machinery (Aalto et al., 1993), has been shown to improve heterologous laccase production in S. cerevisiae (Larsson et al., 2001). The addition of copper into the culture medium has also proved to be important for heterologous laccase production in Pichia pastoris and Aspergillus spp. (O'Callaghan et al., 2002; Larrondo et al., 2003a; Liu et al., 2003). In contrast to homologous laccase production, in which copper addition often affects laccase gene expression, the increased laccase production by copper addition is probably related to improved folding of the active laccase in heterologous production (Larrondo et al., 2003a). The importance of adequate copper concentration for proper laccase folding was further corroborated by studies in which two genes related to copper-trafficking in Trametes versicolor were overexpressed in S. cerevisiae expressing T. versicolor lacIII gene; the heterologous laccase production by S. cerevisiae was improved up to 20-fold (Uldschmid et al. 2003). The effect was suggested to result from more efficient transport of copper to the Golgi compartment (Uldschmid et al., 2003). Directed evolution has also been used for improving heterologous laccase production. Mutations in the Myceliophthora thermophila laccase gene resulted in the highest reported laccase production level in S. cerevisiae, 18 mg 1⁻¹ (Bulter et al., 2003).

**Purification and characterization of laccase**

Production of extracellular laccase is a common feature of many higher Basidiomycetes fungi, particularly those associated with wood decay or the terminal stages of decomposition of leaf litter. Most of the white rot fungi produce laccase in multiple isoforms (Perry et al., 1993; Buswell et al., 1995; Fu et al., 1997; Palmieri et al., 2000). Laccases have been purified over the 30 years by many researchers from various fungi in view of characterization. Several purification steps are required to obtain a preparation of free of both pigment and other contaminating proteins. Multiple steps like ultra filtration, precipitation using ammonium sulphate or organic solvents, ion exchange and size exclusion chromatography have been used for the purification of laccases from the culture filtrate.
Electrophoretic separation of crude and purified laccase revealed the presence of multiple isoforms in many fungi. The laccase activity was detected in polyacrylamide gel after the protein separation by incubating the gel in laccase substrates like guiacol (Wood, 1980; Coll et al., 1993), ABTS (Niku-Paavola et al., 1990; D’Souza, 1999) p-phenylenediamine (Wood, 1980, Perumal, 1997) and syringaldazine (Youn et al., 1995).

Wood (1980) reported that laccase protein was composed of several polypeptide when analyzed by SDS-PAGE. Number of laccase isoforms varied between fungal species. *Pycnoporus cinnabarinus* (Eggert et al., 1996; Schliphake et al., 2000). *Chaetomium thermophyllum* (Chefetz et al., 1998), *Coriolopsis gallica* (Calvo et al., 1998). *Cyathus stercorarius* (Sethuraman et al., 1999), *Marsmius quercophilus* (Dedeyan et al., 2000). *Trametes pubenses* (Galpaup and Haltrich, 2001) produced only one isoform of laccase. But other fungal strains namely Basidiomycete strain PMI (Coll et al., 1993) and *Ceriporiopsis subvermispora* (Fukushima and Kirk, 1995) produced two isoforms of laccases. Whereas, in *P. chrysosporium* (Leisola et al., 1984), *Pleurotus sajor caju* (Fu et al., 1997), *P. ostreatus* (Palmieri et al., 2000) and *Pycnoporus sanguninus* (Pointing et al., 2000) produced multiple isoforms of laccase.

The molecular weight of the fungal laccase varies from 60kDa to 80kDa. However, a very high molecular weight (390 kDa) laccase isoform was detected in *Podospora anserine*. Many excreted proteins from fungi and other organisms have been shown to be glycoproteins. The carbohydrate content of laccase varies from 6.5 to 80%. The highest amount carbohydrate (80%) was detected in the laccase of in *Botrydis cinerea* laccase (Marbech and Mayer, 1985).

The optimum pH for the purified laccases of fungi varied between 3 and 6.5 and optimal temperatures from 25 to 80°C. The laccases from *Coriolus hirsutus* and *Coriolus zonatus* are reported as the most thermo-stable laccases, reported so far, showing the thermal transition at 87°C and 92°C (Koroleva et al., 2001). This dramatic loss of
enzymatic activity in the temperature ranging from 50 to 65°C caused by a release of Type 2 copper ion. Type 1 copper site as well as the type 3 site are completely disintegrated at temperatures higher than 70°C. Thus, the crucial event for integrity of the copper takes place between 60 and 70°C, whereas over all protein structure is still maintained (Koroleva et al., 2001).

