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2.1. Phenolic Pollution

One of the major problems faced by the world today is the environmental contamination arising as the result of industrialization and extensive use of pesticides. Contaminating chemicals can exert detrimental effects to the health and safety of people and the environment (U.S.EPA 1980). The persistent nature of many pollutants contributes to the potential risks and difficulty of remediation (Pascazi 2008).

Most of the hazardous pollutants are phenolic in nature and persists in the environment. Phenolic compounds are listed as a priority pollutant by the US EPA (code U188). Phenols enter the surface water from a variety of industries like pulp and paper mill, leather, polymer, oil refinery, coal carbonization, wood processing and preservation plants, coir industrial effluents etc to site a few (Paula and Young 1998; U.S.EPA, 1980). The presence of phenol in water imparts carbolic odor to receiving water bodies and can cause toxic effects on aquatic flora and fauna (Ghadhi and Sangodkar 1995; Nair et al. 2008). Some of these compounds such as pentachlorophenols, hydroxy polycarbonated biphenyls (PCBs), benzene, toluene, xylene, trinitrotoluene, tetrachlorophenol, hexachlorobenzene, bisphenol A and nonylphenol are known to have endocrine disrupting, carcinogenic and or mutagenic effects (Braun et al. 1977; Hardell et al. 1981; Paulmurugan et al. 2004; Prasanth et al. 2010, Prasanth et al. 2012). By-product contaminants in pentachlorophenol used in wood preservatives
include various dioxins and furans (Environmental Health Criteria 71, Pentachlorophenol, WHO, Geneva, 1987). These chemicals are without any doubt the most toxic chemicals ever known to mankind (Pascazi 2008; Environmental Health Criteria 71, Pentachlorophenol, WHO, Geneva, 1987). Natural sources of phenol include forest fire, natural run off from urban area where asphalt is used as the binding material and natural decay of lignocellulosic material (Nair et al. 2008).

One of the potential sources of phenolic pollution of the backwaters/ estuaries of Kerala is coir industry. The coastal region of Kerala State, India produces 60% of the total world supply of white coir fiber. Coir can be extracted from the coconut husk by retting or by mechanical extraction. Coconut husks are suspended in a river or water-filled pit for up to ten months- a process known as retting. Retting is environmentally hazardous due to the release of the toxic compounds such as polyphenols. The coir industry retting effluent is discharged from retting ground once in two months at a rate of 1000 m$^3$ with high salinity and BOD (Ravindranath and Sharma, 1995). Direct landfill of coir processing effluents has created various problems such as leachate, rich in polyphenols polluting ground and surface waters (Das and Saema 1998). Polyphenols are major constituents, representing as much as 75-76 g kg$^{-1}$ of husk material (Jayashankar and Bhat, 1966). Polyphenols come mostly from lignin during its biodegradation and plays a key role in the formation of humic substances (Nelliyat 2003, Van Schie and Young 1998; Arts et al. 2005). Phenolic acids
such as ferulic, p-coumaric, p-hydroxy benzoic and gentisic were identified by Gomes and Mavinkurve (1982) from the Goa estuarine complex. Deepa (2001) reported the presence of phenolic compounds namely resorcinol, catechol and phenol are present in the coir fibre steep liquor. The toxic effects of polyphenols to flora and fauna of estuaries make their survival difficult (Ravindranath and Sarma 1995). Paulmurugan and co workers (2004) reported elevated concentration of phenol in the retting zones (0.47 mg l\(^{-1}\)) compared to non retting zones (0 to 0.11 mg l\(^{-1}\)).

2.2. Microbial Enzymes in Phenolic Bioremediation

Despite the toxicity of phenols and its derivatives, numerous microorganisms are capable of degrading these compounds through the action of variety of enzymes (Kidwai et al. 2012; Nair et al. 2008). These enzymes may include hydroxylases (phenol 2-monooxygenase), peroxidases and phenoloxidases which include polyphenols oxidases or laccase.

