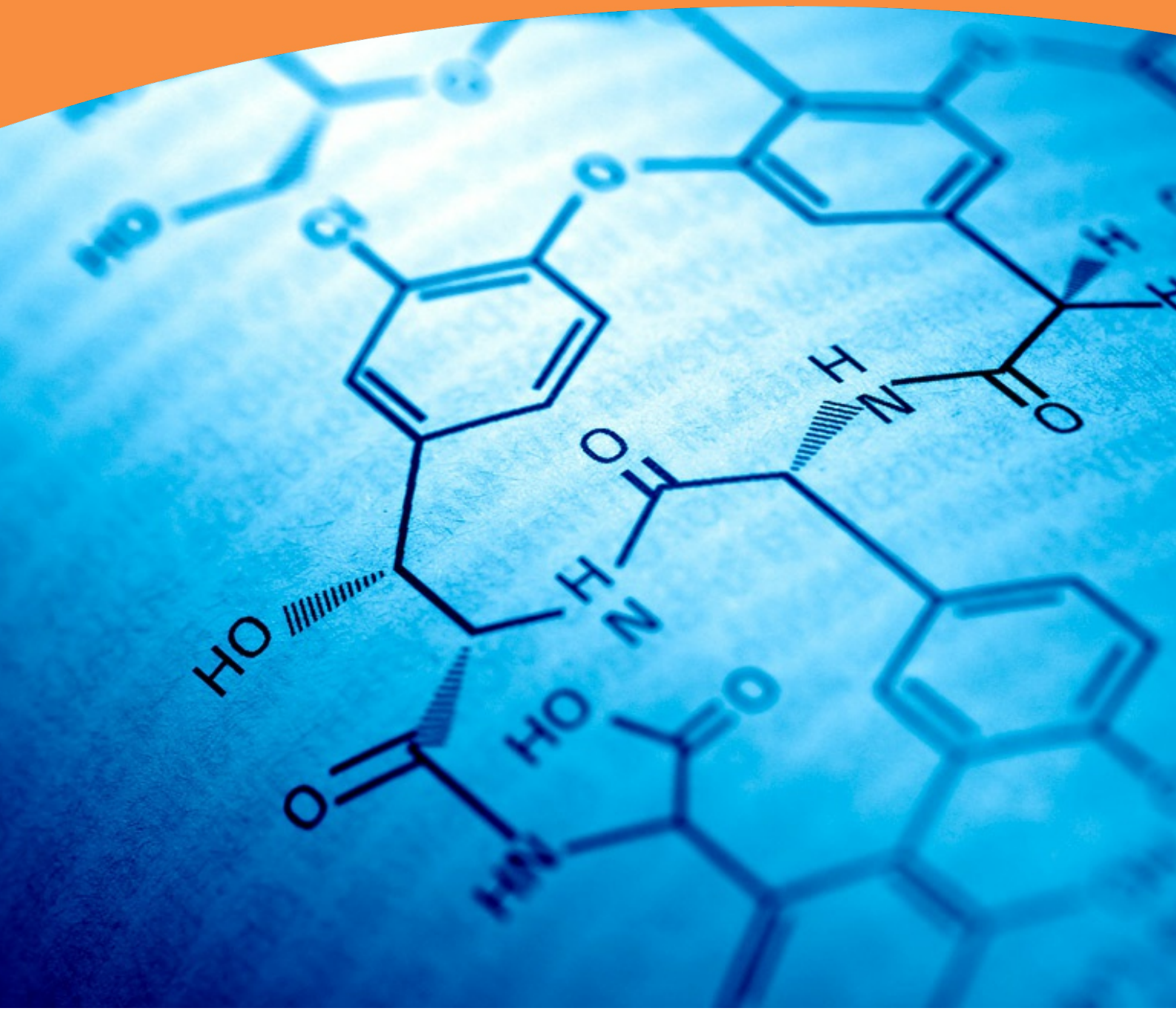
Laccases and their applications

Pratima Bajpai, PhD



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PREFACE

The demand of laccase enzymes in industry and biotechnology is ever increasing due to their use in a variety of processes. These enzymes have potential applications in a large number of fields, including pulp and paper, textile, cosmetics, pharmaceutical sectors, chemicals, food & fuel and many more. Also, these enzymes are directly involved in the degradation of many xenobiotic compounds and dyes, soil bioremediation, removal of toxic pollutants such as herbicides, pesticides, dye degradation and removal of endocrine disruptors. Their capacities to remove xenobiotic compounds and produce polymeric products make them very useful for bioremediation application. Laccases are the main ligninolytic enzymes belonging to the blue multi-copper oxidases group which participate in the ring cleavage of certain complex aromatic compounds, degradation of polymers and crosslinking of monomers. These enzymes are produced from bacteria, fungi and plants and have been mostly characterized in fungi than in higher plants. These enzymes have been also utilized in the manufacture of anti-cancer drugs. Recently, laccases have also been applied to nanobiotechnology. Laccase technology has been applied to almost the whole production chain of paper products starting from pulping to recovery of secondary fibers and also effluent treatment. Emerging research areas include the use of laccase for adhesion enhancement in binderless wood boards and the tailoring of lignocellulosic materials by laccase-assisted biografting of phenols and other compounds. This e-book covers the occurrence, mode of action, production and cultivation techniques, immobilization as well as potential applications of laccases in different industries and their potential application in the nanobiotechnology area.

Pratima Bajpai

Pulp and Paper Consultant

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1 GENERAL BACKGROUND AND INTRODUCTION

The demand for laccase enzymes has increased in the recent years due to their potential applications in the diverse biotechnological areas (Madhavi and Lele, 2009; Kunamneni et al., 2008). Laccases have a broad range of specificity, and is highly versatile in nature. A laccase is an isozyme predominantly present in the microbial community, which is encoded by different genes and expressed in different organelles. It can be readily detected by gel electrophoresis.

Laccase is one of a few enzymes that have been studied since the nineteenth century. Laccase was first reported in 1883 by Yoshida from the exudates of the Japanese lacquer tree *Rhus vernicifera*.

1.1 OCCURRENCE AND PROPERTIES

Laccases are ubiquitous in nature. They have been reported in different molecular forms, i.e., lCC 1, lCC 2, lCC 3 and lCC 4, which are obtained from *Pleurotus ostreatus* (Mansur et al., 2003). Laccases are polyphenol oxidases that catalyze the oxidation of several aromatic compounds, especially those with electron-donating groups, for example phenols and anilines using molecular oxygen as an electron acceptor (Gianfreda et al., 1999).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi copper oxidases capable of catalyzing the oxidation of a wide range of phenolic and non-phenolic aromatic compounds.

Biochemically laccases are monomeric, dimeric or tetrameric glycoproteins. They have four copper atoms and three types of copper (Thurston 1994):

Type 1 copper is responsible for the oxidation of the substrate and also for the blue color of the enzyme, having strong electronic absorbance around 610 nm and detectable electro-paramagnetic resonance (EPR). Type 1 copper displays an EPR spectrum characterized by very narrow hyperfine splitting in the direction parallel to the magnetic field.

Type 2 copper is colorless. It is also detectable by EPR. Type 2 copper has EPR parameters more typical of regular copper complexes

Type 3 copper gives a weak absorbance near the UV spectrum (330 nm) but it is not detectable by EPR. Type 3 copper is almost certainly an antiferromagnetic pair of Cu(II) ions not detectable by EPR at any temperature between that of liquid helium and ambient. Leontievsky et al. (1997) have reported that the Type 2 and Type 3 copper sites are close together and form a trinuclear centre in which binding dioxygen and four-electron reduction to water occur.

Laccases are widely distributed among plants, bacteria and fungi – in different genera of ascomycetes, some deuteromycetes, and mainly in basidiomycetes.

Laccases have the ability to oxidize phenolic and non-phenolic substrates. These enzymes use molecular oxygen to oxidize a variety of aromatic and non-aromatic hydrogen donors via a mechanism involving radicals. These radicals are able to undergo further laccase catalyzed reactions and/or non-enzymatic reactions, such as polymerization and hydrogen abstraction.

Phenolic substrate oxidation by laccases results in the formation of an aryloxy radical which is an active species. This is converted to a quinone in the second stage of the oxidation. The typical substrates of laccases are diphenol oxidases. Monophenols, e.g. guaiacol, sinapic acid can also oxidize, aminophenols, polyamines, lignin, aryl diamines, and inorganic ions, and they may reduce the toxicity of some polycyclic hydrocarbons (Baldrian, 2006). However, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, the substrate which is most commonly used, does not produce quinone and is not pH dependent. So, it is used to calculate the international unit of laccase activity. Laccases play diverse roles in nature (Table 1.1).

Structurally, laccases contain 15–30% carbohydrate and have molecular weight of 60–90 kDa with acidic isoelectric points around pH 4.0, which shows high enzymatic stability (Baldrian, 2006; Duran et al., 2002). Laccases are able to oxidize a wide range of molecules, and nearly 100 different types of compounds have been identified as substrates, which vary from one laccase to another.

Laccase is widely distributed in higher plants and fungi and has been found also in insects and bacteria (Piontek et al., 2002; Xu, 1996; Xu et al., 1996; Sakurai, 1992; Messerschmidt and Huber, 1990). In plants, they are involved in the synthesis of lignin and in the wounding response. Beloqui et al. (2006) reported a novel polyphenol oxidase with laccase like activity from a metagenome expression library from bovine rumen microflora.

Lignin, which provides the structural component of the plant cell wall, is a complex and heterogenous biopolymer that consists of phenyl propanoid units linked by various non-hydrolyzable C-C and C-O bonds (Gellerstedt and Northy 1989). For several years, it was thought that only the ligninolytic system of some white-rot fungi capable of degrading this recalcitrant polymer to a major extent involved lignin peroxidase and manganese peroxidase enzymes (Evans, 1985). Although the latter can only oxidize the phenolic components of lignin, lignin peroxidase, which has a high redox potential, is also capable of breaking the non-phenolic aromatic part. The major limitation of all heme containing peroxidases is their low operational stability, mostly due to their rapid deactivation by hydrogen peroxide. Also, the dependence of Mn^{2+} (for the manganese peroxidase) or veratryl alcohol (for the lignin peroxidase) has further shortcomings for their practical use. On the other hand, laccase alone is not capable of cleaving the non-phenolic bonds of lignin, and it was not considered an important component of the ligninolytic system, in spite of the secretion of large quantities of laccase by these fungi under ligninolytic conditions. However, Paprican (now FP Innovations) researchers (Bourbonnais and Paice, 1990) reported that laccases can catalyze the oxidation of non-phenolic benzyl alcohols in the presence of a redox mediator, such as ABTS. This finding led to the discovery that laccase-mediator systems (LMS) effectively degrade residual lignin in unbleached pulp (Call, 1994). Laccases produced by some wood-rot fungi from the genus Basidiomycete play an important role in the biodegradation of lignin (Coll et al., 1993). These enzymes have the ability to oxidize recalcitrant aromatic compounds with redox potentials exceeding their own with the help of chemical or natural mediators (Camarero et al., 2005; Xu, 1996). Because of their broad substrate specificity and wide reaction capabilities, the laccase and the LMS possess show great biotechnological potential. Promising applications include textile-dye bleaching, pulp bleaching, food improvement, bioremediation of soils and water, polymer synthesis and the development of biosensors and biofuel cells (Kunamneni et al. 2008; Kierulff, 1997; Palonen and Viikari , 2004; Minussi et al., 2002; Li et al., 1999; Wesenberg et al., 2003; Marzoorati et al., 2005; Trudeau et al., 1997; Tayhas et al., 1999; Madhavi and Lele, 2009).

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Roles of laccases
Plants Lignification of xylem tissues Wound healing Defense against external conditions
Fungi Delignification Sporulation Pigment production Fruiting body formation Plant pathogenesis
Bacteria Melanin production Spore coat resistance Morphogenesis

Table 1.1 Roles of laccases

2 LACCASES: SOURCES

Laccases are most widely distributed in fungi and higher plants and are also found in plants and insects (Baldrian, 2006; Benfield et al. 1964; Xu, 1999; Kumar and Sonkar, 2013; Mishra et al., 2015; Lakshmi et al. 2015; Rajeswari, 2015).

2.1 FUNGAL SOURCES

Till recently, fungal laccases account for the major group of laccases characterized with respect to the number when compared to bacterial laccases.

In 1896, laccase was demonstrated to be present in fungi for the first time by both Bertrand and lLaborde. Laccase from fungusi *Monocillium indicum* was the first laccase to be characterized from an ascomycete showing peroxidase activity.

Fungal laccases have roles in morphogenesis, fungal plant-pathogen/host interaction, stress defence, delignification, sporulation, pigment production, fruiting body formation (Thurston, 1994; Yaver et al., 2001). Fungal laccases have higher redox potential than that of plant or bacterial laccases. Fungi from the deuteromycetes, ascomycetes, and basidiomycetes are the known producers of laccases (Baldrian 2006; Aisemberg et al., 1989; Sadhasivam et al., 2008; Hao et al., 2007; Morozova et al., 2007; Wood, 1980; Perry et al. 1993; Ullrich et al., 2005; Mishra et al., 2015; Kumar and Sonkar, 2013; Arora and Sharma, 2010; Alexandre et al., 1999). Table 2.1 shows laccases from different fungal sources.

2.2 BACTERIAL SOURCES

Although there are also some reports about laccase activity in bacteria (Alexandre and Zhulin, 2000; Martins et al., 2002; Claus, 2003; Givaudan et al., 2004), it does not seem probable that laccases are common enzymes from certain prokaryotic groups. Bacterial laccase-like proteins are intracellular or periplasmic proteins (Claus, 2003).

The first bacterial laccase was reported from *Azospirillum lipoferum* in 1993 by Givaudan et al. which was involved in melanin synthesis.

Table 2.2 shows laccases from different bacterial sources (Sharma et al., 2007; Octavio et al. 2006; Rosconi et al., 2005;Solano et al., 2000; Reiss et al., 2011; Martins et al., 2002; Arias et al., 2003; Suzuki et al., 2003; Francis and Tebo, 2001; Fitz-Gibbon et al., 2002; Santo et al., 2013; Bains et al., 2003). Alexandre and Zhulin (2000) and Ausec et al. (2011) reported that laccases are widespread in bacteria.

The biological functions of bacterial laccase are (Martins et al., 2002; Endo et al., 2002; Francis and Tebo, 2001; Huang et al., 2013; Santo et al., 2013; Freeman et al., 1993; Roberts et al., 2002; Bains et al., 2003):

- In spore pigment formation
- UV resistance
- Melananization
- Oxidation of metals
- Degradation of lignin and polyethylene
- Antibiotic synthesis
- Copper resistance and detoxification of phenolic compounds

2.3 PLANT SOURCES

The first laccase discovered in 1883 was from *Rhus vernicifera* sap containing derivatives of catechols called as urushiol. Apart from *Rhus* genus of Anacardiacaea family, laccase was reported in other plants, namely mango, horse chestnut, tobacco, peach, pine, prune, sycamore poplar and mung bean (Xu et al., 1999; Lehman et al. 1974; Bligny and Douce 1983; De Marco and Roubelakis-Angelakis 1997; Ranocha et al. 1999). Laccases are also found in cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965). Recently laccase has been expressed in the embryo of maize (*Zea mays*). Plant laccases were found to have optimum pH values around 5 to 7 (Robinson et al., 1993) and have high carbohydrate content of up to 43% compared to fungal laccases (Dwivedi et al., 2011). In plants, laccase plays a major role in lignin polymerization (Gavnholt and Larsen, 2002), wound healing and iron metabolism (McCaig et al., 2005). Plant laccases participate in the radical-based mechanisms of lignin polymer formation (Sterjiades et al., 1992; Liu et al., 1994; Boudet, 2000; Ranocha et al., 2002; Hoopes and Dean, 2004). The plant laccases have not been characterized or used extensively despite their wide occurrence, because their detection and purification is often difficult, as the crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha et al. 1999).

2.4 INSECT SOURCES

Laccases are also found to be present in several insects of genera that include *Drosophilia, Lucilia, Manduca, Bombyx, Calliphora, Diploptera, Musca, Oryctes, Papilio, Phormia, Rhodnius, Sarcophaga, Schistocerca,* and *Tenebrio* (Xu, 1999; Lakshmi et al., 2015; Mishra et al., 2015; Kumar and Sonkar, 2013; Arora and Sharma, 2010).