The effect of several potential laccase inhibitors has been studied for many fungal laccases. The most effective inhibition was obtained with sodium azide for many laccases (Coll et al., 1993, Eggert et al., 1996; Perumal, 1997; Sethuraman et al., 1999). In Cyathus stercoreus and Pycnoporus cinnabarinus, laccase activity was completely inhibited by sodium azide, dithiothreitol and l-cyste in at low concentration. No inhibition of laccases was noticed with kojic acid, EDTA and acetyl acetone upto 8.8 mM (Sethuraman et al., 1999; Eggert et al., 1996). In contrast, the activity laccase in Coriolus versicolor was completely inhibited by 0.35 μM kojic acid (Murao et al., 2002).

Laccase exhibited phenol oxidase activity towards various lignin related compounds. The laccase of Pleurots ostreatus and other fungal laccases had shown high affinity for phenolic compounds containing methylated and hydroxy groups directly attached to the benzene ring but no affinity towards non-phneolic compounds such as p-trans cinnamic acid, and 3,4-methoxy cinnamic acid (Youn et al., 1995).

**Crystallization**

The blue copper oxidases, including laccases are composed of three domains as revealed by x-ray structure analysis (Malmstrom and Leckner, 1998) and structure based sequence alignment (Murphy et al., 1997). Several attempts have been made for laccase crystallization but due to the micro-heterogeneity of protein, those attempts were unsuccessful (Esser and Minuth, 1971; Eggert et al., 1996). There may be multiple causes of charge heterogeneity in proteins, eg., the de-amidation of liable amide containing amino acids (Solstad and Flatmark, 2000) and variations in the degree of
glycosylation (Macmillan et al., 2001). Enzymatic deglycosylation of laccase was performed in an attempt to improve the diffraction quality of single crystals (Ducros et al., 1997), a procedure which apparently led to the loss of the type two copper. Ducros et al., (1998) crystallized and determined the structure of type 2 copper depleted laccase from Coprinus cinereus. They reported that the laccase is a monomer composed of three cupredoxin-like β-sandwich domines, similar to that found in ascorbate oxidase. Recently, Antorini et al., (2002) successfully crystallized x-ray diffracted the fully glycosylated laccases from two white rot fungus P. cinnabarinus and T. versicolor.

**Laccase applications:**

Oxidation reactions are widely used in industrial processes, for example in the textile, food, wood processing, pharmaceutical and chemical industries. Many of the currently used oxidation methods are not economically or environmentally satisfactory, because they produce unwanted side reactions and the oxidants or reaction catalysts are often toxic. Enzymatic oxidation is a potential alternative to chemical methods, because enzymes are very specific and efficient catalysts, and are ecologically sustainable. Laccases are currently seen as very interesting enzymes for industrial oxidation reactions, because they are capable of oxidizing a wide variety of substrates. In addition, they use readily available molecular oxygen as an electron acceptor instead of expensive cofactors such as NAD(P)⁺.

Laccase are currently studied intensively for many applications and they are already used in large scale in the textile industry. Together with low molecular weight redox-mediator compounds, laccases can generate a desired worn appearance on denim by bleaching indigo dye (Pedersen and Kierulff 1996; Campos et al., 2001). They could also be used for decolorizing dye house effluents, that are hardly decolorized by conventional sewage treatment plants (Abadulla et al., 2000; Wesenberg et al., 2003). In addition to dye house effluents, laccases can decolorize waste waters from olive oil mills (D'Annibale et al., 2000; Dias et al., 2004) and pulp mills (Manzanares et al., 1995) by removing colored phenolic compounds. Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases are able to oxidize toxic
organic pollutants, such as polycyclic aromatic hydrocarbons (Collins et al., 1996) and chlorophenols (Gianfreda et al., 1999; Ahn et al., 2002). The most useful method for this application would probably be inoculating the soil with fungi that are efficient laccase-producers, because the use of isolated enzymes is not economically feasible for soil remediation in large scale.