2.2.1. Phenol 2-monooxygenase (E.C 1.14. 13.7)

Catalyses the degradation of phenol via two different pathways initiated either by ortho or meta cleavage. Catalyzes the chemical reaction

\[
\text{Phenol} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightleftharpoons \text{catechol} + \text{NADP}^+ + \text{H}_2\text{O}
\]
The substrates of this enzyme are phenol, NADPH, H⁺, and O₂, whereas its products are catechol, NADP+, and H₂O. It participates in 3 metabolic pathways: gamma-hexachlorocyclohexane degradation, toluene and xylene degradation. The systematic name of this enzyme class is phenol, NADPH: oxygen oxidoreductase (2-hydroxylating). Other names in common use include phenol hydroxylase, and phenol o-hydroxylase. It employs one cofactor, FAD.

2.2.2. Lignin peroxidases (EC.1.11.1.14)

Peroxidases are one of the major Lignin Degrading Enzymes (LDEs) acting directly or indirectly on lignin (Higuchi 1990). Peroxidases comprise of lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). All of these enzymes can act with low molecular weight mediators to bring about lignin oxidation. Lignin peroxidases have molecular masses of approximately 40 kDa, are glycosylated, and have acidic PIs and pH optima. They contain a single ferric protoporphyrin IX heme moiety and operate via a typical peroxidase catalytic cycle. Enzyme is oxidised by 2O₂ to a two-electron deficient intermediate termed Compound I, which returns into its resting state through two one-electron oxidations of donor substrates. The one-electron deficient intermediate is termed Compound II. The key characteristic of LiP is its capability to oxidise non-phenolic compounds or moieties. LiPs are powerful oxidants that can also oxidise several aromatic ethers and polycyclic aromatics (Kersten et al. 1990; Field et al. 1993). LiP-
catalysed oxidation results in aryl cation radicals that can react as a radical and as a cation, forming a wide variety of degradation fragments (Higuchi 1989).

2.2.3. Manganese peroxidase (E.C. 1.11.1.13)

They have a similar catalytic cycle as LiP, but it utilizes Mn (II) as a substrate. Compound I can oxidise phenolic compounds or Mn (II), but compound II can apparently oxidise only Mn (II) (Wariishi et al. 1988). The product of Mn (II) oxidation, Mn (III), must be chelated by organic acids, such as oxalate or glycolate, which stabilise the ion and promote its release from the enzyme (Glenn and Gold 1985; Glenn et al. 1986; Wariishi et al. 1988; Kishi et al. 1994). However, the Mn (III)-chelate is not a strong oxidant, and can oxidise only the phenolic moiety of the lignin (Wariishi et al. 1991; Tuor et al. 1992). Studies indicate that MnP may oxidise Mn(II) without H2O2 with decomposition of malonic acid, and concomitant production of peroxyl radicals (Hofrichter et al. 1998).

2.2.4. Versatile peroxidase (E.C. 1.11.1.16)

Oxidizes a variety of substrates including Mn+2, phenolic and nonphenolic lignin dimmers, veretfly alcohol, dimethoxybenzenes, azodyes, substituted phenols and hydroquinones (Heinfling et al. 1998; Martinez 2002; Martinez et al. 2004). Thus it combines the substrate specificity characteristics of LiP, MnP and cytochrome c peroxidases. VP has a Mn-binding site similar to MnP and an
exposed tryptophan residue homologues to that involved in veratryl alcohol oxidation by LiP. Phenoloxidases comprise of tyrosinase (monophenol monooxygenase, (EC 1.14.18.1), catechol oxidase (EC 1.10.3.1) and Laccase (Lcc, EC 1.10.3.2).

2.2.5. Tyrosinase (EC 1.14.18.1)

Tyrosinase (monophenol monooxygenase) is involved in the phenomenon of browning of fruits and is present in mushrooms, apples and potatoes. This enzyme catalyzes phenol oxidation with molecular oxygen through two distinct reactions: the ortho-hydroxylation of phenol producing catechols and its dehydrogenation producing quinones. Quinones are extremely unstable in aqueous solutions, reacting nonenzymatically to produce brownish insoluble polymers (Mayer and Harel 1979). These polymers can be removed from solution through many processes such as adsorption, precipitation and flotation.

2.2.6. Catechol oxidase (EC 1.10.3.1)

Catechol oxidase catalyses the oxidation of phenols such as catechol. Catechol oxidase is a copper-containing enzyme whose activity is similar to that of tyrosinase. Catechol oxidase carries out the oxidation of phenols such as catechol, using dioxygen (O2). In the presence of catechol, benzoquinone is formed. Hydrogens removed from catechol combine with oxygen to form water. This reaction, producing the brown compound benzoquinone, is a form
of enzymatic browning exhibited in many foods upon exposure to oxygen (Solomon et al. 2001).