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Fungi
Agaricus bisporus
Agaricus blazei
Agrocybe praecox
Albatrella dispansus
Armillaria mellea
Aspergillus nidulans
Betulina
Botrytis cinerea
Cantharellus cibarius
Ceriporiopsis subvermispora
Cerrena maxima
Cerrena unicolor

Fungi
Chaetomium termophilum
Chalara paradoxa
Colletotrichum graminicola
Coniothyrium minitans
Coprinus cinereus
Coprinus friesii
Coriolopsis fulvocinnerea
Coriolopsis gallica
Coriolopsis rigida
Coriolposis polyzona
Coriolus hirsutus
Coriolus maxima
Coriolus zonatus
Cryptococcus neoformans
Cyathus stercoreus
Daedalea quercina
Dichomitus squalens
Fomes fomentarius
Gaeumannomyces graminis
Ganoderma lucidum
Ganoderma tsugae
Hericium echinaceum
Junghuhnia separabilima
Lactarius piperatus
Lentinus edodes

Fungi
Lentinus tigrinus
Lenzites
Magnaporthe grisea
*Marasmius quercophilus_*
Mauginiella sp.
Melanocarpus albomyces
Monocillium indicum
Myrothecium verrucaria
Neurispora crassa
Ophiostoma novo-ulmi
Panaeolus papilionaceus
Panaeolus sphinctrinus
Panus tigrinus
Pestalotiopsis sp.
Phanerochaete chrysosporium
Phanerochaete flavido
Phellinus noxius
Phellinus ribis
Phlebia radiata
Phlebia tremellosa
Pholiota mutabilis
Physisporinus rivulosus
Picnoporus cinnabarius
Pleurotus eryngii
Pleurotus florida

Fungi
Pleurotus ostreatus
Pleurotus pulmonarius
Pleurotus sajor-caju
Pleurotu seryngii
Podospora anserine
Polyporus anceps
Polyporus anisoporus
Polyporus pinsitus
Pycnoporus cinnabarinus
Pycnoporus coccineus
Rhizoctonia solani
Rigidoporus lignosus

Fungi
Russula delica
Schizophyllum commune
Sclerotium rolfsii
Stropharia coronilla
Stropharia rugosoannulata
Trametes gallica
Trametes ochracea
Thelephora terrestris
Trametes (Coriolus, Polyporus) versicolor
Trametes gallica
Trametes hirsuta
Trametes multicolor
Trametes ochracea
Trametes pubescens
Trametes sanguinea
Trametes trogii
Trametes villosa
Trichoderma atroviride
Trichoderma harzianum
Trichoderma viride
Tricholoma giganteum
Volvariella volvacea

Table 2.1: Laccases from different fungal sources

Bacteria
Alpha-proteobacterium
Gama-proteobacterium
Aquifex aceolicus
Azospirillum lipoferum
Bacillus halodurans,
Bacillus subtilis
Escherchia coli
Leptotrix discophora
Marinomonas mediterranea
Oceano bacilusiheynesis
Pseudomonas aerophillum,
Pseudomonas fluorescens,
Pseudomonas maltophila,
Pseudomonas putida,
Pseudomonas syringae
Rhodococcus sp.
Sterptomyces cyaneus
Streptomyces antibioticus
Streptomyces griseus
Streptomyces lavendulae
Streptomyces psammoticus
Thermus thermophillus
Xanthomonas campesteris

Table 2.2: Lacasses from different bacterial sources

3 LACCASES: PROPERTIES

3.1 STRUCTURAL AND CATALYTIC PROPERTIES

Laccases belong to the multi-copper enzyme family and are phenol-*oxidases* that have a distinct redox ability to catalyze the oxidation of a wide range of aromatic substrates (Solomon et al., 1996, 2001; Messerschmidt, 1997; Babu et al. 2012).

Laccase catalysis involves reduction of the type 1 copper by reducing substrate; internal transfer of electron from the type 1 to the type 2 and type 3 copper and finally reduction of oxygen to water at the type 2 and type 3 copper site.

Properties of purified laccases have been reported by many researchers (Baldrian, 2006; Kunamneni et al., 2007; Madhavi and Lele, 2009; Babu et al. 2012; Chandra and Chowdhary, 2015). Laccases are monomeric, dimeric and tetrameric glycoproteins and generally have fewer saccharide compounds (10–25%) in fungal and bacterial enzymes than in plant enzymes. The carbohydrates are 10–45% of the total molecular weight. These are covalently linked. Due to this property the enzymes show high stability. The carbohydrate compound contains monosaccharides such as hexoamines, glucose, fucose, mannose, galactose, and arabinose (Rogalski and Leonowicz 2004). Mannose is one of the main components of the carbohydrates attached to laccases. The molecular weight of a laccase is in the range of 50–97 kDa. The molecular weights of laccases in *Bacillus pumilus* were estimated to be 58 and 64.8 kDa. Glycosylation plays an important role in copper retention, thermal stability, susceptibility to proteolytic degradation, and secretion. Glycosylation content and composition of glycoprotein vary with growth medium composition (Li et al., 1999; Pickard and Hashimoto, 1988). Laccase enzymes show considerable heterogeneity upon purification. The sugar composition has been analyzed in several microorganisms, such as *Podospora ansenna, Botrytis cinerea*, *Trametes hirsuta*, *Trametes ochracea, Cerrena maxima, Coriolopsis fulvocinerea* and *Melanocarpus albomyces* (Call and Mucke, 1997; Shleev et al., 2004; Piontek et al., 2002).

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidized varies from one laccase to another.

Laccases enzymes catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, ascorbate and aromatic amines with the concomitant four-electron reduction of oxygen to water (Thurston, 1994). These enzymes have broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups is found in lignin (Youn et al., 1995). Kinetic data of laccases from different sources were reported (Yaropolov et al., 1994). Km values are found to be similar for the co-substrate dissolved oxygen but Vmax varies with the source of laccase. The turnover is heterogeneous over a broad range depending on the enzyme source, substrate and the type of reaction. The kinetic constants differ in their pH dependence. Km is pH-independent for both substrate and co-substrate, whereas Kcat is pH-dependent (Kunamneni et al., 2007).

The pH optima of laccases are highly dependent on the substrate. With ABTS as substrate, the pH optima are more acidic and are found in the range 3.0–5.0 (Heinzkill et al., 1998). In general, laccase activity has a bell shaped profile with an optimal pH that varies considerably. This variation could be due to changes in the reaction caused by the substrate, oxygen or the enzyme (Xu, 1997). Fungal laccases have isoelectric points ranging from 3 to 7, whereas plant laccase isoelectric point values range to 9 (Babu et al. 2012). The major difference between the two enzymes is that fungal enzymes have their pH optima between pH 3.6 and 5.2, whereas laccase from *Rhus vernicifera* have pH optima in the range of 6.8 to 7.4. The low pH optima of the fungal enzyme may be due to the reason that they are well adapted to grow under acidic conditions, whereas the plant laccase being intracellular have their pH optima closer to the physiological range. The differences in pH optima may be due to the difference in physiological functions. These enzymes also differ in their function in addition to their variation in pH. Fungal enzyme is responsible in mechanism for removing toxic phenols from the medium in which these fungi grow under natural conditions, whereas the plant enzymes are involved in synthetic process such as lignin formation (Benfield et al. 1964).

The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion binding to the T2 and T3 coppers results in an inhibition of the laccase activity because of the disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects may play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Xu, 1997). Nyanhongo et al. (2002) reported that laccase produced by *Trametes modesta* was fully active at pH 4.0 and very stable at pH 4.5 but its half-life reduced to 125 minutes at pH 3.0. The optimal temperature of laccase can differ greatly from one strain to another. The laccases isolated from a strain of *Marasmius quercophilus* (Farnet et al., 2000) were found to be stable for 1 hour at 60°C. Farnet et al. (2000) further found that pre-incubation of enzymes at 40°C and 50°C significantly increased laccase activity. Another method that can be used for increasing the stability of laccase is to immobilise the enzyme on glass powder by air-drying (Ruiz et al., 2000). This method also has potential for the enzyme to be used on the glass powder matrix in specific applications in biotechnology where stability is needed (Ruiz et al., 2000).

The laccase from *P. ostreatus* is fully active in the temperature range of 40–60 °C, showing maximum activity at 50 °C. The activity remains unchanged after prolonged incubation at 40 °C for more than 4 hours (Palmieri et al., *1993*). Nyanhongo et al. (2002) showed that laccase produced by *T. modesta* was found to be fully active at 50 °C and was very stable at 40 °C but half-life reduced to 120 minutes at temperature of 60 °C.

In general, laccases respond similarly to several inhibitors of enzyme activity (Bollag and Leonowicz, 1984). Many ions such as halides, azides, cyanide, thiocyanide, fluoride and hydroxide bind to the type 2 and type 3 copper. This results in the interruption of internal electron transfer and therefore inhibition of activity. Other inhibitors include fatty acids, metal ions, sulfhydryl reagents, hydroxyl glycine, kojic acid, and cationic quaternary ammonium detergents, the reactions with which may involve amino acid residue modifications, confirmational changes or chelation with copper (Gianfreda et al., *1999*; Call and Mucke, 1997).

Many fungi producing laccases secrete isoforms of the same enzyme (Leontievsky et al 1997). Archibald et al. (1997) have reported that these isozymes originate from the same or different genes encoding for the laccase enzyme. The number of isozymes present differs between species and also within species depending on whether they are induced or non-induced (Assavanig et al., 1992). They differ significantly in their stability, optimal temperature and pH and affinity for different substrates (Assavanig et al., 1992; Heinzkill et al., 1998). Moreover, these different isozymes can modulate different roles in the physiology of different species or in the same species under different conditions (Assavanig et al., 1992). *Cerrena unicolor* secreted two laccase isoforms with different characteristics during the growth in a synthetic low-nutrient nitrogen/glucose medium (Michniewicz et al., 2006). Several laccase encoding gene sequences have been reported from various ligninolytic fungi. These sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is shown by sequence comparisons (Bourbonnais et al., 1995).

The catalysis performed by all members of this family is guaranteed by the presence of different copper centres in the enzyme molecule. In particular, all blue multi-copper oxidases are characterized by the presence of at least one type-1 copper, together with at least three additional copper ions: one type- 2 and two type-3 copper ions, arranged in a tri-nuclear cluster. The different copper centres can be identified on the basis of their spectroscopic properties (Messerschmidt, 1997; Leontievsky et al., 1997).

The three-dimensional structure of five fungal laccases has been reported: *Coprinus cinereus* (in a copper type-2-depleted form), *T. versicolor*, *P. cinnabarinus*, *M. albomyces* and *R. lignosus* (Ducros et al., 1998; Bertrand et al., 2002; Piontek et al., 2002; Antorini et al., 2002; Garavaglia et al., 2004; Hakulinen et al., 2002), the latter four enzymes with a full complement of copper ions. Moreover, the three-dimensional structure of the CoA laccase from *Bacillus subtilis* endospore has been reported by Enguita et al. (2003, 2004). Inspite of the amount of information on laccases and other blue multi-copper oxidases, neither the exact electron transfer pathway nor the details of dioxygen reduction in blue multi-copper oxidases are completely understood (Garavaglia et al., 2004). A detailed structural comparison between a low redox potential *C. cinereus* laccase and a high redox potential *T. versicolor* laccase showed that structural differences of the Cu1 coordination possibly account for the different redox potential values (Piontek et al., 2002). This was later on confirmed by the study of *R. lignosus* laccase with a high redox potential (Garavaglia et al., 2004). Unlike the laccases described above, the enzyme from *P. ribis* with catalytic properties typical for laccases does not belong to the blue copper proteins because it lacks Cu1 and contains one manganese atom per molecule. The structural differences are perhaps also responsible for the relatively high pH optimum for ABTS oxidation (Min et al., 2001). The 'white' laccase POXA1 from *P. ostreatus* contains only one copper atom, together with one iron atom and two zinc atoms per molecule (Palmieri et al., 1997). According to Baldrian (2006), laccases are a more structurally heterogeneous group of proteins than expected.

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4 LACCASES: PRODUCTION

Laccases are extracellular enzymes and their production usually occurs during the secondary metabolism of different fungi (Kunamneni et al., 2007) although a large number of studies report intracellular laccases too. Several factors can influence laccase production. These include type of cultivation techniques, carbon source, nitrogen source, and concentration of microelements (Brijwani et al., 2010a; Elisashvili et al., 2008a,b; Elisashvili and Kachlishvili, 2009; Kunamneni et al., 2007).

4.1 PRODUCTION METHODS

Submerged fermentation and solid state modes of fermentation are used intensely for the production of laccases. Submerged fermentation, though, leads the solid state fermentation for industrial production of laccase. Future efforts in improving the solid state fermentation bioreactor designs can make this method more potent and competitive.

The successful application of laccases for various industrial applications would require production of high amounts of laccases at low cost. Many production strategies can be adopted along with media and process optimization for achieving better process economics. Media optimization and use of appropriate inducers could bring additional benefits of higher production with expenditure of minimum resources.

4.1.1 SUBMERGED FERMENTATION

In submerged fermentation, microorganisms are cultivated in liquid medium containing appropriate nutrients with high oxygen concentrations when operated in aerobic conditions. One of the main challenges in fungal submerged fermentations is viscosity of broth. Mycelium formation during growth of fungal cells can also hinder impeller action causing blockades which result in oxygen and mass transfer limitations. Different strategies have been used to deal with mass transfer and oxygen limitations. Cell immobilization is one technique to solve problems associated with broth viscosity, and mass transfer and oxygen transfer (Rodriguez Couto et al., 2004a,b; Schliephake et al., 2000; Luke and Burton, 2001; Galhaup et al., 2002). Rodriguez Couto et al. (2003, 2004a,b 2006) examined different types of synthetic materials as carriers for the immobilization of the white rot fungus *Trametes hirsuta* in fixed bed bioreactors operated in batch. They tested different materials and found that use of stainless steel sponge resulted in highest laccase activities. Agitation is also found to affect laccase production. Hess et al. (2002) found that laccase production by *Trametes multicolor* reduced significantly when the fungus was grown in stirred tank reactor, probably because of damage to mycelia. Mohorcic et al. (2004) found that it was possible to grow the white rot fungus *Bjerkandera adusta* in a stirred tank reactor after its immobilization on a plastic net, although very low activities were obtained. On contrary, Tavares et al. (2006) observed that agitation did not play an important role in laccase production by *T. versicolor*. Fed-batch operation is also found to be effective for producing laccase. Galhaup et al. (2002) obtained a higher laccase activity and found that use of fed-batch mode increased the laccase production of *T. pubescens* by two times.

Several studies have been conducted on laccase production using submerged fermentation with different microorganisms at different scales and the addition of inducers and the use of immobilization supports. With *Trametes* genus – *T. pubescens*, *T. hirsuta*, *T. versicolor* – high laccase activities were obtained (Galhaup et al., 2002; Font et al., 2003; Tavares et al., 2006; Rodriguez Couto et al., 2006). In all these studies, inducers were added to the culture medium. Galhaup et al. (2002) obtained a maximum laccase activity of 740,000 U/L by growing *T. pubescens* in a 20-L stirred-tank reactor with an agitation speed of 100 rpm and with 2 mM copper ions. Rodríguez Couto et al. (2006) supplemented the medium with glycerol and copper ions and obtained a maximum laccase activity of 19,400 U/L by growing *T. hirsuta* in a 6-L airlift reactor. Tavares et al. (2006) reported a maximum laccase activity of 11,403 U/L with *T. versicolor* in a stirred-tank reactor of 1 liter in a medium supplemented with 30 µM of xylidine. Font et al. (2003) obtained a maximum laccase activity of 16,000 U/L by free cells of *T. versicolor* in a 0.5-L pulsed-bed reactor. *Galerina* sp. produced high laccase activity under optimized conditions in batch submerged fermentation (Gulden et al., 2005). Bahrin et al. (2010) used rice straw in a solid-state fermentation process using *Pleurotus sajor-caju*, for producing laccase. The highest laccase activity of 224.93 U/mg was obtained from the crude extract after 9 days of fermentation. Laccase was concentrated by ultrafiltration and purified using DEAE-Sepharose anion exchange chromatography. The peak fraction obtained was then loaded into Sephacryl S200-HR gel filtration chromatography. Laccase was purified by 43-fold to a specific activity of 19335 U/mg, with an overall protein recovery of 18.6%. It appeared as a single band on SDS-PAGE with an apparent molecular weight of 53 kDa.

Submerged cultivation can be carried out by utilizing inexpensive materials considered as "waste" and which are produced in large amounts (Dong et al., 2005; Revankar and Lele, 2006). These materials can contain considerable concentrations of soluble carbohydrates, nitrogen, minerals, vitamins and even inducers for enzyme production.

4.1.2 SOLID STATE FERMENTATION

Solid state fermentation (SSF) process occurs in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support (Pandey et al., 1999). SSF is shown to be especially suitable for the production of enzymes by filamentous fungi because they mimic the conditions under which the fungi grow naturally (Moo-Young et al., 1983; Pandey et al., 1999). The use of natural solid substrates, especially lignocellulosic agricultural residues as growth substrates has been studied for various enzymes like cellulases including laccases (Brijwani et al., 2010a,b; Rodriguez Couto and Sanromán 2005). The presence of lignin and cellulose/hemicellulose act as natural inducers and most of these residues are rich in sugar promoting better fungal growth and thereby making the process more economical (Toca-Herrera et al., 2007). The major disadvantage with SSF is lack of any established bioreactor designs. There are several bioreactor designs reported in the literature that have addressed the main limitations of mass and heat transfer in solid media. But still, a lot of progress is to be made.

Different types of bioreactor configurations have been studied for production of laccase. Rodriguez Couto et al. (2004a) studied three bioreactor configurations – immersion, expanded bed and tray for laccase production by *T. versicolor* using an inert (nylon) and non inert support (barley bran). They found that the tray configuration resulted in high laccase production. Rodriguez Couto et al. (2004b) also compared tray and immersion configurations for production of laccase by *T. hirsuta* using grape seeds as substrate. In this case also, tray configuration gave the best results. In a similar study by Rosales et al. (2007) tray configuration produced higher laccase activity in *T. hirsuta* cultures grown on orange peels.

Several studies on laccase production in SSF using agro-industrial wastes have already been reported. Gómez et al. (2005) observed that barley bran was the best lignocellulosic waste for producing laccase by solid state fermentation of *C. rigida*. The higher porosity and roughness of barley bran makes easier the attachment of the fungus to the support. Oil palm frond parenchyma tissue was used as a solid substrate for the production of laccase via SSF using the white rot fungus *Pycnoporus sanguineus*. Maximum laccase activity was achieved on day eight (950 U/m3) (Annuar et al., 2010). Laccase activity of 1570 U/L (on day 20) was obtained with *Trametes pubescens* in SSF with banana skin as support substrate. The most important characteristics that affect adhesive behavior of filamentous fungi to the support are hydrophobicity and surface charge. The higher hydrophobicity and its higher carbohydrate content cause the attachment of the fungus to the carrier easily (Osma et al., 2007). The use of rice straw as one of agricultural wastes was suitable for laccase production as it contains substances acting as inducers for laccase. Rodriguez Couto and Sanroman (2005) stated that using organic wastes rich in cellulose stimulated laccase production. The scarcity of bioreactor designs to perform solid-state processes along with the advantages offered by such processes promote the necessity of developing new bioreactor configurations or modifying the existing designs. These bioreactor designs are able to operate in continuous mode with high enzyme productivity for prolonged periods of time without operational problems and also allow the scale-up of the process. Rivela et al. (2000) developed a new bioreactor design for the production of ligninolytic enzymes under SSF conditions named immersion bioreactor. They obtained high ligninolytic activities. The bioreactor was able to operate in continuous mode (Rodriguez Couto and Toca Herrera, 2006). Dominguez et al. (2001) developed a rotating drum reactor for the production of ligninolytic enzymes under SSF conditions. This bioreactor was able to operate in batch and continuous mode. Also, Böhmer et al. (2006) reported the advantages of adapting the temporary immersion RITA®-System (Récipient à Immersion Temporaire Automatique) as a bioreactor for laccase production by white-rot fungi and its use for discoloration of synthetic dye.