The involvement of fungal laccases in lignin biodegradation has raised interest in the use of laccases in lignocellulose processing. The proposed applications include pulp bleaching (Bourbonnais and Paice 1992; Call and Mucke 1997) and fiber modification (Felby et al., 1997; Chandra and Ragauskas 2002). Laccases are able to de lignify pulp when they are used together with mediators (Bourbonnais and Paice 1992; Call and Mucke 1997). The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates (Bourbonnais and Paice 1990; Bourbonnais and Paice 1992; Call and Mucke 1997). Although the laccase-mediator system has been studied extensively, there are still unresolved problems concerned with mediator recycling, cost and toxicity. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be used in the enzymatic adhesion of fibers in the manufacturing of lignocellulose-based composite materials, such as fiber boards. Laccase has been proposed to activate the fiberbound lignin during manufacturing of the composites, and boards with good mechanical properties have been obtained without toxic synthetic adhesives by using laccases (Felby et al., 1997; Hilttermann et al., 2001). Another possibility is to functionalize lignocellulosic fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolic acid derivatives onto kraft pulp fibers (Lund and Ragauskas 2001; Chandra and Ragauskas 2002). This ability could be used in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties, such as hydrophobicity or charge.
Because laccases are able to catalyze electron-transfer reactions via a direct mechanism, i.e. without additional cofactors, their use has also been studied in biosensors that detect various phenolic compounds (Ghindilis et al., 1992; Lisdat et al., 1997; Kuly and Vidzijuaitė 2003), oxygen (Gardiol et al., 1996) or azides (Leech and Daigle 1998). In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems (Chen et al., 2001).

In the food industry laccases have potential in wine, fruit juice and beer stabilization by removing the polyphenols that cause haze formation and discoloration (Cantarelli et al., 1989; Giovanelli and Ravasini 1993; Minussi et al., 2002). In addition, laccases can be used in baking to improve the mixing properties of the dough and the structure of the baking product (Si 1993; Labat et al., 2001). 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 (Roure et al., 1992; Aaslyng et al., 1996; Xu 1999). In the future laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative de protection (Semenov et al., 1993) and production of complex polymers and medical agents (Xu 1999; Mai et al. 2000; Uyama and Kobayashi 2002; Kurisawa et al., 2003; Nicotra et al., 2004).

Dye decolorization and bioremediation of dye effluent and dye contaminated soil by white rot fungi

A dye is a substance used to impart colour to fabrics, food and other objects for their beautification. Based on the chemical composition, synthetic dyes are classified as azo dyes, nitro dyes, triphenylmethane dyes, phthalocyanin dyes, indigoid dyes and anthraquinone dyes. Based on the application and usage, the dyes are classified as acid dyes, basic dyes, reactive dyes, polyazo dyes, vat dyes, azoic or naphthal dyes and disperse dyes.

Synthetic dyes are used extensively for textile dyeing, paper printing and colour photography and as additives in petroleum products. About 10 000 different dyes and pigments exist and over $7 \times 10^5$ tons of these dyes are produced annually (Vaidya and Datye, 1982). These dyes released mostly into the aquatic environment primarily from
textile and dyestuff industries (Ankliker, 1979). Azo dyes constitute the largest class of
dyes used and their mutagenic, carcinogenic and toxic potential have been extensively
studied along with the risk of occupational cancer associated with their use (Joachim et
al., 1985; Gonazoles et al., 1988). Their fate in the environment is uncertain, depending
on many unknown factors (Chung and Steven, 1993). Almost 15 % of the total dyes
consumed in dyeing process may be found in wastewater (Zollinger, 1987). Therefore, it
is hard to remove them from effluent by means of conventional biological wastewater
treatments, for example activated sludge treatment.

Pollution problems caused by dyes

The major environmental problem of colorants is the removal of dyes from
effluents (Zollinger, 1987). The untreated effluents of these industries may be highly
coloured and thus particularly dangerous when discharged into open water bodies. The
concentration of dye may be much less than 1 ppm but the dye is visible even at that
lesser concentration.