2.2.7. Laccase (EC 1.10.3.2)

Laccase (benzenediol:oxygen oxidoreductase) is one of a small group of enzyme called the large blue copper proteins or blue copper oxidases. The other members of this group are the plant ascorbate oxidases, the mammalian plasma protein ceruloplasmin and phenoxazinone synthase from *Streptomyces antibioticus* (Freeman et al. 1993; Thurston 1994; Xu 1996). The ability of laccases to oxidize phenolic compounds and reduce molecular oxygen to water has led to intensive studies of these enzymes (Camarero et al. 2012; Li et al. 2012; Nagai et al. 2002; Murugesan et al. 2006; Lu et al. 2007; Liersa et al. 2007; Piscitelli et al. 2010; Tamaki et al. 2010). This thesis is an investigation into the production of laccase by the fungi involved in coir retting.

2.3. Occurrence of laccases

Laccase is widely distributed in higher plants, fungi and bacteria (Ausec et al, 2011; Ihssen et al. 2011; Messerschmidt and Huber, 1990; Reiss et al. 2011; Sirim et al. 2011) and has also been found in insects (Catalano et al. 2011). Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Levine 1965; Thurston 1994) from which the designation laccase was derived. In 1896 laccase was demonstrated to be present in fungi for the first time by both
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Bertrand and Laborde (Bertrand 1896; Laborde 1896). The occurrence of laccases in higher plants appears to be far more limited than in fungi. Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine 1965). Laccase production occurs in various fungi over a wide range of taxa. Among them, basidiomycetes are considered to be efficient laccase producers, especially white rot fungi. Most of the laccases studied thus far are of fungal origin, especially from white-rot fungi, such as *Pleurotus ostreatus* (Palmieri *et al.* 2000), *Trametes versicolor* (Bourbonnais *et al.* 1995), *Lentinula edodes* (Nagai *et al.* 2002) and *Cerrena unicolor* (D’souza-Tichlo *et al.* 2009) to site a few. In the fungi, Ascomycetes and Deuteromycetes have not been a clear focus for lignin degradation studies as much as the white-rot Basidiomycetes. Laccase from *Monocillium indicum* was the first laccase to be characterized from an Ascomycete showing peroxidative activity (Thakker *et al.* 1992). Recently ascomycete fungi are also recognized as laccase producers (Kiiskinen *et al.* 2002; Liersa *et al.* 2007; Sadashivam *et al.* 2008).

2.4. Physiological Functions of Laccase

2.4.1. Lignification and Delignification

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). Laccases from white-rot fungi, such as *Trametes*
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).

2.4.2. Morphogenesis

Aspergillus nidulans has two laccases with different functions have been characterized. The product of thei A gene is a laccase uniquely involved in formation of the green colour of the conidium (Clutterbuck 1972). A second laccase is localized in the hulk cells and cleistothecial primordia and again is involved in pigment synthesis (Hermann et al. 1983). In a number of fungi such as Daldinia concentrica and Lentinus edodes, laccase activity is associated with pigment formation in structures that are more rigid than a simple mycelial aggregate. Fruiting body formation may involve phenol oxidase catalysed formation of extracellular pigments coupled to oxidative polymerization of cell wall components strengthening cell-to-cell adhesion (BuLock 1967; Leatham and Stahmann 1981). Laccases have also been proposed to participate in fungal morphogenesis in Armillaria spp. (Worrall et al. 1986), Lentinus edodes (Leatham and Stahmann 1981) and Volvariella volvacea (Chen et al. 2004). 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). Bacillus subtilis produces a thermostable CotA laccase which
participates in pigment production in the endospore coat (Martins\textit{et al.} 2002).

\textbf{2.4.3. Sclerotization}

Laccases or laccase-like activities have been found in some insects, where they have been suggested to be active in cuticle sclerotization (Sugumaran\textit{et al.} 1992; Dittmer\textit{et al.} 2004).

\textbf{2.4.4. Pathogenesis}

In plant-pathogenic fungi, laccases are important virulence factors. The grapevine grey mould, \textit{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\textit{et al.} 1988). Laccases have also been shown to be important for pathogenesis in the chestnut blight fungus \textit{Cryphonectria parasitica} (Rigling and van Alfen 1991; Choi\textit{et al.} 1992; Mayer and Staples 2002) and in the human pathogen \textit{Cryptococcus neoformans} (Williamson 1994).