During recent years, efforts are being made to develop strategies for maintaining the process under optimum condition, which can significantly increase the enzyme production. In SSF, since the fungi grow on heterogeneous substrate appropriation are important for designing and optimizing SSF processes for hyper production of fungal metabolites of industrial importance (Gomez et al., 2005). For effective laccase expression, it is important to optimize all the culture conditions and composition for production media for facilitating economic design of the full-scale fermentation operation system. The one factor-at-time strategy of improving fermentation conditions is the most often used operation in biotechnology for achieving high cell density, high yields of the enzyme or desired metabolic product in the microbial system. This strategy is not only time consuming, but also ignores the combined interactions between physico-chemical parameters. Optimization of medium by the conventional method involves changing one independent variable such as the pH, temperature, nutrient etc. and keeping the other factors constant. The conventional method for multifactor experimental design are time-consuming and not able to detect the true optimum, particularly due to the interactions among the factors (Liu and Tzeng, 1998) and frequently does not guarantee the determination of optimal conditions. In fermentation process, the operational variables interact and influence each other's effect on the response, it is important that the optimization methods account for its interactions so that a set of optimal experimental condition can be determined (Silva et al., 2005). By the use of different techniques, this limitation of a single factor optimization process can be eliminated. One of the techniques is response surface methodology. This technique is a collection of experimental strategies, mathematical methods and statistical inference and is used to explain the combined effects of all the factors in a fermentation process (Annaduari and Sivakumar, 2000). Several statistical designs are currently available to predict the behavior of a reaction through response surface methodology (Liu and Tzeng 1998; Elibol 2001; Khuri and Cornell 1987).

4.2 SUBSTRATES FOR LACCASE PRODUCTION

Several substrates have been used for laccase production (Table 4.1). Increased production of extracellular laccases in several species of white rot fungi when grown on natural substrates such as cotton stalk, molasses waste water, wheat bran and barley bran has been reported by several researchers (Ardon et al.1996; Kahraman, and Gurdal, 2002; Souza et al., 2002; Rodriguez Couto et al., 2002; Risna, and Suhirman, 2002). Use of agricultural and industrial wastes for laccase production is an effective way for reducing the production costs (Risna, and Suhirman, 2002).

4.3 EFFECT OF CARBON SOURCES ON LACCASE PRODUCTION

Carbon, nitrogen and copper sources regulate the level of gene transcription for laccase expression and are the main nutritional parameters studied for laccase production (Collins and Dobson, 1997). The carbon source in the medium plays an important role in production of ligninolytic enzyme. Glucose showed the highest potential for the production of laccase (Lee et al., 2006). Glucose is the easiest carbon source for fungi to metabolize but its effect on laccase production depends on the fungal strain. High concentrations of glucose are inhibitory to laccase production in various fungal strains (Lee et al. 2004). Use of excess of sucrose also reduced the production of laccase by blocking its induction and allowed constitutive production of enzyme. By the use of polymeric substrates such as cellulose, this problem was solved (Lee et al., 2004). Glucose has been found to increase the production in *Galerina* sp. HC1, but it inhibited the production in *Trametes pubescens* (Galhaup et al., 2002) and *Phlebia* sp. (Arora and Rampal, 2000; Daljit et al., 2002). Mansur et al. (1997) reported that fructose induced 100-fold increase in laccase production of Basidiomycete species I-62. Glucose and cellobiose were rapidly used by *Trametes pubescens* and produced high laccase activity (Galhaup et al., 2002). Fructose was shown to be a good carbon source for laccase production in *Pleurotus sajor-caju* (Bettin et al., 2009); cellobiose in *T. pubescens* (Galhaup et al., 2002); lactose or glycerol in *Pseudotrametes gibbosa, Coriolus versicolor* and *Fomes fomentarius* (Revankar and Lele, 2006) and cellobiose in *T. pubescens* (Galhaup et al., 2002). Replacement of crystalline cellulose or xylan by cellobiose increased the laccase activity of *C. unicolor* by 21 and 70-fold respectively (Elisashvili et al., 2008a). Furthermore, in *T. versicolor,* use of barley bran increased almost 50-fold laccase activity compared to the control culture with glucose (Moldes et al., 2004). *Pleurotus eryngii* and *P. ostreatus* strain No. 493 showed the highest laccase activity when grapevine sawdust and mandarine peels were used as carbon sources (Stajic et al., 2006). *T. versicolor* was found to produce high laccase activity when mandarin peels were used (Mikiashvili, et al., 2004).

4.4 EFFECT OF NITROGEN SOURCES ON LACCASE PRODUCTION

The ligninolytic systems of white-rot fungi are activated during the secondary metabolic phase of the fungus and are triggered by depletion of nitrogen (Keyser et al., 1978). Monteiro and De Carvalho (1998) noted high laccase activity with semi-continuous production in shake culture using a low carbon to nitrogen ratio (7.8 g/g). Buswell et al. (1995) found that laccases were produced at high nitrogen concentrations although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Elishashvili et al. (2001) observed highest laccase activity in *C. unicolor* IBB 62 in a medium with ammonium sulphate as the nitrogen source. D'Souza-Ticlo et al. (2006) showed that well defined organic nitrogen sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production. Heinzkill et al. (1998) also reported a higher yield of laccase using nitrogen rich media rather than the nitrogen-limited media usually employed for induction of oxidoreductases. On the other hand, while low nitrogen levels (as yeast extract) improves the laccase production in *Pleurotus ostreatus* (Prasad et al., 2005), *Coriolus versicolor (*Revankar and Lele, 2006) and *Pycnoporus sanguineus* (Pointing, 2001); high concentrations are needed for *Trametes pubescens* (Galhaup et al., 2002), *Trametes gallica* (Dong et al., 2005) and *Galerina* sp. HC1. Casein, another nitrogen source, was successfully used for the production of laccase in *Pleurotus sajor-caju, Trametes versicolor* and *Coriolopsis polyzona;* the laccase production was significantly improved when ammonium nitrate, ammonium sulfate, potassium nitrate and peptone were used as supplementary nitrogen sources (Bettin et al., 2009; Elisashvili et al., 2008).

4.5 EFFECT OF PH ON LACCASE PRODUCTION

The information on effect of pH and temperature effects on laccase production is scarce, but most reports show initial pH between 4.5 and 6.0 is suitable for enzyme production (Thurston, 1994). Nyanhongo et al. (1998) reported that an initial pH of 7.0 was the best for optimal growth and laccase production by a newly isolated strain of *T. modesta*.

4.6 EFFECT OF TEMPERATURE ON LACCASE PRODUCTION

The optimum temperature for laccase production is between 25 °C and 30 °C (Pointing et al., 2000). The optimal temperature for fruiting body formation and laccase production is 25 °C in the presence of light but 30 °C when the cultures are incubated in the dark (Messerschmidt and Huber 1990). In general, the fungi were cultivated at temperatures between 25 °C and 30 °C for optimal laccase production (Monteiro and De Carvalho 1998). When cultivated at temperatures higher than 30 °C, the activity of ligninolytic enzymes was reduced (Nyanhongo 1998).

4.7 EFFECT OF INDUCERS ON LACCASE PRODUCTION

Laccase production can be enhanced by the presence of inducers mainly aromatic or phenolic compounds related to lignin or lignin derivatives such as veratryl alcohol, guaiacol, gallic acid, ferulic acid and ethanol (Muthukumarasamy and Murugan, 2014). Also, laccase production can be considerably stimulated in the presence of inducing substances like ethanol, veratryl alcohol, 2,5-xylidine, guaiacol and ferulic acid. Production of laccase in γ-*proteobacterium* JB increased 13-fold due to addition of copper sulphate after the onset of growth. Similarly, Malachite Green, Ethidium bromide, Phenol Red and Thymol Blue also increased the laccase production by 17-, 19-, 4- and 2-fold (Kanam et al., 2004).

Zhang et al. (2010) reported that laccase activity was not significantly affected by the presence of Mg^{2+}, Zn^{2+}, Cu^{2+} ions and EDTA at the concentrations of 6.25–50 mM but was reduced by Ca^{2+} at 25–50 mM, Al^{2+} and Fe^{2+} at a concentration of 6.25–50 mM. These researchers also reported that *Lentinula edodes* laccase was inhibited in the presence of 1 mM Ca^{2+} (70%) and Zn^{2+} (64%) and was increased by 40% in the presence of 10 mM Cu^{2+}.

Palmieri et al. (2000) reported that the addition of copper sulphate in the production media resulted in 50–fold increase in laccase activity when compared to a basal medium without using any copper sulphate. Similarly, oxidation of manganese ions was found to play an important role in the function of lignolytic complex of wood degradation, because it was able to efficiently oxidize certain types of non-phenolic compounds of lignin (Gorbacheva et al., 2009).

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Substrates
Wheat straw
Rice straw
Wheat bran
Rice bran
Maize bran
Gram bran
Sugar cane bagasse
Rice husk
Soy hull
Sago hampas
Grapevine trimmings dust
Corncobs
Coconut coir pith
Saw dust
Banana waste
Tea waste
Cassava waste
Palm oil mill waste
Aspen pulp
Sugar beet pulp
Sweet sorghum pulp
Apple pomace
Peanut meal
Rapeseed cake
Coconut oil cake

Substrates
Mustard oil cake
Cassava flour
Wheat flour
Corn flour
Steamed rice
Steam pre-treated willow
Starch

Table 4.1: Substrates used for laccase production

5 MECHANISM OF ACTION OF LACCASES

Laccases have four copper atoms in their active site which participate in oxygen reduction and water production (Figure 5.1) (Placido and Capareda, 2015).

Laccases attack only the phenolic subunits of lignin. This leads to aryl-alkyl cleavage, Ca oxidation and Ca–Cb cleavage (Figures 5.2). Laccases catalyse reduction of the type 1 copper by reducing substrate; internal electron transfer from the type1 to the type 2 and type 3 copper; reduction of oxygen to water at the type 2 and type 3 copper site (Kunamneni et al., 2007, 2008a; Madhavi and Lele, 2009; Brijwani et al., 2010). The oxidation of a reducing substrate by laccase enzyme involves the loss of a single electron and the formation of a cation radical. This radical is generally unstable and may undergo further laccase-catalyzed oxidation to form quinone from phenol or nonenzymatic reactions such as hydration, disproportion or polymerization (Xu, 1999). The transfer of electron from substrate to type 1 copper is probably controlled by redox potential difference. A lower oxidation potential of substrate or a higher redox potential of laccase often results in a higher substrate oxidation rate. It appears that the binding pocket of reducing substrate is shallow and has limited stearic effect on simple phenol substrate. In contrast, the oxygen binding pocket appears to hinder the access of oxidizing agents other than oxygen. Activation of oxygen likely involves chemical bond formation on the trinuclear copper cluster. Solomon et al. (1996) suggested that the rate of oxidation of substrate is dependent upon its reduction potential. This implies that electron transfer from the substrate to the type 1 site is the rate determining step in turnover. Laccases resemble to other phenol-oxidizing enzymes, which by preference polymerize lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups (Bourbonnais et al. 1995).

The substrate range of laccase can be extended to non-phenolic subunits by the addition of mediators (Figure 5.3) which are also termed as enhancers because its use enhances the catalytic performance of laccases to a very significant extent.

Laccases became much more significant in the area of many biotechnological applications after the discovery of high redox potential mediators.

Laccases are generally classified as low-, medium- or high-redox potential in function of their redox potential at the T1Cu ($E^{0'}_{T1}$) (Mate and Alcalde, 2015). Plant and bacterial laccases form the group of low-redox potential laccases. Fungal laccases include both medium- and high-redox potential enzymes. The medium-redox potential laccases are produced from ascomycetes and basidiomycetes. High-redox potential laccases are produced by basidiomycete white rot fungi. From a biotechnological viewpoint, high-redox potential generated much interest because a wider range of substrates can be oxidized (Rodgers et al., 2010). Table 5.1 shows redox potential of laccases from different sources (Xu et al., 1996).

Laccase can oxidize only phenolic fragments of lignin due to the random polymer nature of lignin and to its lower redox potential. Small low molecular weight compounds with higher redox potential than laccase itself (more than 900 mV) called mediators may be used to oxidize the non-phenolic part of lignin. A mediator is a small molecule which acts as a sort of electron shuttle. It is oxidized by the enzyme and produces a strongly oxidizing intermediate which is the co-mediator (oxidized mediator). It diffuses away from the enzyme and in turn oxidizes any substrate which is not able to directly enter into the active site due to its size. Alternately, the oxidized mediator could depend on an oxidation mechanism not available to the enzyme, thus extending the range of substrates accessible to it. In the laccase-dependent oxidation of non-phenolic substrates, earlier evidence suggests an electron-transfer (ET) mechanism with mediator ABTS, towards substrates having a low oxidation potential. Instead of that, a radical hydrogen atom transfer route may operate with N-OH type of mediators, if weak C-H bonds are present in the substrate. Figure 5.4 shows the differences between the oxidation mechanisms followed by ABTS radicals (Electron Transfer route) and HBT radicals (Hydrogen Atom Transfer route) in LMS for oxidation of non-phenolic substrates (Kunamneni et al., 2008b; Galli and Gentili, 2004).

Several mediators have been reported in the literature. Some of these are ABTS, 1-hydroxybenzotriazole (HBT), benzotriazole (BT), chlorpromazine (CPZ), promazine (PZ), remazol brilliant blue (RBB), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS), violuric acid (VA), and 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS) but the most commonly used are the ABTS and the 1-hydroxybenzotriazole (HBT). Figure 5.5 shows the chemical structures of some mediators.

The laccase mediator system (LMS) was originally developed for solving problems in bio-bleaching of wood pulps and was first reported by Bourbonnais and Paice (1990) with the use of ABTS as the mediator. According to Bourbonnais et al. (1995), the delignification of kraft pulp by laccase can be promoted by a number of external synthetic, low molecular mass dyes, or other aromatic hydrogen donors. ABTS was the first mediator shown to be effective in the delignification of kraft pulp and lignin transformation by laccase. Call and Mucke (1997) reported Lignozyme process which uses laccase mediator system for delignification of kraft pulp.

Various laccases readily oxidize ABTS by free radicals to the cation radical ABTS+ and the concentration of the intensely colored, green-blue cation radical can be correlated to the enzyme activity. It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding di-cations can be obtained. The redox potentials of ABTS+• and ABTS2+ were found to be 0.680 and 1.09 V respectively.

The reaction mechanism mediated by ABTS seems to proceed as follows:

Laccase is activated by oxygen and the mediator is oxidized by the enzyme. The oxidized mediator diffuses into pulp and oxidizes lignin, breaking it into smaller fragments, which are easily removed from the pulp by means of alkaline extraction. The application of the laccase mediator system for bleaching of hardwood kraft pulp resulted in a reduction of kappa number, demethylation, and depolymerization of kraft lignin (Archibald et al., 1997; Reid, 1991).

HBT belongs to the *N*-heterocyclic compounds bearing *N*-OH groups mediators. Consuming oxygen HBT is converted by the enzyme into the active intermediate, which is oxidized to a reactive radical (R-NO.). The redox potential of HBT has been estimated as 1.1–1.2 V. Laccase mediated catalysis has been used in a wide range of applications, such as delignification of pulp, bleaching of textile dyes, degradation of polycyclic aromatic hydrocarbon, degradation of pesticide or insecticide and organic synthesis. In pulp and paper industry, novel enzymatic bleaching technologies are attracting significant attention due to concerns regarding the environmental impact of the chlorine-based oxidants presently being used in delignification or bleaching. But synthetic mediators are toxic, expensive and usually inactivate the laccase at concentrations above 1 mM. New strategies to overcome this problem are being researched. Natural mediators such as *p*-coumaric acid, 4-hidroxybenzoic acid, syringaldehyde etc. are being explored (Kunamneni et al., 2007).

The LMS was successfully applied for delignification of kraft pulp (Call, 1994; Bourbonnais and Paice, 1996), the oxidation of benzyl alcohols (Johannes et al. 1996), aromatic methyl groups, polycyclic aromatic hydrocarbons (Majcherczyk et al. 1998; Johannes and Majcherczyk 2000; Johannes et al. 1998) and bleaching of textile dyes (Rodriguez et al. 2004; Reyes et al., 1999).