The problems caused by them are (a) sun light penetration of the streams would
be reduced, which is essential for photosynthesis consequently, the ecosystem of the
steam will be seriously affected (Kuo, 1992) (b) Toxicity to fish and mammalian life (c)
Inhibits activity and growth of microorganisms, particularly in high concentration. Some
cationic species (mostly triphenylmethanes) affected the flora and fauna even at lesser
concentrations (Meyer, 1981). (d) Possible chronic risks of colorants and their
intermediates, which are carcinogenic and to a lesser extent sensitizing and allergic (Kuo,
1992). (e) Intestinal cancers are the most common in highly industrialized societies and
possible connection between these tumors and the users of azo dyes has been
investigated. (f) Some dyes are reported to cause cerebral abnormality in fetuses and
skeletal abnormalities.

Several amino substituted azo dyes including 4 – phenyazoaniline and N-methyl
and N, N – dimethyl – 4 – phenylazo anilines are mutagenic as well as carcinogenic. The
carcinogenicity of an azo dye may be due to the dye itself or to aryl amine derivatives
generated during the reductive biotransformation of the azo linkage (Kulla, 1981; Zimmerman et al., 1982). Fujita and Pesiach (1977) reported that azo dyes are reduced to the aryl amines by cytochrome P-450 and a flavin dependent cytosolic reductase in mammals. Gray et al., (1992) indicated that in female and male mice exposed to congo red (II), the utero gonadal development has been adversely affected in both the sexes however, only females displayed reduced fertility. Many synthetic dyes are characterized by high stability in light and during washing which gives them recalcitrance to biodegradation (Cripps et al., 1990) Many authors have described non-specific fungal oxidative enzymes which attach a wide range of anthraquinonic and azo dye outside the cell (Cripps et al., 1990; Ollika et al., 1993).

**Effluent Treatment**

The coloured effluents resulting from different industries contain considerable amount of different pollutants due to which it is not possible to discharge the untreated water either into the water course or into a municipal sewage without causing damage, since it contains a variety of processed chemicals and dyes.

Removal of colour from industrial effluent is a major problem and tighter constraints on discharges are forming waste creators and managers to consider new options for effluent treatment and disposal (Parker, 1995). Hence decolorizations as well as the methods of decolorization are important. Methods of effluent treatment for dyes may be classified into three main categories: physical, chemical and biological as follows.

**Methods of effluents treatment**

<table>
<thead>
<tr>
<th>Physical</th>
<th>Chemical</th>
<th>Biological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>Neutralization</td>
<td>Stabilization</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>Reduction</td>
<td>Aerated lagoons</td>
</tr>
<tr>
<td>Floatation</td>
<td>Oxidation</td>
<td>Trickling filter</td>
</tr>
</tbody>
</table>
Flocculation | Electrolysis | Activated sludge
---|---|---
Coagulation | Ion exchange | Anaerobic digestion
Foam fraction | Wet air oxidation | Bioaugmentation
Polymer flocculation | - | -
Reverse Osmosis | - | -
Ultrafiltration | - | -
Ionization radiation | - | -

Unfortunately, the physical and chemical methods of effluent treatment have high operating costs and of limited applicability (Waters, 1984; Cooper, 1993). An effective and inexpensive biological treatment system would be of great value.

**Biological treatment of dyes**

An organic chemical introduced into a terrestrial or aquatic ecosystem may be subjected to enzymatic or non-enzymatic reactions brought about by inhabitants of the environment (Alexander and Lustigman, 1966). This method is most effective and economical for the treatment of organic and certain inorganic wastes. Biological removal of degradable organics involves a sequence of steps including mass transfer, adsorption, absorption and biochemical enzymatic reactions (Rao and Dutta, 1978).

Many synthetic dyes are recalcitrant to biological degradation under conditions normally found in wastewater treatment plants (Shaul et al., 1991; Cripps et al., 1990). Such recalcitrance is desirable for a commercial textile dye under typical usage conditions (Seshadri et al., 1994). The metabolism of pure dyes has been extensively studied but little has been published on biological treatments of industrial dye effluents (Abadulla et al., 2000; Wesenberg et al., 2002).

Many microbes have been isolated which can catalyse anaerobic reductive fission of the azo linkage resulting in formation of colourless aromatic amines (Chung and...
Steven, 1993). These can be highly toxic and carcinogenic and anaerobic degradation of many of these aromatic intermediates have not been reported further, on exposure to air, some of these products may be oxidized to highly coloured compounds (Knapp and Newby, 1995).