\textbf{2.4.5. Plant Defense}

Laccases have been shown to be involved in the first steps of healing in wounded leaves (De Marco and Roubelakis-Angelakis 1997, Mayer and Staples 2002). The presence of laccase in the resin ducts of narcardiaceae suggests its role in defense against predators and pathogenesis by fungi and bacteria.

\textbf{2.5. The reactions catalyzed by laccase}
Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidized varies from one laccase to another. These enzymes catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four-electron reduction of oxygen to water (Thurston et al. 1994).

Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin (Paula and Young, 1998). A laccase which possess HIV-1 Reverse Transcriptase inhibitory activity from the broth of mycelial culture of the mushroom *Lentinus tigrinus* was recently reported (Xu et al. 2012). Kinetic data of laccases from different sources were reported (Yaropolov et al. 1994). $K_m$ values are similar for the co-substrate dissolved oxygen (about 5-10 M), but $V_{max}$ varies with the source of laccase (50–300 M/s). The turnover is heterogeneous over a broad range depending on the source of enzyme and substrate/type of reaction. The kinetic constants differ in their dependence on pH. $K_m$ is pH-independent for both substrate and co-substrate, while $K_{cat}$ is pH-dependent (Yaropolov et al. 1994).

Laccase has considerable overlap in their substrate affinities with tyrosinase and catechol oxidase (Burke and Cairney, 2002). Simple diphenols like hydroquinone and catechol are good substrates (for most laccases, but not all), but guaiacol and 2,6-dimethoxyphenol
are often better. However laccase can be differentiated from other phenol oxidases on the basis of their substrate specificity (Walker and McCallion 1980). Only tyrosinases can oxidize L-thyrosine and only laccase have the ability to oxidize syringaldazine in the absence of hydrogen peroxide as this compound is also oxidized by the manganese-dependent peroxidases (Eggert et al. 1996; Thurston 1994). Laccase can oxidize guaiacol but catechol oxidase cannot. Secondly, laccases are remarkably non-specific to their reducing substrate (Wood 1980, De Vries et al. 1986). Thus laccase oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but does not oxidize tyrosine (Thurston 1994). At present we are unaware of the full range of laccase substrates and still less the range of compounds that laccase activity can affect either directly or indirectly.

2.6. Structure and mode of action of laccase

The functional units of the enzyme laccase comprises three cupredoxin-like domains A, B and C and they are about equal in size and equally important for the catalytic activity (Ducros et al. 1998; Piontek et al. 2002; Hakulinen et al. 2002) (Figure 2.1). The substrate-binding site is located in a cleft between domains B and 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 that is trigonally coordinated to two histidines and a cysteine (His-
Cys-His motif) as shown in Figure 2.2. The coordination bond between T1 and Sulphur of Cys is highly covalent, which provides a strong absorption peak at around 600 nm and gives laccases their typical blue color, due to charge transfer between Copper atom and the Sulphur (Shin et al. 1996). T1 has also a distant axial ligand, usually a Leu or Phe residue in fungal laccases (Ducros et al. 1998; Piontek et al. 2002) and a Met residue in the Bacillus subtilis CotA laccase and in other multicopper oxidases (Enguita et al. 2003). The trinuclear cluster contains one type-2 (T2) and two type-3 (T3) coppers (Messerschmidt 2003). The T2 copper is coordinated by two and the T3 copper by six conserved histidines as shown in Figure 2.2 (Bertrand et al. 2002; Piontek et al. 2002).

When oxidized by a laccase, the 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 polymerization (Thurston 1994). During the process, the substrate donates an electron to the T1 copper. The reduction of oxygen takes place in the trinuclear copper center, which is, located about 12 Å away from T1 (Bertrand et al. 2002; Piontek et al. 2002).