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	Laccase redox potential* (V vs. NHE)
Polyporus pinsitus	0.79
Rhizoctonia solani	0.73
Rhus vernicifera	0.44
Scytalidium thermophilum	0.53
Mytheliophthora thermophila	0.48

Table 5.1: Redox potential of laccases from different sources
**Laccase redox potentials (Eo) at pH 5.3*

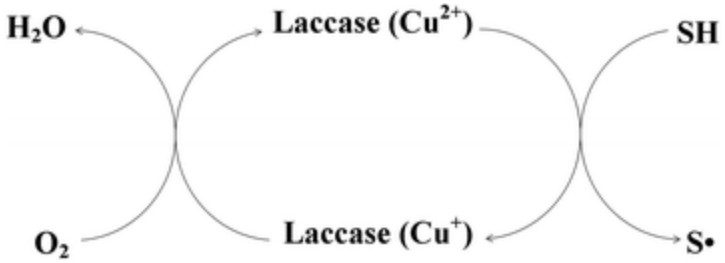

Figure 5.1: Laccase redox mechanism (Based on Placido and Capareda, 2015)
SH reduced substrate • oxidized substrate

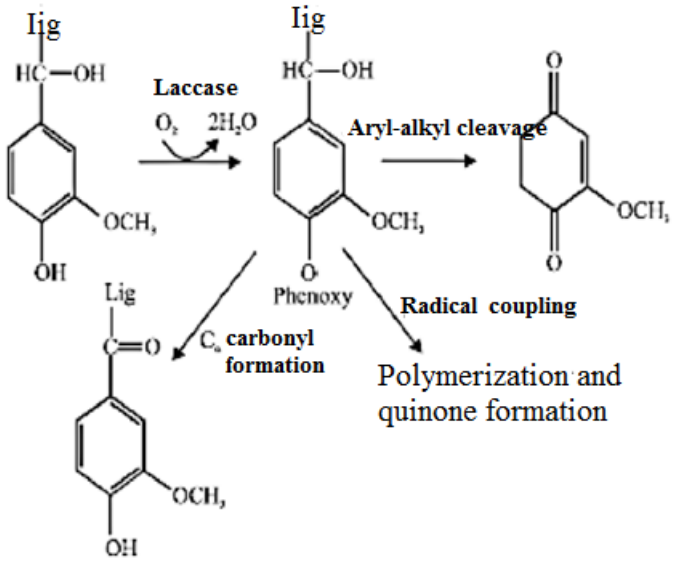

Figure 5.2: Oxidation of phenolic subunits of lignin by laccase (Based on Archibald et al., 1997)

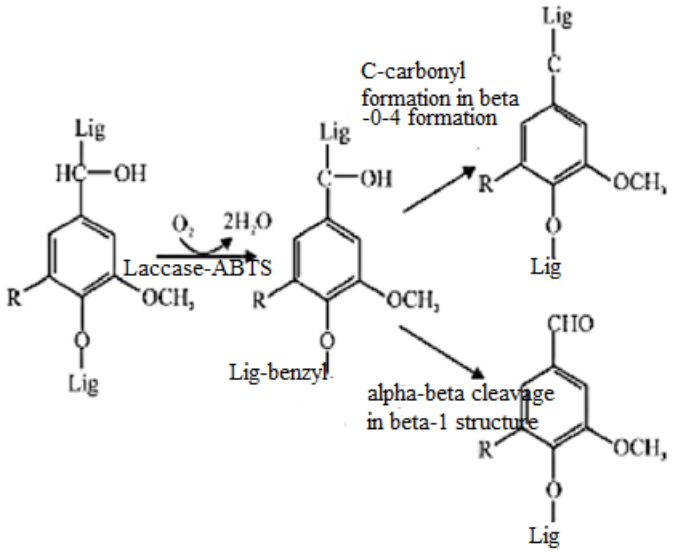

Figure 5.3: Oxidation of nonphenolic lignin model compounds by a Laccase Mediator System (Based on Archibald et al., 1997)

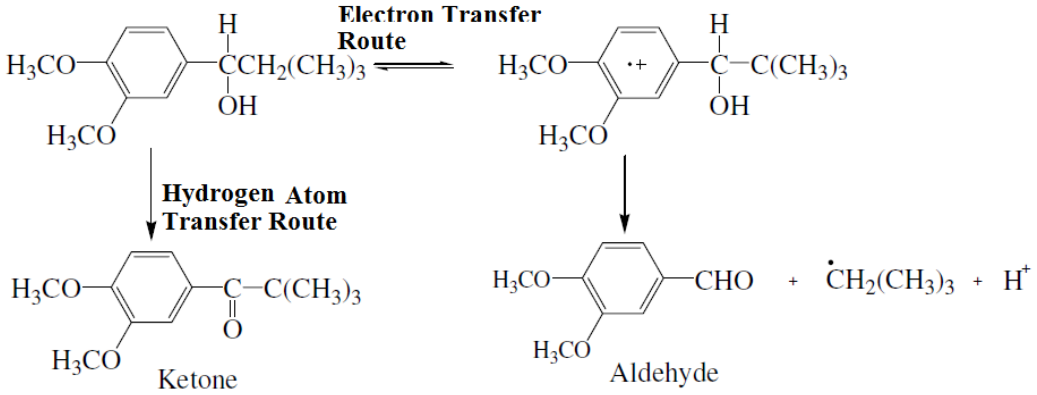

Figure 5.4: Oxidation mechanisms followed by ABTS radicals (Electron Transfer route) and HBT radicals (Hydrogen Atom Transfer route) in LMS for oxidation of non-phenolic substrates (Based on Kunamneni et al., 2008b and Galli and Gentili, 2004)

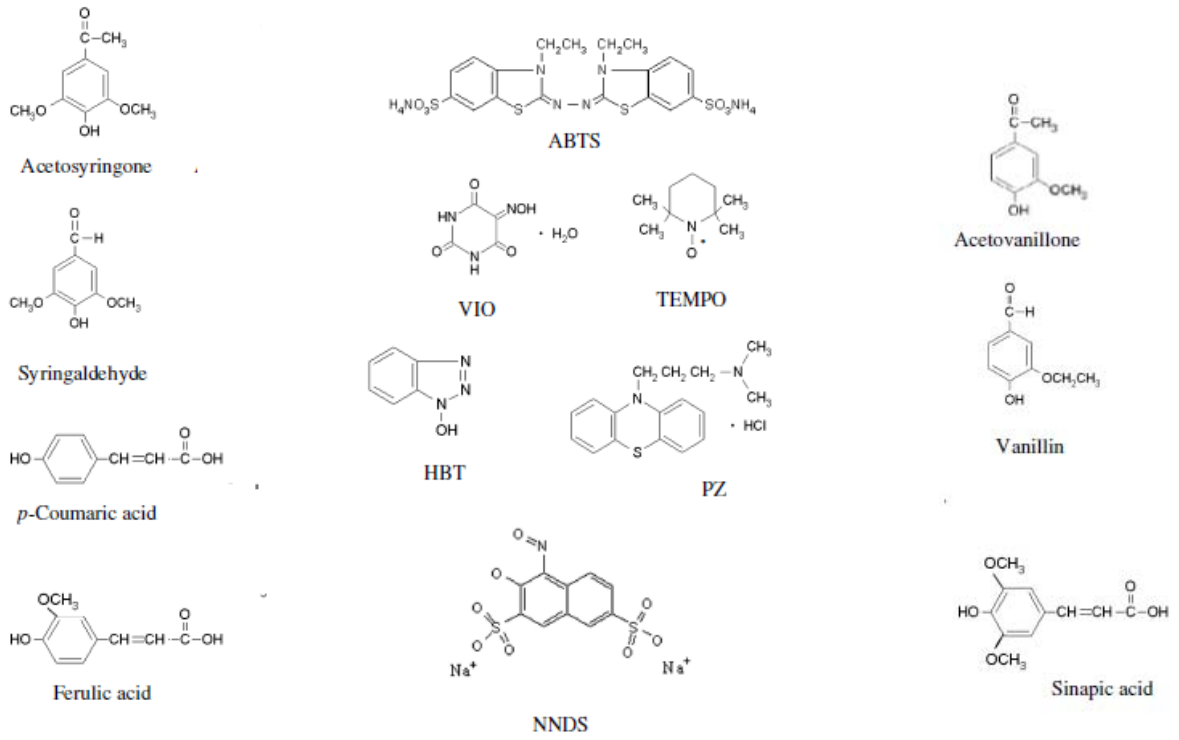

Figure 5.5: Chemical structures of some mediators

6 LACCASES: APPLICATIONS

Laccases are of great interest to industry, and have been used in many processes (Kunamneni et al., 2007, 2008a,b; Rodrıguez Couto and Toca Herrera, 2006; Shraddha et al., 2011; Singh Arora and Sharma, 2010; Widsten and Kandelbauer, 2008; Virk et al., 2012; Osma et al., 2010; Kudanga et al., 2011; Riva, 2006; Mikolasch and Schauer, 2009; Witayakran and Ragauskas, 2009a; Viswanath et al., 2014; Zucca et al., 2016; Bajpai et al., 2006a,b; Madhavi and Lele, 2009; Pezzella et al., 2015; Mate and Alclade, 2016; Cannatelli and Ragauskas, 2017).

Laccases are currently considered by many as an ideal green catalyst. There is an increased interest in the use of laccases to replace conventional chemical processes in the forest, textile and pharmaceutical sectors. These enzymes also have possible applications in several other sectors such as the food, cosmetics, paint, organic synthesis and bioremediation. Laccases have also a place in the production of bioethanol from lignocellulose materials. Indeed, the potential use of laccases for industrial and biotechnological applications is a thriving area of research.

Table 6.1 shows the breakdown in different sectors (Mate and Alclade, 2016). Table 6.2 shows some of the commercially available laccases. Among the commercially available laccases, there are bacterial laccases heterologously expressed in *Escherichia coli*, as well as laccases from filamentous fungi (*Aspergillus* sp.) and from several basidiomycete species including *Agaricus bisporus*, and *Trametes versicolor* (Mate and Alclade, 2016; Piscitelli et al., 2013).

6.1 FOREST INDUSTRY

The forest products sector includes companies involved in growing, harvesting and/or processing wood and wood fibre, manufacturing pulp, paper and paperboard products, and producing engineered and traditional type of wood products. Major challenges facing the pulp and paper industry in Europe and North America are increasing production costs, severe competition from new pulp and paper producers in Latin America and Asia, and complying with stringent environmental legislation (Viikari, 2002; Ragauskas, 2002; Stanko and Angus, 2006). Major issues in the pulp and paper industry include environment friendly bleaching, energy saving in pulping and bleaching processes, recycling of fibres, reduced generation of effluents and detoxification for saving fresh water and protecting the natural water bodies. Manufacturers of medium density fibreboard (MDF) and particleboard (PB), structural panels, solid wood joints, are under pressure to reduce production costs and harmful emissions of formaldehyde from the adhesives, and to improve the recyclability of product (Sellers, 2001; Maloney, 1996). Innovative approaches are needed to meet these challenges to reduce the amount of binder while maintaining the product quality. Another topic related to wood products is the chemical modification of their surface and bulk properties to improve their durability, range of application, and compatibility with other materials for use in hybrid products such as wood–plastic composites (Sellers, 2001). The manufacturing process of fibreboards includes pulping, so the pulping cost may also be an issue for fibreboards.

Laccase is one of the most important enzymes in terms of application versatility in the forest products industry (Mayer and Staples, 2002; Yaropolov et al., 1994; Leonowicz et al., 2001). Laccases with high redox potential can be used in almost the entire paper product production chain. In the forest product industry laccases are being explored for functionalization of cellulose fibres. Also, lignocellulosic materials with new resistance and stability properties are designed by means of phenolic compound grafts catalyzed by laccase. Laccases have been examined for improving compression degree in wood based panels by "in situ" enzyme lignin coupling without using any toxic adhesives containing formaldehyde. The forest products industry is increasingly turning to enzyme technology to meet the above challenges (Viikari, 2002; Ragauskas, 2002; Bajpai et al., 1999; 2012b; Borch et al., 2003).

Laccase is applicable to virtually the total production chain of paper products from pulping to recovery of secondary fibres and effluent treatment. Indeed, most of the published research and applications of laccase in the forest products industry relate to the pulp and paper sector, where particular emphasis has been placed on the use of laccase in biobleaching and mill water treatment. Emerging research areas focus on tailoring of lignocellulosic materials by laccase-assisted biografting of phenols and other compounds, and the use of laccase for adhesion enhancement in binderless wood boards.

The substrate range of laccase is not limited to phenols (Wells et al., 2006). It can be used with an oxidation mediator to oxidize non-phenolic substrates. Table 6.3 shows the application of laccase in forest products industry where the objective is to either remove or co-polymerize lignin.

6.1.1 PULPING

The lignin-degrading fungi or enzymes are used for the pretreatment of lignocellulosic raw material. This process is referred to as biopulping. The treatment is generally carried out before pulping, and has also been used between the primary and secondary refiners in thermo-mechanical pulping (TMP) (Burton, 2001). The recent biopulping research has mainly focused on the use of the white-rot fungus *Ceriporiopsis subvermispora* (Blanchette et al., 1991; Akhtar, 1994; Ferraz et al., 2002; Hunt et al., 2004; Mendonc et al., 2004) which produces enzymes laccase and manganese peroxidase. Also, several other white-rot fungi and actinobacteria, expressing these enzymes, have been studied for biopulping (Leatham et al., 1990; Setliff et al., 1990; Kashino et al., 1993; Patel et al., 1994; Akhtar et al., 1997; Behrendt et al., 2000; Hatakka et al., 2002; Hernández et al., 2005; Selvam et al., 2006). Table 6.4 shows advantages of biopulping.

Although the bulk of biopulping research on lignocellulosics has focused on the use of the white-rot fungi, some researchers have also used laccase mediator system (LMS) to study biopulping (Dyer and Ragauskas, 2004; Petit-Conil et al., 2002; Vaheri et al., 1991). Pine chips were treated with laccase together with mediator ABTS, HBT, or violuric acid (VA) by Dyer and Ragauskas (2004) before kraft pulping. Laccase/HBT was found to be the most effective LMS for increasing delignification and pulp yield. Petit-Conil et al. (2002) treated spruce chips with laccases obtained from three fungi with a mediator HBT before TMP. Use of laccase/HBT saved refiner energy with two of the laccases by up to 20%, but the use of third laccase increased it. The effect on pulp properties in terms of brightness and mechanical strength properties was mostly positive. The improved pulp properties were attributed to a modification of surface chemistry of the fibre and increased external fibrillation and bonding potential. A reduction of 15% in peroxide consumption during subsequent bleaching to comparable brightness was obtained with one of the laccases without using mediator compared to bleaching without laccase pretreatment. In a patent by Vaheri et al. (1991), use of laccase pretreatment for reducing energy consumption during mechanical pulping was described. The treatment also enhanced the pulp strength properties and blue reflectance factor. Laccases can depolymerize lignin and delignify wood pulps due to its property of removing potentially toxic phenols which are generated during degradation of lignin (Virk et al. 2012). First laccase acts on small phenolic lignin fragments which react with the lignin polymer, and then results into its degradation. Fourthermore, pretreatment of wood chips with ligninolytic fungi increases the pulp strength and the energy requirement for mechanical pulping is also reduced. *Cryptococcus albidus* producing laccase enzyme was effective in reducing the lignin content of eucalyptus wood and found suitable for biopulping (Singhal et al. 2005). Pretreatment of hardwood with *Phlebia tremellosa* (laccase producer) produced an 80% increase in the tensile strength. In *Phlebia brevispora* another laccase producer, energy requirement was reduced by 47% by incubating aspen chips for 4 weeks.

LMS is currently marketed by MetGenm Kaarina Finland for increasing throughput in mechanical pulping, for enhancing paper strength properties and reducing pitch problems (MetZyme® LIGNO™) (Mate and Alclade, 2016).

6.1.2 BLEACHING

Pulp bleaching is generally achieved by treating pulps with chlorine-based chemicals. This results in the formation of chlorinated aliphatic and aromatic compounds that could be carcinogenic, toxic and mutagenic (Bajpai, 2012a; Taspinar and Kolankaya 1998). In the recent years, it has been replaced by elemental chlorine free (ECF) and totally chlorine free bleaching (TCF) sequences (Stanko and Angus, 2006; Bajpai, 2012a). TCF processes use oxygen based- bleaching agents, mainly oxygen, alkaline hydrogen peroxide , and ozone, whereas ECF bleaching may also make use of chlorine chemicals other than elemental chlorine mainly chlorine dioxide (Bajpai, 2012a). ECF is found to be more effective than TCF in terms of bleaching effect. In North America, ECF bleaching is mostly used whereas in European mills, TCF bleaching is more common. In the recent years, intensive studies have been performed to develop enzymatic, environmentally benign, bleaching technologies. Research into use of enzymes is being driven to reduce overall production costs and improve work safety and also to meet the stringent environmental restrictions concerning the fresh water consumption in mills and effluent toxicity (Bajpai et al., 1999, 2012b). Debate is ongoing concerning the impact of ECF bleaching on the aquatic environment and whether the perceived benefits achieved with TCF bleaching are warranted in view of somewhat lower quality of TCF pulps and the higher production cost (Stanko and Angus, 2006). For the mills considering to starting TCF pulping, the challenge is to bridge the cost gaps as well as the quality between ECF and TCF pulps. Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content (Luisa et al., 1996). Oxygen delignification process was commercially introduced in the last years to replace conventional and chlorine-based methods which are highly polluting. In spite of this method, use of laccase enzyme can provide milder and cleaner strategies of delignification that does not have any adverse impact on cellulose (Barreca et al., 2003; Gamelas et al., 2005; Shi, 2006; Xu et al., 2006). Laccases are able to delignify pulp when they are used together with mediators (Bajpai, 2012a; 2012b). Small natural low molecular weight compounds having high redox potential more than 900 mV, called mediators may be used to oxidize the non-phenolic residues from the oxygen delignification (Bourbonnais et al., 1997). The mediator gets oxidized by laccase and the oxidized mediator further oxidizes subunits of lignin that otherwise would not be laccase substrates (Bourbonnais and Paice, 1990; Call and Mucke, 1997). Although LMS has been studied extensively, there are still unresolved problems concerning with mediator recycling, cost and toxicity. However, some environmental benefits are realized and the fact that LMS could be easily implemented in the existing bleaching sequences is a major advantage that could possibly lead to a partial replacement of chlorine dioxide in pulp mills. Furthermore, the application of laccases in kraft pulp bleaching may result in higher pulp yields and energy savings.

Most studies on the development of LMS-biobleaching methods have been directed toward kraft pulps. Call and Mucke (1997) developed a biobleaching process called Lignozym®, which is based on laccase/HBT. This process performed well in pilot plant trials (Table 6.5). To effectively delignify, mediators should be stable and have electron redox potential (Eo) of at least 0.7V (Bourbonnais et al., 1997, Xu et al., 2002).

The most effective mediators discovered until now are N-heterocycles bearing N-OH-groups, the most important being HBT, VA and N-hydroxy acetanilide (NHA). Figure 6.1 shows the chemical structure of these most effective mediators.