Glenn and Gold (1983) first demonstrated that ligninolytic cultures of *Phanerochaete chrysosporium* decolorize several polymeric dyes. Decolorization of the azo dyes such as orange II, tropeolin O, congo red, acid red 114, acid red 88, biebrich scarlet, direct blue 15, chrysophenine, tetrazine, and yellow 9 (Cripps et al., 1990; Raffi., 1990; Paszcznski et al., 1991) triphenyl methane dyes, basic green 4, crystal violet, brilliant green, cresol red, bromophenol blue and para rose anilines (Bumpus and Brock, 1988) by various fungi has been reported. Colleen et al. (1990) has reported the degradation of azo and heterocyclic dyes or orange II, tropeolin O, congo red and azure B by *P. chrysosporium* culture medium. The degradation of azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes by *P. chrysosporium* was most extensively studied (Bumpus and Brock, 1988; Cripps et al., 1990; King and Neto, 1991; Ollika et al., 1993).

Cripps et al., (1990) reported that *P.chrysosporium* removed 87 to 93 % of orange II, tropeolin O and congo red and the mycelial mats were visibly coloured after 5 days of incubation. Further they have reported that congo red was resistant to decolorization by *Phanerochaete chrysosporium* in nitrogen limited cultures and remained tightly bound to the fungal mycelium after 12 days of incubation. N-limited ligninolytic cultures of *Trametes versicolor* have been reported to degrade PCBs, anthracene, fluorine, phenanthrene, and dichloroaniline (Morgan et al., (1992) showed that 54 % decolorization of congo red occurred in the presence of a crude preparation of lignin peroxidase and hydrogen peroxide. Heinfling et al., (1997) have screened 18 fungal strains for their potential to decolorize commercially used reactive textile dyes (reactive orange 96, reactive violet 5 and reactive black 5) and two phthalocyanine dyes (reactive blue 15 and reactive blue 38). They reported that only *Trametes versicolor*, *Bjerkandera adusta* and *Phanerochaete chrysosporium* were able to decolorize all the dyes. The dye
HRB 8 was decolorized rapidly within 24 th to below 40 % by Bjekandera adusta and Trametes versicolor and 95 percent decolorization was achieved within 4 days.

Knapp and Newby (1995) observed that in many cases adsorption of dye is in the microbial cell surface primary mechanism of decolorization. Enzymes such as Lip, MnP and laccase are involved in the decolorization of the dyes (Vyas and Molitoris, 1995). Kim et al., (1996) reported the presence of $\text{H}_2\text{O}_2$ dependent RBBR decolourizing enzymatic activity in the culture filtrate of Pleurotus ostreatus in chemically defined medium. Young and Yu (1997) suggested the binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation by extracellular and intracellular enzymes as reasons for the colour removal. The dye-saturated mycelium can be regenerated and used for repeated dye adsorption. They have further stated that the dyes were not decolorized by manganese dependent peroxidase (MnP) while above 80 % colour was removed by Ligninase catalyzed oxidation. Dyes with different structures are decolorized at different intrinsic enzymatic rates and high dye concentration results in slower decolorization rate (Abadulla , et al., 2000). The dye was adsorbed to the mycelial pellets in both ligninolytic and non-ligninolytic cultures. Wang and Yu (1998) reported the adsorption of acid green 27, acid violet 7 and indigo carmine dyes on living and dead mycelium of Trametes versicolor. The physical desorption and enzymatic degradation of the adsorbed dye molecules were also investigated and reported that the enzymatic degradation of adsorbed dyes was the major mechanism in which the regeneration of dye adsorption capacity of the mycelium was achieved.