One catalytic cycle involves the transfer of altogether four electrons, which are carried from T1 to the T2/T3 cluster presumably through a conserved His-Cys-His tripeptide (Bertrand et al. 2002; Messerschmidt 2003; Piontek et al. 2002).
Figure 2.1 Three-dimensional structure of *M. albomyces* laccase. Domains A, B, and C are colored red, green and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks (Hakulinen *et al.* 2002)

The bonds of the natural substrate, lignin, that are cleaved by laccase include, Cα-oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (Figure 2.3 a).
Figure 2.2: The laccase active site- The Type 1 copper is coordinate with two histidine ligands and two sulphurs one of methionine and the other of cysteine. The Type 2 center is 3-coordinate with two histidine ligands and water as ligands. The Type 3 coppers are each 4-coordinate, having three histidines ligands and bridging hydroxide (Duran et al. 2002)

Laccases are similar to other phenol-oxidising enzymes, which preferably polymerise lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups (Bourbonnais et al. 1995). But unlike other ligninolytic enzymes, laccase can oxidize only phenolic fragments of lignin due to the random polymer nature of lignin and to the laccase lower redox potential (Kersten et al. 1990; Evans et al. 2001). Beside these direct reactions, laccases catalyze the indirect oxidation of chemicals that are not phenols or amines. In that case, they need the presence of a redox mediator, which can be of natural or synthetic origin.
Figure 2.3 Oxidation of (a) phenolic subunits of lignin by laccase and (b) non-phenolic lignin model compounds by a laccase mediator system (Kunamneni et al., 2007).

Low molecular weight compounds with high redox potential than laccase itself (> 900 mV) can act as mediators (Figure 2.3) to oxidize the non-phenolic part of lignin (Eggert, 1990).

A mediator acts as a sort of ‘electron shuttle’: once it is oxidized by the enzyme generating a strongly oxidizing intermediate, the co-mediator (oxidized mediator), it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size could not directly enter into the active site. Furthermore, the use mediators allows the oxidation of polymers by side-stepping the inherent steric hindrance problems (enzyme and polymer do not have to interact in a direct manner) (Banci et al., 1999).
**Figure 2.4.** Catalytic cycle of a laccase-mediator oxidation system (Banci et al. 1999)

**Glycosylation:** Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Glycosylation of fungal laccase is believed to play a role in secretion, susceptibility to proteolytic degradation, copper retention and thermal stability (Call et al. 1997). The molecular mass of the monomer ranges from about 50 to 100 kDa. An important feature is a covalently-linked carbohydrate moiety (10–45% of total molecular mass), which may contribute to the high stability of the enzyme (Archibald et al. 1997; Call et al. 1997; Piontek et al. 2002; Shleev et al. 2004).

**Isozymes:** Many laccase producing fungi secrete isozymes which may originate from the same or different genes encoding for the laccase enzyme (Archibald et al. 1997). The number of isozymes present differs between species and also within species depending on nutrient medium composition and presence and absence of inducers (D’souza-Tichlo et al. 2009; Michniewicz et al. 2006; Assavanig et al. 1992). Furthermore, these different isozymes can modulate the physiology of the producing strain under different conditions (Assavanig et al. 1992).

## 2.7. Production of fungal laccases
Laccases occur as extracellular glyco-proteins, which allows for rapid removal from fungal biomass. Laccase activity was detected in the cultures of a wide range of fungi, from Ascomycetes to Basidiomycetes, and from wood- and litter-decomposing fungi to ectomycorrhizal fungi (Bollag and Leonowicz, 1984). One of the major limitations for the large-scale applications of fungal laccases is the low production rates by both wild type and recombinant fungal strains (Galhaup and Haltrich 2001). Laccase producing fungi constitutively produce low concentrations of various laccases even when cultivated in submerged or stationary conditions. Many studies have shown that higher concentrations can be induced by the addition of various compounds such as ABTS, guaiacol, 2,5-xylidine, syringaldehyde, veratryl alcohol, ferulic acid etc (Assavanig et al. 1992; D’souza-Tichlo et al. 2009; Michniewicz et al. 2006; Quaratino et al. 2007). Laccase induction in fungi is generally influenced by environmental conditions and medium composition (Assavanig et al. 1992; D’souza-Tichlo et al. 2009; Galhaup and Haltrich 2001; Lu et al. 2007; Liersa et al. 2007; Murugesan et al. 2006; Nagai et al. 2002; Sole et al. 2012; Tamaki et al. 2010). There are various response element sites in the promoter regions of laccase genes that can be induced by certain xenobiotic compounds, heavy metals or heatshock treatment (Faraco et al. 2002).