In a comparative study on the efficiency of HBT, VA and NHA to mediate laccase bleaching of unbleached softwood kraft pulp, VA was found to be the best mediator in terms of delignification and extent of oxidation of residual lignin (Chakar and Ragauskas, 2004). Sealey and Ragauskas (1998) found the residual lignins of pre- and post-oxygen stage delignified kraft pulps subjected to laccase/NHA treatment to contain more O-4 linkages than those of the original oxygen delignified pulps. The survival of the O-4 linkages is inconsistent with the model compound studied by Kawai (1999) with laccase/HBT. A prerequisite for LMS used for biobleaching is that they are highly selective toward lignin and do not have any adverse effect on pulp viscosity. The high rate of carbohydrate degradation observed with certain mediators such as 2,2,6,6-tetramethylpiperidine-Noxyl (TEMPO) renders them unsuitable for biobleaching while other mediators such as VA, NHA, and HBT show high selectivity (Barreca et al., 2004, Chakar and Ragauskas, 2000; Poppius-Levlin et al., 1999, 2001). The LMS treatment followed by alkaline extraction results in significant delignification of chemical pulps (Bourbonnais and Paice, 1996), it must be incorporated into an ECF or TCF-bleaching sequence for obtaining fully bleached pulps. Investigations have shown that laccase/NHA (Paice et al., 2002), laccase/HBT (Kandioller and Christov 2001; Sealey et al., 2000), and laccase/polyoxometalate (Gamelas et al., 2007) can substantially reduce the demand of bleaching chemicals for chemical pulp or allow bleaching to lower kappa numbers and higher brightness.

Lignozym later introduced a new mediator, NHA that is biodegradable and has been claimed to be cost-effective (Amann 1997). Biobleaching with NHA also allows the enzyme to maintain about 80% of its original activity after treatment for an hour, whereas biobleaching with HBT have an adverse effect on enzyme activity.

Fu et al. (2000) bleached *Eucalyptus urophylla* Kraft pulp with laccase in the presence of NHA and achieved 43% reduction in kappa number after alkali extraction. The addition of surfactant improved the dissolution of lignin, and hence improved the pulp brightness and also the activity of the laccase. The effectiveness of HBT and NHA in LMS has been confirmed (Chakar and Ragauskas 2000). Higher levels of delignification were achieved with HBT compared to NHA.

Arias et al. (2003) have reported that application of the laccase from *Streptomyces cyaneus* in the presence of ABTS to bleaching of eucalyptus Kraft pulps resulted in a significant reduction in the kappa number by 2.3 U and an increase in the brightness by 2.2%, (as determined by the International Standard Organization test) of pulps, showing the suitability of *Streptomycetes* laccases for industrial applications. A comparison of *T. versicolor* laccase with various mediators – ABTS, HBT, Remazol blue, nitroso-napthols and phenothiazines has shown that HBT produced the most extensive delignification, but it also deactivated the enzyme and, therefore, required a higher dose of enzyme.

Poppius-Levlin et al. (1997) subjected three different chemical pulps, i.e., a unbleached Kraft pine pulp, oxygen-delignified (two stage) Kraft pine pulp, and birch formic acid/peroxyformic acid (MILOX) pulps to laccase-HBT and laccase-ABTS treatments. HBT was found to be more effective than ABTS in delignifying pulp and gave higher pulp brightness. All the laccase/HBT-treated pulps showed better response to alkaline hydrogen peroxide bleaching, and as a consequence oxygen-delignified pulp and MILOX pulp obtained full brightness in one and two stages, respectively. Even the Kraft pine pulp reached a final brightness of 83%. Furthermore, a xylanase stage before or with laccase/HBT slightly improved the effect of laccase/HBT and resulted in a higher final brightness after peroxide bleaching than without using the xylanase treatment.

Kandioller and Christov (2001) used HBT, NHA, VA and potassium-octacyanomolybdate (IV) as mediators in combination with *T. versicolor* laccase at various dosages to delignify and bleach the pulps. Kappa number reduced between 5.6% and 64.3%, depending on the type of pulp, enzyme and mediator charge, following alkaline extraction. On all pulps, VA was the most effective mediator in terms of kappa number reduction. In terms of brightness increase after L-E treatment, VA was most efficient on bagasse soda pulp and hardwood sulfite dissolving pulp. HBT was the most efficient mediator in terms of brightness gain on bagasse soda pulp (4.0 points) and hardwood soda-AQ pulp (1.5 points), whereas VA was most efficient on hardwood sulfite dissolving pulp (1.1 points). Chlorine dioxide savings were obtained on all the three pulps: hardwood sulfite dissolving pulp (55% savings), hardwood soda-AQ pulp (50%) and bagasse soda pulp (50%).

Camarero et al. (2004) compared three fungal laccases (from *P. cinnabarinus, T. versicolor* and *P. eryngii*) and two mediators, ABTS and HBT. *P. cinnabarinus* and *T. versicolor* laccases in the presence of HBT showed the best results in terms of high brightness and reduced lignin content. The former laccase also resulted in largest removal of residual lignin and the best preservation of cellulose as shown by analytical pyrolysis, and was chosen for subsequent TCF bleaching. Up to 90% delignification and strong brightness increase were obtained after an LM treatment followed by hydrogen peroxide bleaching. This TCF sequence was further improved by applying hydrogen peroxide under pressurized oxygen.

Chandra et al. (2001) have reported that the bleaching of high kappa Kraft pulps with an LMS showed 42.6–61.1% delignification following extraction with peroxide when VA was used as the mediator. The pulp yield, after the alkaline extraction, was +99.9%. A comparison of mediator efficiency showed that VA was superior to N-hydroxybenzotriazole or N-acetyl-N-phenyl hydroxylamine for delignification of high lignin content pulps. Molecular modeling of these three mediators shows an elevated impaired electron density for VA over the other mediators, perhaps accounting for its improved performance. Structural analysis of the residual lignin after the laccase treatments showed that the laccase-stage oxidizes basically the phenolic structures of lignin. Full sequence ECF bleaching of high- and low-kappa SW Kraft pulps after an L(EoP) or L-L(EoP) showed that the pulps could be readily bleached to +85 Tappi brightness.

Bajpai et al., (2006b) examined the delignification efficiency of different laccase enzymes on the eucalyptus Kraft pulp. The laccase enzyme from *Trametes versicolor* showing the highest delignification efficiency was used in the ECF sequence for improving the pulp bleachability. Significant reduction in chlorine dioxide consumption was also obtained. More reduction in chlorine dioxide consumption was obtained when the same laccase treated pulp was subjected to an acid treatment after the extraction stage followed by the DEPD sequence. ECF bleaching was also conducted using the xylanase-laccase treated pulp. Xylanase treatment was incorporated to the laccase mediator system in the elemental-chlorine free bleaching both sequentially and simultaneously. The bleaching sequence DEPD followed and in both the cases, the reduction in chlorine dioxide consumption was higher as compared to the control. The chlorine dioxide consumption was reduced further when xylanase-laccase treated pulp was given an additional acid treatment. The final pulp properties of the treated pulps were comparable to the control pulp.

Call et al. (2004) studied chlorine dioxide saving potential of different enzymatic treatments. Treatments were performed with an extended hydrolase-mediated oxidation system (HOS), and an improved laccase oxidation system (LASox). Before chlorine dioxide sequences, these enzymatic treatments, achieved a kappa reduction of 40%, with only a small reduction in viscosity. After the chlorine dioxide sequences, the 88.5% ISO brightness target was easily achieved. Strength properties of untreated and enzyme treated pulps were equivalent. A saving of around half of the chlorine dioxide charge at good strength properties, and at a comparable cost, was possible. One disadvantage was some loss in hemicellulose content (2%), probably due to the presence of impurities in the hydrolase (lipase) formulation used. Nonetheless, this enzymatic approach appears to have high potential for delignification and chlorine dioxide saving.

In Paprican (now FP Innovations), the use of transition metal complexes as catalytic mediators in the enzymatic delignification and bleaching of Kraft pulps was studied by Paice et al. (2001) and Bourbonnais et al. (2000). Softwood Kraft pulp delignified with oxygen was treated with laccase in the presence of potassium octacynomolybdate which is a transition metal complex. At all charges of mediator, pulp delignification was higher in comparison to control pulps. However, there was slight loss of pulp viscosity at the highest dosage of mediator. Treatment of hardwood Kraft pulp under the similar reaction conditions produced similar results. The molybdenum mediator was found to be recycled after pulp delignification and could be reused with the same efficiency as the fresh mediator. LMS was also found to be effective in removing hexenuronic acid from Kraft pulp (Fagerstrom et al., 2001).

Codexis Inc., United States has patented binary mediator system which bleaches cellulose efficiently when used in combination with laccase (Cheng et al., 2003). It includes prooxidants and prodegraders, and a laccase enzyme from the fungus *Aspergillus*. The prooxidants used are ascorbic, salicylic, and nicotinic acids; their salts; lignin sulfonates; and mixtures of these substances. They are efficient laccase enhancers by themselves, in both oxidation of some dyes (e.g., Chicago blue) and delignification of paper pulp. The prodegraders used are urea, thiourea, sulfaminic acid, sulfamide, guanidine, methylsulfonic acid, and their mixtures. These substances are not laccase enhancers by themselves. However, simultaneous use of prooxidants and prodegraders results in more efficient paper pulp delignification as compared with prooxidants not supplemented with prodegraders.

The cost of synthetic mediators tends to be prohibitive for implementation in biobleaching. This has generated interest in natural mediators obtained from plants or as industrial by-products. One of the first natural laccase mediators found was syringaldehyde (Kawai et al., 1989). Potentially cost-effective lignin-derived natural mediators – p-coumaric acid, syringaldehyde, and acetosyringone – obtained from spent pulping liquors and plant materials, were studied by Camarero et al. (2007). Figure 6.2 shows the chemical structure of few natural mediators for laccases.

6.1.3 FIBRE MODIFICATION

Laccase and LMS can modify the fibre and improve its properties (both physical and chemical) by enzymatic activation of fibres containing high lignin-content (Mocciutti et al. 2008; Chen et al. 2012). Lund and Felby (2001) found that wet tensile strength properties were significantly improved when the unbleached high-yield kraft pulp was modified with LMS. However, they did not observe any effect on the dry tensile strength. Mohandass et al. (2008) reported that laccase oxidation of pulp fibres increased the amount of carboxyl groups contained on the original fibres. This modification improved fibre swelling and flexibility and was found advantageous in the bonding of pulp fibres in paper to increase paper strength (Witayakran and Ragauskas 2009b). Chen et al. (2012) observed that carboxyl group content and physical strength properties of old corrugated container pulp increased with laccase or a laccase-HBT system (Chen et al., 2012). Wong et al. (2000) found that laccase treatment of mechanical pulp improved paper strength by increasing the interfibre bonding.

Compared to laccase alone, LMS are more effective in terms of increasing strength of kraft paper produced from unbleached pulp. With a low dose of laccase/HBT, Wong et al. (1999) improved the strength of paper produced from high-yield kraft pulp. The wet strength of kraft paper produced from unbleached high-yield pulp increased with LMS treatment with different mediators (Lund and Felby, 2001). LMS combined with heat treatment improved wet strength more than the LMS treatment alone. This was ascribed to polymerization of lignin in the handsheets, and to crosslinking of phenoxy radicals in adjacent fibres. These researchers observed that treatment with laccase alone had only a very little effect on the wet strength of the pulp, whereas addition of lignin rich extractives significantly increased the wet strength after the enzyme treatment (Lund and Felby, 2001).

Lund and Felby (2000) patented a process for producing corrugated paperboard or corrugated containers with LMS to produce a paper material with improved wet strength. The wet strength of paper materials was improved without using wet strength resins which makes the product more easily re-used.

Hansen et al. (1995) patented a process for producing linerboard or corrugated medium having increased strength. These researchers found that the strength of the linerboard/corrugated medium could be increased by treating the pulp suspension with a phenol-oxidizing enzyme system in the stock preparation section prior to the paper machine. This strengthening is attributed to cross-linking of the lignin present at the surface of the individual pulp fibres.

By laccase-assisted polymerization of vanillic acid, Yamaguchi et al. (1994) prepared dehydrogenative polymer and deposited on laccase-treated TMP. The water resistance and tensile strength of TMP paper increased. This was ascribed to coupling reactions between fibre lignin and dehydrogenative polymer, and also to an increase in fibre contact area. In another study (Yamaguchi et al., 1992), dehydrogenative polymers with laccase from various phenols including tannic acid, vanillic acid, and catechol were prepared and precipitated on TMP. This improved the ply bond strength.

6.1.4 PITCH CONTROL

Lipophilic extractives from wood and other lignocellulosic materials are often referred to as wood resins. These cause the pitch deposits in the pulp and paper manufacturing processes. Pitch presents a serious problem for the paper industry. Wood resins include the following:

- Alkanes
- Fatty alcohols
- Fatty acids
- Resin acids
- Sterols
- Terpenoids
- Conjugated sterols
- Triglycerides
- Waxes

Table 6.7 presents examples of prominent types of lipophilic wood extractives causing pitch problems. Pitch problem makes the paper machine operation very difficult and inefficient and reduce the quality and benefits (Back, 2000; Allen, 1975, Allen 2000a, b). The problems are mentioned below (Back and Allen, 2000):

- Reduced production levels
- Higher equipment maintenance costs
- Higher operating costs
- Increased incidence of defects in the finished products

The effluents containing wood extractives are found to be toxic and harmful to the environment (Bajpai, 2012b; Leach and Thakore 1976; Liss et al. 1997). Otero et al. (2000) have reported that pitch problems are greater in mills having a high degree of water circuit closure. Both microbial and enzymatic products for wood and pulp respectively have been commercialized. Laccase is one of the enzymes useful for solving pitch problems; it has been shown to modify lipophilic extractives (Bajpai, 1999; Bajpai et al., 1999; Dube et al., 2009).

Promising results have been reported by the use of oxidative enzymes, particularly laccases in the presence of redox mediators which are effective on several lipophilic extractives such as fatty acids, resin acids, free and conjugated sterols and triglycerides (Gutiérrez et al., 2006a,b,c). With laccases, a double benefit can be obtained from their application on pulps in the presence of redox mediators. These compounds enable laccase to remove residual lignin, as well as extensive degradation of pulp extractives including the most recalcitrant compounds, such as sterols and resin acids. Karlsson et al. (2001) first reported reactivity of *Trametes* species laccase on polyunsaturated fatty acids and conjugated resin acids. Similar action of laccase on trilinolein was reported by Zhang et al. (2002a). In the reaction with trilinolein, the major oxidation products identified were monohydroperoxides, bishydroperoxides and epoxides. Similarly, a reduction over 30% of lipophilic extractives present in softwood pulp from TMP pulping and process waters was also reported (Buchert et al., 2002; Dubé et al., 2009; Zhang et al., 2000, 2005). Paice (2005) reported about 85% removal of extractives from mechanical pulp by laccase treatment (Figure 6.3).

Gutiérrez et al. (2006b,c) reported the use of LMS for the removal of lipophilic extractives present in pulps from different origins. Laccase from *Pycnoporus cinnabarinus* in the presence of the mediator HBT was very efficient in removing free and conjugated sterols by 95–100% from eucalyptus kraft pulp; triglycerides, sterols and resin acids by 65–100% from spruce TMP pulp; and fatty alcohols, alkanes and sterols by 40–100% from flax soda pulp. The removal of lipids by laccase-HBT resulted in the formation of several oxidized derivatives that were absent or presented low abundances in the initial pulps. In spite of this, the total lipid content in pulps reduced significantly, and the problematic compounds were completely removed. In another study, the enzymatic treatment was applied as an additional stage of an industrial-type TCF sequence for bleaching eucalyptus kraft pulp (Gutiérrez et al., 2006a) showing the complete removal of free and conjugated sitosterol. Pulp brightness was also improved due to the simultaneous removal of lignin by LMS treatment. Molina et al. (2008) conducted further research on the chemistry of the reactions of LMS with model lipids representative for the main lipophilic extractives present in hardwood, softwood and non-wood paper pulps. The reaction products were identified and quantified during the treatment for understanding the degradation patterns. About 60–100% decrease of the initial amount of unsaturated compounds was seen at the end of 2 h laccase-HBT treatment. Similarly, a reduction of 20–40% of these unsaturated lipids was seen after treatment with laccase alone except in the cases of abietic acid which reduced by 95%, and cholesteryl palmitate and sitosterol that were not affected. This confirmed that laccase alone reduced the concentration of some unsaturated lipids (Karlsson et al., 2001; Zhang et al., 2002b). But with LMS, the most rapid and extensive lipid modification was achieved (Molina et al., 2008). Model unsaturated lipids were largely oxidized and the major products identified were epoxy and hydroxy-fatty acids from fatty acids, and free and esterified 7-ketosterols and steroid ketones from sterols and sterol esters.

Gutiérrez et al. (2007) treated unbleached eucalyptus kraft pulp with a fungal laccase in the presence of acetosyringone, syringaldehyde, and p-coumaric acid as mediators. The enzymatic treatment using syringaldehyde as mediator caused the highest removal by more than 90% of free and conjugated sitosterol, similar to that obtained with HBT, followed by acetosyringone which showed over 60% removal, whereas p-coumaric acid was not much effective. Moreover, recalcitrant oxidized steroids which survived in laccase-HBT treatment could be removed when using these natural mediators. Pulp brightness also increased by the laccase treatment in the presence of the above phenols followed by the hydrogen peroxide stage due to the simultaneous removal of lignin.

6.1.5 DEINKING OF RECYCLED FIBRE

Paper recycling activities are increasing due to growing environmental awareness, robust overseas markets and domestic demand. Wastepaper recycling enables the following:

- Substitution of virgin pulp with recycled fibres
- Reduces the exploitation of old forests
- Reduces disposal problems

Table 6.8 shows the benefits of using recycled fibre.

Recycling technologies have been improved by the developments in pulping, flotation deinking, cleaning, screening & bleaching, and also by efforts to boost overall yield, encouraging developments of products based on recycled materials.

The difficulty in dealing with secondary fibre is the removal of contaminants, particularly ink. Some paper grades, such as newspapers can be deinked with relative ease by conventional deinking processes. Non-impact-printed papers are more difficult to deink. Colour printing via offset lithography is expanding in the United States; other countries are also expected to follow. The cross-linking inks used in this process are also difficult to remove by conventional methods. Mixed office waste is a source of high quality fibre that can be used for fine papers and many other products, if the deinking process can be improved. Ink removal continues to be a major technical problem to greater use of recycled paper. Conventional deinking processes use large quantities of chemicals. Enzyme assisted deinking is an environmentally friendly alternative to conventional deinking processes (Anon, 2010; Bajpai, 1999; Bajpai and Bajpai, 1998; Bajpai, et al., 1999; Ma and Jian, 2002; Mohammed, 2010; Puneet et al., 2010; Xu et al., 2004).