Kim and Shoda (1999) purified and characterized a novel peroxidase (Dyp) from Geotrichum candidum that is responsible for the decolorizing activity of nine of the 21 types of dyes. Swamy and Ramsay (1999) reported that in the white rot fungus Trametes versicolor, the enzyme lignin peroxidase (LiP) was not detected during decolorization of the azo dye of amaranth instead laccase and manganese peroxidase (MnP) were detected in the decolorizing cultures. They did not exclude the involvement of extracellular MnP and laccases in decolorization of the dye, but also pointed out the demand for bio-mass associated factors during the removal of the dye colour. However, others have shown
good correlation between dye decolorization and peroxidase and H$_2$O$_2$ production by fungus (Swamy and Ramsay, 1999) Ollika et al., (1993) found that dyes belonging to four different groups (Polymeric, azo, heterocyclic and triphenyl methane) were decolorized by lignin peroxidase but in some cases veratryl alcohol was required for decolorization. Biodegradability of dyes depends on the presence of very specific changes in their molecular structure (Paszczynski and Crawford, 1995). Dyes that are anthraquinonic derivatives, remazol brilliant blue R and number of polymeric dyes synthesized from RBBR serve as substrate for the lignin degrading enzymatic system of wood decaying fungi. Their decolorization has also been used as an indicator and measure of the lignolytic activity (Freitag and Morrell, 1992). It was found that some azo and anthraquinonic dyes were good substrates for assaying the LiP, MnP and laccase. Abdhulla et al., 2000 described the decolorization of anthraquinic dyes which was faster than that of azo dyes by both Trametes hirsute and its purified laccase.

Studies on mineralization of several dyes have revealed that most of the dyes investigated were degraded extensively only in low nitrogen concentrations both in solid and liquid medium (Swamy and Ramsay, 1999; Kapdan et al., 2000; Hatvani and Mecs, 2002). Hatvani and Mecs, (2002) reported Lentinus edodes decolorized the dyes in the medium without nitrogen source but this was greatly enhanced in the presence of low concentration of an inorganic or organic nitrogen source.

The sequence adsorption and degradation of dye molecules on living fungal hyphae may provide a mechanism for feasible application of white rot fungi in a continuous treatment of industrial effluent (Wang and Yu, 1998).

**Immobilization of Enzymes**

The development of methods for removing and transforming toxic compounds has increased in recent years. Biodegradation appears a promising technology, particularly the use of oxidative enzymes as biocatalyst, included in a microorganism or used as free enzyme. Laccase has received particular attention because of its ability to catalyze the oxidation of a wide spectrum of molecules containing an aromatic ring substituted with
electron withdrawing groups (d’ Annebale et al., 1999). Enzyme immobilization usually allows a good preservation of enzyme activity over a long period (d’ Annibale et al., 1999). The efficiency of enzyme extract is enhanced by selective adsorption when immobilized, as reported by Tatsumi et al., (1996), in the removal of chlorophenols from wastewaters by peroxidase immobilized on magnetite. In most cases, laccases are immobilized on porous beads. Xenobiotics are degraded in bed-packed column reactors. However immobilization of enzymes on a membrane and the use of filtration offer advantages. First it allows the simultaneous downstream separation of the transformation products, when they are insoluble, secondly flow rates can be higher than with packed beads, because all the substrate flows through the support instead of diffusing in the bead pores. Some of the intended applications such as kraft pulp bleaching, dye effluent using laccase involve high pH. However, a laccase from Corpinus cinereus recently has been proved to be active at elevated pH range (Schneider et al., 1990). Being added to alkaline detergents, the laccases are able to oxidize various textile dyes to bleach the undesirable colour in washing solution.

The potential advantages of enzymatic treatment included application to (1) biorefractory compounds (2) operation at high and low concentrations of contaminants (3) operation over a wide range of pH, temperature and salinity (4) absence of shock loading effects (5) absence of delays associated with the acclimatization of biomass (6) reduction in sludge volume and the ease and simplicity in controlling the process (Nicell et al., 1993).

Application of immobilized laccase

Several industrial dyes were decolorized biocatalytically by extracellular enzymes (Rodriguez et al.,) Laccase has been used as a possible candidate for the treatment of effluent from the bleaching plant (Milstein et al., 1988; Lankinen et al., 1991). Royer et al., (1991) reported that the laccase obtained from Corious versicolor efficiently decolorized pulp effluent in immobilized form than the free form. The detoxification effect of laccases in reaction with xenobiotics was shown using intact fungal cultures (Kadim et al., 1999). Isolated laccases (Bollag et al., 1988; Imura et al., 1996; Ullah et
al., 2000) and immobilized laccase in different substrates (Shuttleworth and Bollag, 1986; Davis and Burns, 1993) had shown advantages in increased stability, resistance to unfavorable conditions of reaction and shifted catalytic properties.