Studies suggest that the addition of copper sulphate to the cultivation media can result in enhanced laccase production compared to a basal medium (Dominguez et al. 2007; Palmieri
The carbon sources in the medium play an important role in ligninolytic enzyme production. Mansur and co-authors (Mansur 1997) showed that fructose induced 100-fold increase in laccase production of *Basidiomycete sp.* I-62. Glucose and cellobiose were efficiently and rapidly utilized by *Trametes pubescens* with high laccase activity (Galhaup *et al.* 2002). Similarly, the replacement of crystalline cellulose or xylan by cellobiose increased laccase activity of *C.unicolor* by 21- and 70-fold, respectively (Elisashvili *et al.* 2002). Furthermore, in *T. versicolor* lignocellulosic material (barly bran) increased almost 50-fold laccase activity compared to the control culture with glucose (Moldes *et al.* 2004). In the medium with the best carbon sources (mandarine peels and grapevine sawdust), both *Pleurotus eryngii* and *P. ostreatus* strain No. 493, showed the highest laccase activity (Stajic *et al.* 2006). Glucose showed the highest potential for the production of laccase (Lee *et al.* 2007). In contrast high concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase production (Eggert *et al.* 1998). An increase in the amount of glucose in the media resulted in a delay of the laccase production (Monteiro and De Carvalho 1998). An excess of sucrose or glucose in the cultivation media can reduce the production of laccase, as these components allow constitutive production of the enzyme, but repress its induction when applicable (Bollag and Leonowicz 1984). White-rot fungi ligninolytic systems are mainly activated during the secondary metabolic phase of the fungus and are often triggered by nitrogen depletion. Monteiro and De
Carvalho (Monteiro and De Carvalho 1998) reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7.8 g/g). Laccases were produced at high nitrogen concentrations (Buswell et al. 1995) although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. There is not much information available on the influence of pH on laccase production, but when fungi are grown in a medium of which the pH is optimal for growth (pH 5.0), the laccase will be produced in excess (Kunamneni et al. 2007). Most reports indicated initial pH levels set between pH 4.5 - 6.0 prior to inoculation, but the levels are not controlled during most cultivation (Vasconcelos et al. 2000; Zhao et al. 2010). Nyanhongo et al. reported that an initial pH of 7.0 was the best for optimal growth and laccase production by T.modesta (Nyanhongo et al. 2002). It has been found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light, but 30°C for laccase production when the cultures are incubated in the dark. In general the fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production (Vasconcelos et al. 2000; Zhao et al. 2010).

2.8. Laccase immobilization

Enzymes exhibit a number of features that make their use advantageous over conventional chemical catalysts. One of the main drawbacks to using free enzymes for detoxifying waste streams is their instability in different pH environments, non-reuse
of free enzymes and their high cost of production and purification. Many of these undesirable limitations may be overcome by the use of immobilized enzymes (Mateo et al. 2007). In order to increase the potential use of laccase in wastewater treatment processes, their immobilizations are necessary (Couto et al. 2004; Delanoy et al. 2005; Peralta-Zamora et al. 2003).

Immobilization is achieved by fixing enzymes to or within solid supports, as a result of which heterogeneous immobilized enzyme systems are obtained. By mimicking the natural mode of occurrence in living cells, where enzymes for the most cases are attached to cellular membranes, the systems stabilize the structure of enzymes, hence their activities. There are six main methods used for the immobilization of biocatalysts. These are: entrapment in polymer matrixes, adsorption of the charged biocatalyst onto oppositely charged support materials, covalent attachment to chemically activated supports, encapsulation inside semi-permeable membranes, aggregation of the biocatalyst particles into flocs or biospecific attachment to supports by means of lectins, etc., Combinations of these techniques can also be used, such as adsorption of the biocatalyst to a charged support followed by cross-linking place. In the immobilized form enzymes are more robust and more resistant to environmental changes allowing easy recovery and multiple reuse (Krajewska et al. 2004). Compared with the free enzyme, the immobilized enzyme has usually its activity lowered and the Michaelis constant increased (Durán et al. 2002). These alterations result from structural changes introduced to the
enzyme by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution. Enzymes may be immobilized by a variety of methods (adsorption, entrapment, crosslinking and covalent bonding) mainly based on chemical and/or physical mechanisms. Entrapment in alginate beads is one of the simplest methods of enzyme immobilization (Kierstan and Bucke 1977), but in some circumstances the low physical stability of the beads in the presence of chelating agents and large/small pore size of the alginate beads can be problematical.