Research has been conducted into deinking of recycled wood-based fibres using laccase and LMS. Li et al. (2000) performed oxidative bleaching of yellow paper with hydrogen peroxide, pressurized oxygen, and LMS. It was found that a LMS treatment, using violuric acid as a mediator, was able to increase the brightness of an unspecified yellow dyed recycled paper from TAPPI brightness 43.5 to 55.0 after biobleaching, achieving significant dye removal. Selvam et al. (2005) reported that laccase treatment reduced the kappa number and increased the brightness of waste photocopy paper.

Hager et al. (2002) investigated whether LMS would be suitable for deinking processes (such as ink detachment and dirt speck reduction) that are run in neutral conditions under atmospheric pressure. They tested two laccases (from *Trametes villosa* and *Myceliophtora thermophila*) and three mediators: ABTS, HBT and violuric acid. Treatment with laccase or laccase with mediator lowered the brightness of artificially aged prints, deinked pulp, and unprinted recycling papers. Subsequent tests indicated that laccase treatment resulted in yellowing of lignin in groundwood and thermomechanical pulp. This yellowing could not be reversed by subsequent alkaline extraction, peroxide bleaching or FAS bleaching. These results suggest that laccase mediator bleaching would not benefit a mill recycling lightly coloured papers containing a high percentage of groundwood or thermomechanical pulp. A recycle mill treating highly coloured papers with a low percentage of groundwood and thermomechanical pulp may still benefit. Also, the neutral pH and/or atmospheric oxygen conditions may have affected laccase selectivity. Patents on deinking and bleaching of waste paper with laccase and other enzymes include those by Call (1991b) and Franks et al. (2000). In a study by Arjona et al. (2007), a bleaching sequence included laccase-mediator system stage (L), a hydrogen peroxide stage (P) and a sodium hydrosulfite stage (Y) on a mixture of different coloured writing and printing papers. After the application of L-P-Y sequence, a pulp with optical properties near to eucalyptus totally bleached pulp was obtained. The L-P-Y sequence reaches a colour removal of 90% and saves chemicals in the final stages.

Xu et al. (2004) studied the deinking of old newspaper (ONP) using cellulase or hemicellulase in conjunction with LMS. The synergistic use of the two enzymes led to the production of pulps with superior brightness and strength compared to those prepared using only one of the enzymes. ONP deinked using cellulase and the LMS had a brightness of 55.9% ISO after bleaching with hydrogen peroxide, a breaking length of 2.13 km and a tear index of 6.43 mN m^2/g. The respective increases in brightness were 2.4 percentage points and 3.8 percentage points compared to the use of cellulase alone and laccase system alone. The breaking length was 30% higher, pulp brightness (after hydrogen peroxide bleaching) of 60.4% ISO, breaking length of 1.94 km and a tear index of 6.54 mN m^2/g. The respective increases in brightness were 2.7% and 8.3% compared to the use of hemicellulase alone and laccase system alone. The breaking length was 20% higher than obtained with the use of hemicellulase alone.

6.1.6 TREATMENT OF MILL PROCESS WATER AND EFFLUENT

Strict environmental regulations are forcing pulp and paper producers to reuse their process water for reducing their fresh water consumption and effluent discharge (Bajpai, 2012a). The closure of water circuits in recycled paper mills leads to the accumulation of dissolved and colloidal material in the process water. These compounds originate from the recycled paper used as raw material; they include anionic trash, secondary stickies, pitch, microorganisms, odour components, salts and calcium hardness (Bajpai and Bajpai 1999). DCS are primarily constituents of wood, such as hemicelluloses, pectins, lipophilic extractives and lignin. Typical negative effects of these materials are presented in Table 6.9 (Habets et al. 1997). Other problems have arisen gradually so that mills have adapted to them or have learnt to live with them. In many cases, the mills use furnish of higher quality than normal, which means that it is relatively clean of contaminants and contains fibres with relatively high freeness (Barton et al. 1995).

It is important to remove harmful white water constituents before the water is recirculated back into the production process. This is accomplished by using evaporators; but they have high operating costs and the investment is also high (Wiseman and Ogden 1996). Because of the lack of economically viable in-mill treatment technology, the white water finally is discharged as wastewater when the concentration of harmful constituents increases to an unacceptable level. Biological in-mill white water treatments are being performed where the part of the contaminants are consumed by microorganisms (Widsten et al., 2004a; Alexandersson and Malmqvist, 2005; Latorre et al., 2007). The biological treatment of white water improves clarification significantly, as organic compounds stabilising the fines in suspension are consumed by the bacteria which are present in the bioreactor. The main carbon sources are the carbohydrates and extractives. These are responsible for poor first-pass retention, poor dewatering on the wire and bad smell in the product. Use of internal biological treatment will improve the efficacy of conventional wastewater treatment systems. It will also eliminate the possibility of secondary solids, as the carbon source is consumed in the bioreactor. The clear filtrate from the pulping should be cooled before biological treatment if the temperature exceeds 30–35°C. Also, it should be supplemented with phosphorous and nitrogen. The pH should also be adjusted to neutral. Several types of bioreactors and methods are available and the selection of appropriate technology will depend on a particular case. Most mills are already using biological effluent treatment, which can

easily be used for the same. In order to make the biologically treated water useful in the process by replacing fresh water, suspended solids and possibly colour must be removed. This can be achieved by the use of ultrafiltration. It has been shown that the flux through the ultrafiltration membrane increases considerably compared with water before biological treatment. This enables higher capacity for a given filtration area at constant pressure drop and temperature. The bacterial biomass partially binds chlorides. This has been shown by the fact that the chloride content in dry matter was found to be 1,500–3,000 ppm for biomass grown in a white water where the chloride concentration was 20–40 ppm. However, in the conditions prevailing in biological treatment, heavy metals are normally precipitated as hydroxides, provided the complexing additives such as DTPA and EDTA are not used in excess. The biodegradability of these substances remains questionable and can be evaluated using existing bioreactors. With a combination of biological treatment and efficient after-treatment such as membrane or chemical precipitation, it is possible to replace fresh water in the process. However, a few important requirements should be considered.

(i) Bioprocesses need nutrients in the form of nitrogen and phosphorous. These elements have to be added before the biological treatment in such a manner that the concentration in the treated water is lower than in the incoming wastewater.
(ii) Biological processes take place at neutral pH.
(iii) After-treatment is needed to separate secondary suspended solids (bacterial cells). With proper operation, the treated water will be free from components that cause biological activity, retention and dewatering disturbances, and thus will be of acceptable quality for recycling.

As discussed earlier, laccase is able to oxidize certain lipophilic extractives in wood raw material and pulp. Zhang et al. (2001) investigated the potential of both commercial and purified laccases to modify model extractive compounds found in thermomechanical pulp (TMP)/newsprint process waters. The model compounds used were representative of the fatty acids, resin acids and triglycerides found in mill process waters. The compounds were modified by the laccase treatments or significantly degraded. When reaction mechanisms were investigated and reaction products analysed, it was found that the laccases primarily interact with specific types of lipophilic extractives. Zhang et al. (2005) were able to reduce pitch by 50% with laccase treatment of TMP white water. In addition, the amount of hydrophilic extractives, including lignans and lignin, was reduced by 90%. In another investigation, Zhang et al. (2002b) treated mill TMP whitewater with a culture filtrate of the laccase-producing white rot fungus *Trametes versicolor*. The treatment removed more than 90% of the esterified fatty acids and lignans, and 40% of the resin acids and free fatty acids.

Widsten et al. (2004a) purified TMP newsprint white water using an aerobic in-mill biokidney followed by microfiltration and laccase treatment. They evaluated removal efficiency by chemical analyses of the white waters and by testing the properties of paper made with the white waters. The microfiltration alone was able to reduce total lipophilic extractives and lignans by 24%, whereas the combination of laccase treatment and microfiltration removed 82% of them, including all the lignans. The lignans were probably polymerized to lignin-like material (Zhang et al., 2002b; Widsten et al., 2004a), whose amount was reduced by 15% and 44% in respective microfiltration treatments without and with laccase pretreatment. These results show that laccase can be used to reduce the problems caused by wood-based white water contaminants. Most studies on the application of laccase to water treatment at pulp and paper mills focus on wastewater.

A number of substances in white water affect both the production process and the quality of the paper produced. These compounds are usually divided into two categories: dissolved substances and colloidal substances (Mittal et al., 2006). Dissolved substances include lignans, polysaccharides and ions; colloidal substances comprise lignin and lipophilic extractives. These compounds accumulate when white water systems are closed and they affect the production process and the final product (Miranda et al., 2006).

A combined fungal-enzyme system has shown promise as one way of reducing the detrimental substances present within closed water systems, while treatments with different enzyme preparations has shown that fungal laccases play an important role in removing white water extractives. The study investigated the potential of both commercial and purified laccases to modify model extractive compounds found in thermomechanical pulp (TMP)/newsprint process waters (Zhang et al. 2001). The model compounds used were representative of the fatty acids, resin acids and triglycerides found in mill process waters. The compounds were significantly degraded or modified by the laccase treatments. When reaction mechanisms were investigated and reaction products analysed, it was found that the laccases primarily interact with specific types of lipophilic extractives.

Hakulinen (1988) published a review on the use of enzymes for waste water treatment in the pulp and paper industry. Forss et al (1987) examined the use of laccase for effluent treatment. The pulp bleaching waste water was aerated in the presence of laccase for one hour at pH 4.8 and then flocculated with aluminum sulfate. High removal efficiencies for chlorinated phenols, guaiacols, vanilins and catechols were obtained.

Roy-Arcand and Archibald (1991) studied direct dechlorination of chlorophenolic compounds in pulp and paper mill effluent produced by laccases from *T. versicolor* and found that all the major laccases, produced by *T. versicolor*, were able to partially dechlorinate a variety of chlorophenolics.

A method for wastewater treatment containing non-degradable phenolic compounds and degradable non-phenolic compounds was patented by Field (1986). This method consisted of an oxidative treatment for reducing or eliminating toxicity of the phenolic compounds followed by an anaerobic purification. This oxidative pretreatment could be performed with the use of laccase enzymes and it reduced COD by one thousand fold.

Call (1991a) patented a process on the use of laccase for waste water treatment. It was found that waste water generated from delignification and bleaching could be treated with laccases in the presence of aromatic compounds, nonaromatic oxidants and reductants. Almost complete polymerization of the lignins was obtained which was 20–50% above the values achieved with the use of laccase alone. About 70–90% lignin was converted into insoluble form, which could be removed by flocculation and filtration.

Milstein et al. (1988) reported the removal of chlorophenols and chlorolignins generated from bleaching effluents by using combined chemical and biological treatments. The organic matter from chlorination, extraction stage effluents or a mixture of both stages, was precipitated as a water insoluble complex by the use of polyethyleneimide. Reduction in AOX was 84% and 73% by laccase treatment of chlorination and extractions stage effluents, respectively. Most of the mono- and dichlorophenols were co-precipitated with the bleaching effluents. The colour and COD were reduced by 92 and, 65% respectively for the chlorination effluent and by 76 and, 70 for the extraction effluent. No significant reduction in BOD of treated effluent was found but fish toxicity was substantially reduced. Enzyme treatment resulted in coprecipitation of the bulk mono-and dichlorophenols from the liquors of the chlorination and extraction bleaching stages.

Lyr (1963) reported that laccase of *T. versicolor* partially dechlorinates PCP and Hammel and Tordone (1988) found that peroxidase from *P. chrysosporium* was able to partially dechlorinate PCP and 2,4,6-trichlorophenol.

Though the use of enzyme based treatments offers some distinct advantages over physical and chemical methods in that only catalytic amounts of reagents are needed, biochemical instability and difficulty in reusing the enzyme are its disadvantages. Immobilization of the enzymes is required for biochemical stability and reuse of the enzymes. Carbon immobilized laccase was used by Davis and Burns (1992) to decolourize extraction stage effluent at the rate of 115 PCU/enzyme unit/hour. The removal rate was found to increase with the increasing effluent concentration.

Laccase is able to remove toxicity from wastewater containing chlorophenols by catalyzing their polymerization via radical coupling (Milstein et al., 1988; Dec et al., 2003). The coupling products could be removed from the wastewater by precipitation (Milstein et al., 1988). The chlorophenols also undergo partial dechlorination with loss of chlorine ions from the aromatic carbons involved in the coupling reaction (Dec et al., 2003), reducing the toxicity of the chlorophenols (Dec and Bollag 1994). Chlorophenols can also cross-couple and precipitate with other phenols present in wastewater, which may enhance their removal efficiency (Bollag et al., 1988; Cho et al., 2007). Laccase has been used for removing AOX from wood pulp bleaching effluents.

Forss et al. (1989) aerated bleaching wastewater in the presence of laccase. The removal of chlorophenols upon subsequent flocculation ranged from 86% to 99%. Besides chlorophenols, laccase could help remove nonchlorinated wood-based phenols present in pulp and paper mill effluents. Lignin and lignin derivatives, recalcitrant lignans produced during the papermaking process, actually impart a strong dark colour to black liquor and other spent pulping effluents and contribute greatly to their toxicity (Garg and Modi, 1999). Conventional biological and physicochemical wastewater treatment methods to remove colour from spent pulping liquors before their discharge to water bodies are not effective and/or expensive. However, the bulk of the non-chlorinated phenolic pollutants present in mill wastewaters can be effectively removed by relatively low-cost treatments with ligninolytic white-rot fungi which produce lignin peroxidase and manganese peroxidase enzymes in addition to or instead of laccase. The most widely investigated laccase producing fungus is *Trametes versicolor* in terms of mill wastewater treatment (Garg and Modi, 1999).

In black liquor treatment with *T. versicolor,* Font et al. (2003) found laccase activity but no LiP or MnP activities, suggesting that laccase may be largely responsible for the removal of phenols in mill effluents treated with *T. versicolor*. Colour removal with *T. versicolor* ranged from 50 to 92% and phenol removal ranged from 23 to 70% in different studies (Font et al., 2003; Livernoche et al., 1983 Royer et al., 1985; Davis and Burns, 1990; Bajpai et al., 1993 Martin and Manzanares, 1994; Mehna et al., 1995; Minussi et al., 2006).

6.1.7 BIOGRAFTING

Laccases have the potential to graft several low-molecular weight compounds onto pulp fibres containing high lignin content. These grafts improved physical strength or imparted new properties to the pulp fibres (Aracri et al., 2011; Fillat et al., 2012; Chandra et al., 2004a; Liu et al., 2009; Chen et al. 2010). The grafted molecules could also act as anchor groups for further fibre modifications. Examples of grafted molecules are presented in Table 6.10.

Several studies have shown the feasibility of laccase-catalyzed biografting of phenols to technical lignins:

Chandra et al. (2004b) observed that the tensile and burst strengths of high-kappa kraft pulp increased when gallic acid was grafted onto pulp fibres with laccase. Liu et al. (2009) found that the wet tensile strength properties of unbleached kraft pulp modified with laccase-syringate was two times high in comparison to that modified with laccase alone. Lund and Ragauskas (2001) grafted guaiacol sulfonate, and 4-hydroxyphenylacetic acid (PAA) to kraft lignin. Grafting of guaiacol sulfonate to lignin was also performed by Lund et al. (1998). Chen et al. (2010) reported laccase-mediated grafting of histidine onto old newspaper pulp fibres. An increase in the carboxyl group content and tensile strength of the treated pulp was observed.

Antimicrobial paper was successfully produced by Fillat et al., (2012) through the grafting of low molecular weight phenols onto unbleached flax fibres by laccase treatment.

Ferulic acid is a low-molecular weight phenolic compound. Compared with other low-molecular weight phenols such as gallic acid, vanillic acid, syringate, ferulic acid should be easy to graft to the fibres catalyzed by laccase because of conjugated – Cα=Cβ–structure on side chain with benzyl rings which can make ferulic acid form free radicals easily. Li et al. (2013) modified unbleached kraft pulp with laccase and ferulic acid in an attempt to enhance its physical strength properties. The carboxyl group and surface lignin contents for laccase-ferulic acid-modified pulps increased as compared to the control pulp. *Atomic force microscopy* phase images showed that the laccase-ferulic acid-modified fibre surfaces were covered with large granular substances from the products of ferulic acid grafting and lignin polymerization/condensation reactions. The improvements in strength properties of laccase-ferulic acid-modified pulp could be attributed to the grafting of ferulic acid onto the fibres, the formation of covalent bonding between the fibres via radical coupling and the higher carboxyl group content of the modified fibres.

The grafting of 3-hydroxytyramine to TMP was reported by Gronqvist et al. (2006).

Aside from phenols, compounds containing a vinyl group such as acrylamine in the presence of organic peroxides have been grafted to technical lignins (Milstein et al., 1994; Mai et al., 1999).

The potential of laccase-assisted biografting of phenolic acids for improving strength properties of kraft paper made from high kappa pulps has been reported by Dr. Ragauskas group (Chandra et al., 2003, 2004b; Chandra and Ragauskas, 2001, 2002a,b).

Improvements in paper tensile and burst strength were obtained with PAA (Chandra and Ragauskas, 2001), 4-hydroxybenzoic acid (4-HBA) (Chandra and Ragauskas, 2002a), and gallic acid (Chandra et al., 2004a), while guaiacol sulfonate and tyrosine were ineffective in enhancing paper strength (Chandra and Ragauskas, 2001).

Treatment of kraft pulp or lignin-coated kraft fibres with 4-HBA increased the content of bulk and surface acid groups (Chandra and Ragauskas, 2002b); the studies with lignin-coated fibres also revealed a concomitant reduction in phenolic hydroxyl groups, suggesting the occurrence of coupling reactions between lignin and 4-HBA radicals. The improvements in paper strength properties obtained with PAA, 4-HBA, and gallic acid were ascribed to the capacity of carboxyl groups to promote hydrogen bonding and to cross-linking of phenoxy radicals in the paper sheet (Chandra et al., 2004). Consistency and 4-HBA dose were significant reaction parameters when grafting 4-HBA to kraft pulp (Chandra and Ragauskas, 2001; Chandra and Ragauskas, 2001, 2002a,b; Chandra et al., 2004). Adding 4-HBA periodically increased the grafting, while performing it in an oxygen-pressurized vessel did not increase the incorporation of 4-HBA into kraft pulp (Chandra et al., 2004).