Leotievsky et al., (2001) reported that the immobilized laccase of *Coriolous versicolor* was more active in strongly acidic and alkaline pH, more stable and active at high temperature and more tolerant to the effect of the strong inhibitor, sodium azide. These properties as well as the shift of optimal pH towards alkaline values and the increase in the optimal reaction temperature to at least 60 °C are good reasons to apply such systems to practical purposes.

Chivukula and Ranganathan (1995) reported that purified laccase of *Pyricularia oryzae* oxidized number of phenolic dyes including methyl, methoxy-, chloro-, and nitro- substituted derivatives of 4-4(4′-sulfophenylazo)-phenol. This enzymatic oxidation produced quinines and released molecule nitrogen from azo linklages, thus reducing the formation of toxic aromatic amines. The laccase can act on chromopheric compounds such as remazol brilliant blue R, triphenylmethane dyes suggested a potential application in bleaching or decolorization of industrial process (Schliphake and Lonergan, 1996), Rodrigue et al., (1999), Abdulla et al., (2000) and Schliphake et al., (2000) and Trejo-Hernndoez et al., (2001) reported that immobilize laccase of *Trametes hirusta* and *Pycnoporus cinnabarinus* degrade many commercial textile dyes. Recently, Soares et al., (2002) reported that purified laccase of *Aspergillus niger* for the degradation of several phenolic diazodyes with addition of redox mediator enhanced the degradation rate.

**Effluent Treatment**

The successful application of white rot fungi for decolorization of effluent has been achieved in several white rot fungi. Spadaro et al., (1992) reported that *Phanerochaete chrysosporium* has potential application for the clean up of textile mill effluent. Bakshi et al., (1999) reported 45.6 % of decolorization was achieved by *P.chrysosporium* in synthetic dye effluent. Immobilized laccase of *Corilopsis gallica* effluently decolorized the dye effluent (Reyes et al., 1999). Abadulla et al., (2000)
reported that the textile dye effluent treated with the partially purified laccase reused for dyeing process. Recently, Wesenberg et al., (2002) reported that the white rot fungus *Clitocybula dusenii* partially decolorized the dye wastewater and the fungus produced higher manganese peroxidase and laccase activities when grown in effluent.

**Bioremediation of contaminated soil**

A contaminated site usually has a variety of contaminants and no single set of site characteristics will favour biodegradation of all chemical contaminants. An important factor in determining the suitability of the contaminated site for bioremediations the type of bioremediation to be implemented. Most pollutant degrading microorganisms isolated and characterized in laboratory are now thought to make a minor contribution to bioremediation (Watanabe, 2001). The spent substrate from the mushroom farms served as a good substrate for bioremediation of the contaminated soils (Okeke et al., 1997) and rich source for lignolytic enzymes.

Heavy metals such as arsenic, copper, cadmium, lead and chromium are dangerous contaminants found in a number of industrial and mining sites sewage sludge and landfill leachate (Pradham and Levine, 1992). In view of the high toxicity off the heavy metals, they must be prevented from being discharged into water bodies.

Baldrain et al., (1996, 2000) described the effect of heavy metals on lignolytic enzyme production in relation with remediation of polycyclic aromatic hydrocarbons contaminated soil. The enzyme activities of *Pleurotus ostreatus* in sterile and non-sterile and non-sterile soil. The activites of both MnP and laccase were higher in non-sterile soil colonization. Recently Sant et al., (2002) observed that fungi showed the highest contribution to in situ biodegradation of poly (-3 - hydroxybutryte-co-3-hydroxyvalerate) film in soil.

Bollag and Leonowicz (1984) suggested that it is possible to enhance the natural. Process of xenobiotics binding and incorporation into humus by adding enzymes such laccase to contaminated soils. Bindings of pollutants of humic material would decrease
the amount of pollutant available to internal flora and fauna and reduce the toxicity of the pollutant through the coupling process.

Biodegradation of environmental pollutants by white-rot fungus has been demonstrated for numerous pollutants in both liquid and soil (Barr and Aust, 1994). High amount of lignolytic enzyme production, robust growth, capability of soil colonization and relative resistance to the inhibitory action of soil bacteria and toxic compounds are the most essential characters for a fungal strain to remediate the contaminated soils (Morgan et al., 1993). Novotny et al., (2001) reported that Irpex lacteus and Pleurotus ostreatus are the potential candidates for water and soil bioremediation.