Selection of immobilization conditions is essential to design a system appropriate to each particular purpose and enzyme. Since the methods for the immobilization procedures greatly influence the properties of the resulting biocatalyst, immobilization strategy determines the process specifications for the catalyst (Hartmeier 1988). Attempts have been made to immobilize laccase, such as immobilization on oxirane acrylic beads (D’Annibale et al. 2000), on porosity glass (Rogalsk et al. 1999), on copper alginate gel (Palmieri et al. 1994) activated carbon (Davis and Burns 1992), activated polyvinyl alcohol (PVA) (Yinghui et al. 2002) and on gold and other metals (Mazura et al. 2007).

Laccase is a copper-dependent enzyme and the enzyme immobilized in copper alginate is likely to retain more activity than laccase immobilized using other methods. Laccase of Pleurotus ostreatus and Ganoderma sp was successfully entrapped in copper
alginate beads and decolorized some synthetic dyes efficiently (Palmieri et al. 2005; Teerapatsakul et al. 2008).

2.9. Applications of fungal laccases

2.9.1. Delignification and pulp bleaching

In the industrial grounding of paper the separation and degradation of lignin in the pulp are traditionally obtained using ClO₂ and O₃. The pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also protect the integrity of cellulose (Gamelas et al. 2001; Cuoto and Herrera 2006). More recently, the potential of this enzyme for cross-linking and functionalizing lignocellulose compounds was discovered. Laccases can be used for binding fibre-, particle- and paper-boards (Gubitz et al. 2003).

2.9.2. Bioremediation

Laccases are known to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures (Wang et al. 2002). The degradation of a variety of persistent environmental pollutants, in particular phenols that are present in wastes from several industrial processes, such as coal industry, petroleum refining, production of organic compounds and palm oil production among others (Aggelis et al. 2003; Nuhoglu et al. 2005; Paula et al. 1998). Laccase have found immense application in the biotreatment plant for bioremediation of phenolic rich effluents (Cuoto and
Herrera 2006). Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants (Ehlers et al. 2005). Laccase was found to be responsible for the oxidation and subsequent polymerization of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. Laccases are also used to oxidize polycyclic aromatic hydrocarbons (PAHs), alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of mediators (Niku and Viikari 2000; Mougin et al. 2000; Alcalde et al. 2004; Majecherczyk et al. 2000).

2.9.3. Textile Industry

The use of laccase mediated dyeing of materials with sulfur and reduced vat dyes have been patented (Xu et al. 2000). In addition to their extensive use in pulp delignification, use of laccase for the treatment of textile (Camarero et al. 2005) and bleach-plant effluents (Manzanares et al. 2001) has also been investigated with success. Laccases have extensive application in the oxidation of dyes and their precursors (Abadulla et al. 2000; Mayer and Staples 2002)

2.9.4. For improving the production of ethanol

Laccases are used for improving the production of ethanol from renewable raw materials. Laccases oxidizes the phenolic intermediators of lignocellulosic hydrosylates which otherwise interferes with ethanol fermentation (Larsson et al. 2001)
2.9.5. Organic Synthesis

Recently, increasing interest has arisen on the application of laccase as a new biocatalyst in organic synthesis (Mayer and Staples 2002; Riva 2006). They are extensively used in the enzymatic conversion of chemical intermediates, and production of chemicals from lignin (Rama et al. 1998; Thurston 1994).

2.9.6. Production and treatment of various food products

The use of laccase for the production and treatment of various food products and as biosensor for the estimation of phenol or other enzymes in fruit juice has also been proposed (Gomes et al. 2004). Laccases are applied to certain process that enhance or modify their colour production and treatment of various food products (Gomes et al. 2004).

2.9.7. Prevention of wine decolouration

Laccases are used to remove phenolic compounds from white grape must during wine preparation (Cuoto and Herrera 2006).

2.9.8. Biosensor Development

Laccases are used as biosensor for the detection and estimation of phenol (Kidawi et al., 2012).

2.9.9. Digestive Aids
As tablets to aid digestion of fiber components in food (Gianfreda et al. 1999)

2.9.10. Design of biofuel cells

Laccases also finds applications in other field such as in the design of biofuel cells (Calabrese et al. 2002).