Surface grafting of the cationic dye phenol celestine blue also increased the strength of high-kappa kraft paper (Chandra et al., 2003).

Improvements in the mechanical properties of TMP treated with per acetic acid in the presence of laccase have been reported by Kenealy et al. (2004).

Laccase-assisted modification of colour and bacterial resistance of lignocellulosic fibres has been examined with non-wood (flax) fibres (Schroder et al., 2007).

Grafting of various methoxy phenols and hydroquinone to flax fibres changed the colour of the fibres; the colouration was found to depend on the phenol used. Further, the growth of bacteria in fibres treated with ferulic acid or hydroquinone was significantly reduced. Laccase-assisted functionalization of wood (spruce) chips used for manufacturing PB with 4-hydroxy-3-methoxybenzyl urea has been conducted by Fackler et al. (2008) (Figure 6.4). The urea group is able to react with urea formaldehyde (UF) resin. Using this method, the researchers were able to improve the internal bond strength of PB (density 750 kg/m^3) by 21%. With lower density (650 kg/m^3) PB, the laccase treatment allowed the amount of UF to be reduced by 16%.

6.1.8 PRODUCTION OF WOOD COMPOSITE BOARDS

There is a trend to consider fabrication of green composites due to growing concerns regarding the emission of formaldehyde from wood composites (Nasir et al., 2015; Widsten and Kandelbauer, 2008). Treatment of fibre with LMS oxidizes the lignin component in fibre without affecting the cellulose structure. This results in the generation of free radicals on the fibre surface which act as potential reactive sites for cross-linking reactions in manufacturing of board. Binderless fibreboards produced using these methods can be considered as green composites because the manufacturing process involves no additional resin. In developing countries research is being focused on developing wood composites free from formaldehyde-based adhesives. Laccase is applied to wood composites with following two objectives (Winandy and Rowell, 2005; Tamminen et al., 2010; Moilanen et al., 2011):

Physical modification of fibre – This includes changes in the morphology or crystallinity of the fibre surface improving the mechanical strength and facilitating self-bonding of fibres by mechanical interlocking.

Chemical modification of fibre – This includes the activation of the lignin molecules in the fibres inducing lignin polymerization reactions.

Thielemans et al. (2002) fabricated a binderless board by heating and pressing cellulosic fibres at high temperature. Lignin starts plasticizing at a high temperature above 200 °C and behaves like a thermoplastic resin (Lora and Glasser, 2002).

Hüttermann et al. (2001) produced a binderless particle board using laccase treatment that showed improved tensile strengths but reduced water resistance. Extensive research has been conducted to develop a completely natural fibreboard by treating fibre with laccase, but these methods have not been commercialized (Milstein et al., 1994; Lund and Felby, 2001; Felby et al., 2004; Nasir et al., 2013a; Nasir et al., 2014a,b).

Felby et al. (1997) and Kharazipour et al. (1997) suggested an enzyme-assisted composite fabrication without using any adhesive. Laccase was used to produce free radicals, which helped in either the physical or chemical bonding of fibres by modifying the fibre and also the lignin structure (Kharazipour et al., 1997; Yu et al., 2009).

Felby et al. (2002) produced a laccase-treated binderless board in a pilot-scale. The mechanical strength was found to be good and comparable to the conventional urea formaldehyde-based resin boards but the dimensional stability was poor. When the wax was applied in treated fibre for improving the dimensional stability, it impaired the bonding effect of the enzyme (Felby et al., 2002). Therefore, binderless boards cannot be regarded as commercially viable until they obtain good dimensional stability along with mechanical strength.

The properties of MDF prepared on a laboratory-scale show a notable improvement upon laccase treatment (Felby et al., 1997; Kharazipour et al., 1997; Cao et al., 2007). Also MDF and high density fibreboards (HDF) prepared on a pilot-scale from laccase-treated fibres by the wet- or dry-process showed superior mechanical strength compared to controls meeting the European standards for IB strength, whereas the dimensional stability of the boards needs to be improved to comply with industrial standards (Felby et al., 2002; Felby et al., 2004; Widsten et al., 2003, 2004). Kharazipour et al. (1993) and Qvintus-Leino et al. (2003) have obtained patents relating to binderless wood composite boards fabricated from laccase-treated fibres. Another strategy to substitute synthetic resins in wood composite production is a two-component system in which laccase-treated lignin functions as the adhesive. This method has been applied to the production of particle board (Kharazipour et al., 1998), and the technology has been patented (Huttermann et al., 1998; Viikari et al., 1998). The drawback of using the water-soluble lignosulfonates is that the products display poor dimensional stability (Kharazipour et al., 1998). The extra costs incurred upon using large amounts of technical lignins may be also prohibitive.

6.2 TEXTILE INDUSTRY

The textile industry accounts for two-thirds of the total dyestuff market and uses large volumes of chemicals and water for wet processing of textiles (Banat et al., 1996). Textile dyeing effluents contain recalcitrant dyes which pollute water due to their colour and by the formation of toxic or carcinogenic intermediates such as aromatic amines from azo dyes. The chemical reagents used are very diverse in chemical composition which range from inorganic compounds to polymers and organic products. There are more than 100,000 dyes which are commercially available with over 7×10^5 t of dyestuff produced yearly (Zollinger, 2002). Dyes are mostly resistant to fading on exposure to light, water and also to various chemicals because of their complex chemical structure, and most of them are difficult to decolourize with great difficulty due to their synthetic origin (Lin et al., 2010a,b). Due to their chemical structure, dyes are resistant to decolour when exposed to light, different chemicals and water (Poots and McKay, 1976; McKay, 1979). In the developed countries especially, government legislation is becoming quite stringent, regarding the removal of dyes from industrial effluents (O'Neill et al., 1999). Concern arises, as several dyes are made from benzidine and other aromatic compounds which are known carcinogens (Baughman and Perenich, 1988). Most of the current processes to treat dye wastewater are ineffective and not cost effective (Cooper, 1995; Stephen, 1995). Therefore, the development of processes based on laccases appears to be an attractive solution because of their potential in degrading dyes of diverse chemical structure including synthetic dyes presently used commercially (Abadulla et al., 2000; Blánquez et al., 2004; Hou et al., 2004; Rodríguez Couto et al., 2005, 2006; Rodríguez-Couto, 2012; Setti et al., 1999; Madhavi and Lele, 2009; Upadhyay et al., 2016).

The use of laccase in the textile industry is growing very fast. Laccases are used to decolourize textile effluents, bleach textiles and even to synthetise dyes.

LMS are finding potential application in enzymatic modification of dye bleaching in the textile and dyes industries (Kunamneni et al., 2008; Abadulla et al., 2000; Blanquez et al., 2004; Hou et al., 2004; Wong and Yu, 1999; Rodríguez et al., 2005; Rodríguez and Toca, 2006; Mate and Alclade, 2016). In textile industry, laccases are used for bleaching of denim fabrics and for the increase of the whiteness in the conventional hydrogen peroxide bleaching of cotton (Yavuz et al., 2014; Iracheta-Cárdenas et al., 2016; Tzanov et al., 2003). There are several commercial laccase-based products for denim bleaching marketed by at least 14 companies around the world (Rodríguez-Couto, 2012).

Laccases can also oxidize several types of aromatic compounds (including phenols and anilines) to concomitantly promote non-enzymatic homo- and/or hetero-coupling reactions yielding a colour palette of different valuable dyes for textiles (including azo and phenoxazine dyes) (Sousa et al., 2013; Polak and Jarosz-Wilkolazka, 2012).

Laccases have been used to dye wool fabrics and cotton with hetero-polymeric dyes produced in situ by the oxidative hetero-coupling of colourless precursors and modifiers initiated by laccase (Hadzhiyska et al., 2006; Díaz Blanco et al., 2009).

Laccases have been also used in cleaning formulations to remove the odour on fabrics and the detergents produced during cloth washing (Kunamneni et al., 2008).

LMS has been also applied to reduce the shrinkage of wool (Yoon, 1998). Wool fabrics have been coated with the water insoluble phenolic compound lauryl gallate, using laccase as a grafting biocatalyst (Hossain et al., 2009). The functionalization reaction was conducted in an 80/20 (v/v, %) aqueous-ethanol medium, maintaining a compromise between the conditions at which the laccase remains active and those of substrate solubility. This study opens up new possibilities for the development of textile materials having multifunctional properties (antibacterial, antioxidant and water repellent).

6.3 FOOD INDUSTRY

Laccases show great potential to be used in the food industry (Mate and Alclade, 2016). These are used as food additives in food processing (Osma et al., 2010; Brijwani et al., 2010). The food industry makes use of laccases due to their ability to promote homo- and heteropolymerization reactions. Table 6.11 shows the use of laccases in food industry.

Laccases are being used to a greater extent for production of cost effective and healthy foods. Laccases have the potential to make food processing more economical and environmental friendly.

One of the main applications of laccase in the food industry is wine stabilization. Wines and must are complex mixtures of different chemical compounds such as alcohol, organic acids, salts, and phenolic compounds. Alcohol and organic acids are responsible for the aroma, whereas the taste and the colour depend on the phenolic compounds. The sensorial properties of fresh wines should remain constant until consumption. Oxidative reactions in wines catalyzed by iron, copper, and enzymes which involve aldehydes, amino acids, and proteins, cause turbidity, colour darkening, and aroma and flavor alterations. This oxidation phenomenon is called madeirization. Several methods have been used to prevent flavor alteration and decolourization in wines. Laccase enzymes are able to remove polyphenols selectively for avoiding any undesirable modification of the organoleptic properties of the wine (Osma et al., 2010). Laccase from *M. thermophila* Suberzyme® is being commercialized by Novozymes, Bagsværd, Denmark for the treatment of cork stoppers for wine bottles (Mate and Alclade, 2016). Laccase oxidizes phenols and the released phenoxyl radicals then undergo non-enzymatic homopolymerization, eliminating the generation of 2,4,6–trichloroanisole which is responsible for the cork taste. This oxidative polymerization process also modifies the cork's surface and increases its hydrophobicity and reduces the extraction of substances into the wine (Conrad et al., 2000).

In the brewing industry, haze formation during long-term beer storage represents a continuous problem (Kunamneni et al., 2008). Haze is formed through the interaction between proanthocyanidins and specific haze-active proteins. To solve this issue, laccases have been used to oxidize polyphenols in beer. The polyphenol complexes produced are then removed using filtration or other separation methods.

Laccases have also been used to remove oxygen at the end of the beer production process for increasing the storage life of beer (Alcalde, 2007). Laccase scavenges oxygen, which would otherwise react with fatty acids, amino acids, proteins and alcohols to form off-flavour precursors. Novozymes has developed an enzyme product Flavourstar®. This is based on the laccase produced from *M. thermophila*.

Browning, both enzymatic and chemical, is one of the major faults in beverages. Various methods are available to avoid discolouration and post turbidity of fruit juices. Stabilization of beverages by using, bentonite, gelatin and silica gel, etc., is a common conventional treatment (Gokmen et al., 1998). Use of laccase enzyme is proposed for fruit juice stabilization (Piacquadio et al., 1997). The results reported on laccase apple juice treatments are contradictory. Some juice treated with laccase showed greater susceptibility to browning during storage, and they were less stable than the physical-chemically treated juice (Giovanelli and Ravasini, 1993; Gökmen et al., 1998; Sammartino et al., 1998). Golden Delicious apple juice was treated with conventional method and with free or immobilized laccase on metal chelate regenerable carrier. The apple juice treated with enzyme was less stable than the conventionally treated juice. But use of laccase in combination with cross flow filtration in continuous process without the addition of fining agents resulted in a stable and clear apple juice. Stutz (1993) reported that it is possible to produce clear and stable juices/ concentrates with a lighter colour using ultrafiltration and laccase with out any large additional investment. Treatment with laccase followed by active filtration or ultrafiltration improved colour and flavour stability in comparison to conventional treatments by addition of ascorbic acid and sulfites. Research by Giovanelli and Ravasini (1993) used laccase in combination with filtration in the stabilization of apple juice. Cantarelli (1986) reported that laccase treatment of juice resulted in the removal of a high fraction of polyphenols and enhanced stabilization compared with the conventionally treated juice. Treatment of fruit juices with laccase in combination with a filtration process was found to enhance colour and flavour stability (Cantarelli and Giovanelli, 1990; Maier et al., 1990; Ritter et al., 1992; Stutz, 1993).

Bezerra et al. (2015) have used low-cost carriers for laccase immobilization in the clarification of fruit juice. Laccase produced from *T. versicolor* was immobilized in coconut fibres activated with glutaraldehyde. This was used to clarify apple juice. It lightened the original juice colour by 61% and removed turbidity. A recombinant laccase from *Pleurotus ostreatus* (POXA1b) was immobilized on epoxy activated poly(methacrylate) beads and used in the clarification of several fruit juices, producing a reduction in phenol of up to 45% (Lettera et al., 2016). Furthermore, the laccase-treated juice had comparable flavanone content to the untreated juice but a substantial reduction in vinyl guaiacol which has an off-flavour with a pepper-like aroma.

Laccases have also been used in baking for improving the dough machinability and the softness of the final product (Labat et al., 2000), and also in teas and oil-containing products for enhancing colour and flavour quality, respectively (Bouwens et al., 1997; Petersen et al., 1999). The gelling effects of laccases have been also studied in blackcurrant juice, luncheon meat and milk with added sugar beet pectin (Norsker et al., 2000). The effect of laccase and LMS on stirred milk yoghurt has been studied in a process that mimics that of industrial production (Mokoonlall et al., 2016). The treatment with laccase resulted in protein degradation at the molecular level, whereas the addition of the natural redox mediator vanillin promoted the formation of higher molecular weight oligomers. In the bread making process, it is a practice to add dough improvement additives to the bread dough. This results in improved flavor, texture, volume, and freshness of the bread/dough. Laccase exerts an oxidizing effect on the constituents of dough which improves the strength of gluten structures in baked products. When laccase is used in the dough, improved crumb structure, an increased volume, stability, strength, softness of the baked product and reduced stickiness results. Laccase is found to reduce the dough extensibility in both flour and gluten dough and can also increase the resistance of dough to its maximum (Selinheimo et al., 2006). The addition of laccase to oat flour results in an increased specific volume of loaf.

Jurado et al. (2009) reported that the phenolic compounds are reduced by the induction of laccase, which act as fermentation inhibitor and increases the ethanol production from steam exploded wheat straw. Laccase expressing yeast strains showed an advantage for producing ethanol from lignocelluloses because it enabled faster growth as well as ethanol formation as it was able to convert coniferyl aldehyde at a very fast rate (Larsson et al. 1999).

Laccases because of their ability to degrade phenolic compounds have been studied for bioremediation of food industry waste (Gianfreda et al., 1999). Aromatic compounds constitute one of the major classes of pollutants. These are heavily regulated in many countries (Karam and Nicell, 1997). The presence of these compounds in drinking and irrigation water or in cultivated land represents a significant health hazard. Crecchio et al. (1995) reported that laccase immobilized on organogel supports remove naturally occurring and also xenobiotic aromatic compounds from aqueous suspensions. Lante et al. (2000) reported that laccase immobilized by adsorption on polyethersulphone showed properties useful for reducing phenol concentration in a model wastewater solution.

Some fraction of beer factory waste water represents an important environmental concern because of their high content of polyphenols and dark brown colour. *Coriolopsis gallica*, producer of laccase (white rot fungi), was able to degrade this high tannin containing wastewater. Reduction in polyphenols pyrolysis products, mainly phenol and guaiacol, with the incubation time was observed (Yague et al., 2000). Distillery waste water produces an ecological impact due to high content of soluble organic matter and its intense dark brown colour. *Trametes* sp., a laccase producer removed colour and COD from distillery waste water. Maximum effluent decolourization of 73.3% and chemical oxygen demand reduction of 61.7% was obtained after 7 days of fungal treatment using 20% v/v of distillery wastes in culture medium. Under these conditions, a 35-fold increase in laccase production by this fungus was observed (Gonzalez et al., 2000). Treatment of olive mill wastewater effluent with laccase from *Pleurotus ostreatus* significantly decreased the phenolic content (up to 90%) but no reduction of its toxicity was observed (Maritirani et al., 1996).

6.4 PERSONAL CARE AND MEDICAL APPLICATIONS

Several products generated by laccases are detoxifying, antimicrobial or active personal-care agents. Laccase can be used in the synthesis of complex medical compounds such as, anti-inflammatory, anesthetics, sedatives, antibiotics, etc.

Potential applications of laccases in the personal care and medical field are attracting active research efforts due to their specificity and bio-based nature.

Poison ivy dermatitis which results from skin contact with poison ivy, poison oak, or poison sumac is caused mostly by urushiol (a catechol-derivative toxin). Laccase is able to oxidize, detoxify and polymerize urushiol (Cheong et al., 2010), reducng the effect of poison ivy dermatitis. However, oxidized urushiol which is an o-quinone derivative, is nontoxic.

Laccase can oxidize iodide to produce iodine which is largely used as a disinfectant. It has several advantages over direct iodine application. Iodide salt is more stable and much safer as compared to iodine, in terms of handling, storage and transport. Release of iodine from a laccase iodide system could be easily controlled. This may be used in various medical, industrial, domestic, and personal care applications, such as sterilization of drinking water and swimming pools, and also disinfections of minor wounds.

Laccases have been also used in the cosmetic sector for the manufacturing of personal-care products. Cosmetic and dermatological products containing laccases were patented for skin lightening (Golz-Berner et al., 2004). Laccases have broader applications in the field of hair bleaching/dying. The bleaching and/or dying of hair mostly involve the use of harsh chemicals such as hydrogen peroxide. This can damage hair and irritate the scalp (Morel and Christie, 2011). Laccases can replace hydrogen peroxide as oxidizing agent in the formulation of hair dyes. Novel laccases from the basidiomycete *Flammulina velutipes* and from the actinomycete *Thermobifida fusca* have been examined in the oxidation of dye intermediates used in hair colouring (Saito et al., 2012; Chen et al., 2013). Bhogal et al. (2013) developed a hair colour comprising butein and a combination of a peroxidase with either hydrogen peroxide or a hydrogen peroxide generator; or laccase. A laccase-based system replaces harsh chemicals and operates at milder conditions (in terms of pH and solvents). Laccase catalyzed oxidation, transformation, and cross-linking of various precursors have been reported to result in satisfactory hair dyeing or waving. Laccase provides an easier handle hair care procedure and also improves or complement the cosmetic effect achieved by conventional chemical methods (Xu, 1999; Kainz et al., 2003).

Laccases may find use as deodorants for personal-hygiene products – mouthwash, toothpaste, detergent, soap, and diapers. Protein engineered laccase can also be used to reduce allergenicity. Many body, domestic, and industrial odours are caused by sulfides, thiols, ammonia, amines, short chain fatty acids, or other volatile organic compounds. Laccases have been investigated for deodourant application as these are able to oxidize various thiols and other sufur containing compounds. Laccase system degrades the offensive molecules or even kill the microbes which produces them (Schmid and Urlacher, 2007) whereas conventional deodorants mask the malodor with fragrances. Laccases are also used as catalysts for the manufacture of anticancer drugs (Kunamneni et al., 2008).

Erb-Downward et al. (2008) reported the synthesis of immunomodulatory prostaglandins using laccase. Proliferation of murine leukemia cell line L1210 and human hepatoma cell line HepG2 was found to be inhibited by the use of laccase isolated from *Pleurotus cornucopiae*, and the activity of HIV-1 reverse transcriptase with an IC50 of 22 µM was reduced. Wong et al. (2010) found no mitogenic activity towards mouse splenocytes and hemagglutinating/hemolytic activity toward rabbit erythrocytes. El-Fakharany et al. (2010) reported inhibition of hepatitis C virus entry into peripheral blood cells and hepatoma cells with laccase from oyster mushroom. A laccase, with HIV-1 reverse transcriptase inhibitor activity and antiproliferative activity against HepG2 and MCF7 cells was isolated from the fresh fruiting bodies of the edible mushroom *Agrocybe cylindracea* (Hu et al., 2011).

6.5 BIOREMEDIATION

Environmentally hazardous xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs), phenols and organophosphorus insecticides are known to have carcinogenic and mutagenic effects. These chemicals are persistent and represent major contaminants of soils and waters. The removal of these chemicals is a priority for most environmental agencies (Alcalde et al., 2006; Viswanath et al., 2014) and stringent regulations have been imposed on industries to treat their waste effluents before their final discharge in the environment.

Use of laccases in enzyme bioremediation is generating a lot of interest both in the presence and in the absence of redox mediators. Laccases or laccase-mediator system oxidize the xenobiotic compounds and release a less toxic product having greater bioavailability that can be more readily removed by physical and/or mechanical processes

Laccases have been used to oxidatively detoxify various aromatic xenobiotics and pollutants found in industrial waste and contaminated soil or water. Use of laccases could result in direct degradation or polymerization/immobilization. Examples of direct degradation by laccase are given below:

- Cleavage of aromatic rings
- Direct dechlorination
- Mineralization of polycyclic aromatic hydrocarbons
- Decolourization of pulp or textile mill effluent
- Bleaching of textile dyes

The processes involve polymerization among pollutants themselves or copolymerization with other nontoxic substances such as humic materials. Polymerized pollutants facilitate easy removal by such means as adsorption, sedimentation, or filtration because they mostly become insoluble or immobilized (Xu, 1999). Examples of this include (Majcherczyk et al., 1998; Cañas et al., 2007; Zumárraga et al., 2007; Zeng et al., 2016; Camarero et al., 2005; Wang et al., 2016; Amitai et al., 1998),

- Removal of *PAHs* like anthracene or benzopyrene
- Recalcitrant dyes like crystal violet or Reactive Black 5
- Organophosphorous compounds, such as the nerve agents VX or Russian VX

Furthermore, laccases can oxidize oestrogenic hormones found in effluents from sewage treatment. The oxidation of the oestrogens estrone, 17β-estradiol and 17α-ethynylestradiol by a laccase from white rot fungus *Trametes* sp. Ha1 has been reported by Tanaka et al. (2009). A treatment system was also developed that comprised the laccase and a β-D-glucuronidase to degrade the 17β-estradiol 3-(β-d-glucuronide), efficiently removing this compound and its intermediate 17β-estradiol.

Fungal laccases are used in the decolourization and detoxifications of effluents generated from industries, and also help in wastewater treatment (Chandra and Chowdhary, 2015). They act on both phenolic and nonphenolic lignin-related compounds and also highly recalcitrant environmental pollutants, and can be used effectively for bioremediation and xenobiotic degradation (Viswanath et al., 2014). Potential environmental applications for laccases include the bioremediation of contaminated soils as it is able to oxidize organic pollutants, such as chlorophenols, polycyclic aromatic hydrocarbons, lignin related structures, organophosphorus compounds, phenols and azo dyes which are toxic (Leonowicz and Trojanowski, 1975; Saratale et al., 2011; Khan et al., 2013). Laccase substrates comprise of polycyclic aromatic hydrocarbons, endocrine disrupters, phenols, dyes and pesticides, out of which some can be oxidized by fungal laccases (Majeau et al., 2010). Induction of hydroxyl ion production through quinone redox cycling enabled *Pleurotus eryngii* to oxidize the dye reactive black 5 and phenol, obtaining a high linkup between the rates of pollutant oxidation and hydroxyl ion production (Gomez-Toribio et al., 2009). The biodegradation of a mixture of pentachlorophenol, 2-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol using the laccase produced by the white-rot fungal strain *Trametes pubescens* was examined by Gaitan et al. (2011). The laccase potential from *Marasmius quercophilus* to transform certain alkylphenols (pnonylphenol, p-octylphenol and p-t-octylphenol) was studied by Farnet et al. (2000). *Trametes vesicolour* degraded naproxen in a liquid medium to non-detectable levels after 6 h. In vitro degradation experiments conducted with purified laccase and purified laccase plus mediator 1-hydroxybenzotriazole showed slight and almost complete naproxen degradation (Marco-Urrea et al., 2010). Saparrat et al. (2010) reported the use of laccase for detoxification of water soluble fraction named as "alpeorujo" a by-product obtained in olive oil extraction industry. Zhao et al. (2010) showed that laccase was responsible for biodegradation of dichlorodiphenyltrichloroethane in soil. Bhattacharya et al. (2009) reported laccase mediated biodegradation of 2,4-dichlorophenol. Bolli et al. (2008) reported that laccase treatment impairs bisphenol-A induced cancer cell proliferation. Pozdnyakova et al. (2006) studied the degradation of polycyclic aromatic hydrocarbons like anthracene, fluoranthene, fluorene, perylene, phenanthrene and pyrene with laccase produced by *Pleurotus ostreatus* in the presence of a synthetic mediator. Laccase has the ability to degrade phenolic compounds in industrial waste water (Gianfreda et al., 1999). Major classes of pollutants are phenols and aromatic amines which are highly regulated in many countries (Karam and Nicell, 1997). The presence of such compounds in drinking water, irrigation water or in cultivated land is very dangerous for health. Laccase immobilization on polyethersulphone showed chemical and physical properties potentially useful for decreasing phenol concentration in a model wastewater treatment (Lante et al., 2000). Yague et al. (2000) found that this high tannin containing waste water was degraded by laccase producing white rot fungus *Coriolopsis gallica*. Crecchio et al. (1995) reported that laccase removed naturally occurring and xenobiotic aromatic compounds from aqueous suspensions when it is immobilized on organogel supports.

6.6 ORGANIC SYNTHESIS

Laccases offer significant advantages over traditional chemical oxidants and transition metal catalysts listed below:

- Renewable
- Biodegradable
- Relatively inexpensive
- Highly active in aqueous solvents and under mild conditions
- Broad substrate range which can be further expanded via the use of LMS

Laccases are able to perform exquisite transformations which range from the oxidation of functional groups to the heteromolecular coupling for production of new antibiotics derivatives, or the catalysis of major steps involved in the synthesis of complex natural products.

Laccases have proved to be exciting catalysts in the field of synthetic organic chemistry in recent decades. The versatility of these enzymes in organic synthesis is exemplified by the variety of chemical transformations they are capable of effecting.

Dr. Ragauskas' group used laccases to generate reactive *ortho*- and *para*-quinone species in situ and these species reacted with various nucleophiles derived from carbon, nitrogen, and sulfur, to yield new and existing compounds under environmentally benign conditions (Witayakran, 2008). Their work focused on laccase-catalyzed carbon-carbon bond forming reactions and also laccase-initiated cascade reactions (Cannatelli and Ragauskas, 2013a,b).

Laccases are useful biocatalysts for the pharma sector (Mate and Alclade, 2016); these can catalyze a wide range of synthetic reactions which range from the transformation of antibiotics to the derivatization of amino acids for the synthesis of metabolically stable amino acid analogues (Piscitelli et al., 2012). The laccase-generated phenoxy radicals that are produced upon oxidation of phenolic compounds are able to undergo a variety of reactions which include the following (Nakamura, 1960):

- Radical-radical coupling reactions of monomers for the synthesis of dimers, oligomers, and polymers
- Radical cross-coupling reactions
- In situ generation of *ortho*- and *para*-quinones from the corresponding catechols and hydroquinones, respectively via disproportionation

Laccases have been used for synthesizing complex medical products (Kunamneni et al., 2008) such as:

- Anti-cancer drugs (e.g. Actinomycin, Vinblastine, Mitomycin)
- Immunosuppressors (e.g. Cyclosporin A)
- Antibiotics (e.g. Cephalosporins, Penicillin × dimer)

In addition, laccases have been used to oxidize the steroid hormone 17β-estradiol and stilbenic phytoalexin *trans*-resveratrol, generating dimers or oligomers after coupling of the radical intermediates (Nicotra et al., 2004a,b). Furthermoe, they have been also used in the enzymatic derivatization of amino acids, such as L-phenylalanine, L-tryptophane, or L-lysine (Mogharabi and Faramarzi, 2014).

Catechin polymers have been used to attenuate postprandial hyperlipidaemia and hypercholesterolaemiad. Their synthesis can be catalyzed by *M. thermophila* laccase affecting lipid and cholesterol absorption (Jeon and Imm, 2014). Laccase-catalyzed catechin polymers have stronger inhibitory activity against cholesterol esterase and pancreatic lipase in comparison to the catechin monomer.

Another potential application of laccases is in the oxidation of iodide to generate iodine which is an efficient and inexpensive antimicrobial compound (Xu, 1999). Iodide oxidation by laccase has been proposed to inactivate *Bacillus anthracis* spores (Niederwöhrmeier et al., 2008). Laccase-mediated synthesis of iodine based on an artificial neural network was studied by Schubert et al. (2015) with a genetic algorithm.

The tandem use of laccases and lipases has been reported by Gavezzotti et al. (2011) in the synthesis of enantiomerically enriched dimeric phenols having structures identical to the β-5 dimers found in lignin. Laccase from *T. versicolor* was used to oxidize the commercially available isoeugenol, and the two resulting enantiomers were separated by alcoholysis cleavage using a lipase enzyme. This process led to the isolation of the target compounds with an *ee* of up to 90%. Laccases have also been shown to be able to oxidize alcohols when used with palladium catalysts (Mekmouche et al., 2015). Specifically, the LAC3 laccase from *Trametes* sp. C30 was combined with different water-soluble palladium complexes known to oxidize primary and secondary alcohols under high temperature and pressure conditions. The laccase-palladium complexes were then studied for the aerobic oxidation of veratryl alcohol into veratryl aldehyde at atmospheric pressure and room temperature. As a result, the association of the laccase and the palladium (II) complexes studied improved the catalytic efficacy of the complex by up to seven fold.

It has been also reported that laccase induced radical polymerization of acrylamide with or without mediator (Ikeda et al., 1998). Laccases are also known to polymerize various amino and phenolic compounds (Aktas and Tanyolac, 2003). To improve the production of fuel ethanol from renewable raw materials, laccase from *T. versicolor* was expressed in *S. cerevisiae* for increasing its resistance to phenolic fermentation inhibitors in lignocelluloses hydrolyzates (Larsson et al., 2001). Laccase from *Coriolus hirsutus* was used for synthesis of an indamine dye and conducting polyaniline (Baker et al., 1996; Karamyshev et al., 2003). Laccase from *Pycnoporus cinnabarinus* was used for synthesis of 3-(3, 4-dihydroxyphenyl)-propionic acid derivatives (Mikolasch et al., 2002). Laccase from *Trametes versicolor* was used for synthesis of aromatic aldehydes (Fritz-Langhals and Kunath, 1998), synthesis of substituted imidazoles and dimerization products (Schäfer et al., 2001), cross-linking of a protein (Boumans et al., 2006) and synthesis of 3, 4-dihydro-7, 8-dihydroxy-2H-dibenzofuran-1-ones (Hajdok et al., 2007). It has been reported that laccase induced radical polymerization of acrylamide with or without mediator (Ikeda et al., 1998). Laccase has been also used for the chemo-enzymatic synthesis of lignin graft-copolymers (Gübitz and Paulo, 2003). Laccases are also able to oxidize catechins. These molecules are the condensed structural units of tannins, which are important antioxidants found in vegetables, herbs, and teas. Catechins ability to scavenge free radicals makes them important in the prevention of several diseases such as cancer, inflammatory and cardiovascular. Oxidation of catechin by laccase has yielded products with improved antioxidant capability (Hosny and Rosazza, 2002).

6.7 BIOFUELS

The desire to reduce dependence on the ever declining fossil fuel reserves coupled with the impetus towards green energy is seeing increased research in the area of biofuels as alternative sources of energy. Bioethanol is one of the most essential biofuels produced from lignocellulosic material. This subject has been the focus of many contemporary studies (Naik et al., 2010). Lignocellulose is the most abundant source of organic material in the world. Lignocellulosic feed stocks are renewable, inexpensive and abundant in nature. They do not compete with food production and contribute to reduce the use of fossil fuels, thereby reducing carbon dioxide emission and global warming (Naik et al., 2010). Lignocellulosic feed stocks mainly comprise of two polysaccharides, cellulose and hemicelluloses which can be hydrolyzed to provide monosaccharides by the use of microorganisms in the fermentation processes (Jönsson et al., 2013; Parawira and Tekere, 2011). Cellulose and hemicellulose unfortunately are closely linked by lignin that acts as cementing agent between cellulose fibres (Jönsson et al., 2013). Therefore, lignocellulosic materials are recalcitrant to hydrolysis and need some kind of pretreatment before being converted to bioethanol (Parawira and Tekere, 2011; Schroyen et al., 2014). Their use actually depends on the efficient hydrolysis of polysaccharides. This requires a cost-effective pre-treatment of biomass for removing lignin and expose sugars to hydrolytic enzymes.

The combination of steam explosion and acidic catalyst is one of the most commonly used methods for lignocellulose pretreatment, since it breaks and/or removes a minor part of lignin, depolymerizes cellulose and hemicellulose, and makes the biomass more accessible to the action of hydrolytic enzymes with relatively reduced cost (Jurado et al., 2009). However, this pretreatment produces some soluble inhibitory compounds which result from partial degradation of lignin and sugars, affecting enzymatic hydrolysis and also the fermentation steps, and will reduce the ethanol productivity of the microorganisms as well as the final ethanol yield (Jönsson et al., 2013; Parawira and Tekere, 2011; Jurado et al., 2009). The inhibitory compounds generated during the pretreatment process include weak acids, furan derivatives, and phenolic and other compounds (Jönsson et al., 2013). Several procedures for the removal of these compounds have been suggested. These include biological, physical, and chemical methods. Among which, use of laccase enzymes has been suggested as one of the most promising approach in lignocellulosic biomass detoxification (Parawira and Tekere 2011; Jurado et al., 2009). Laccase has been also studied as pretreatment agents in biofuel production, mainly as a delignifying enzyme (Kudanga and Le Roes-Hill, 2014; Mate and Alclade, 2016).

Laccases have potential applications in detoxification of lignocellulosic biomass after thermochemical pretreatment and production of value added products or biofuels from renewable biomass.

When detoxifying the lignocellulosic hydrolysate, laccase was found to be selective and could almost remove all phenolic monomers. Compared with other strategies, advantages of using laccases are that enzyme preparations show higher catalytic efficiencies, lower energy cost, fewer toxic sub products and shorter treatment time (Parawira and Tekere, 2011; Jurado et al., 2009; Kudanga and Le Roes-Hill, 2014). However, the disadvantage of using laccases are the high enzyme production costs, as the commercial laccases are still expensive despite efforts made to reduce enzyme cost through the use of biotechnology, thus limiting the applications of laccases (Parawira and Tekere, 2011). Screening for laccases having higher lignocellulosic hydrolysate-detoxifying ability can improve the present situation.

A study by Larsson et al. (2001) revealed that a laccase from the white rot fungus *T. versicolor* heterologously expressed in *Saccharomyces cerevisiae* improved the production of ethanol by removing the phenolic compounds (Larsson et al., 2001).

A laccase from the white-rot fungus *Ganoderma lucidum* was identified and studied for detoxifying lignocellulosic hydrolysates and for production of bioethanol. This laccase removed 84% of the phenolic content in corn stover hydrolysate, and when added before cellulase hydrolysis, it increased ethanol yield by 10% (Fang et al., 2015). Another interesting trend in this developing field is the engineering of a full consolidated bioprocessing microorganism by engineering an artificial secretome in yeast which contains the main enzymes of the ligninolytic consortium (Gonzalez-Perez and Alcalde, 2014; Alcalde, 2015).

6.8 NANOBIOTECHNOLOGY

Research in the area of nanotechnology has grown rapidly in recent years. Nanotechnology has its role in all fields of human need. Nanotechnology contributes to the development of smaller and highly efficient biosensors by controlled deposition and specific adsorption of biomolecules on different surfaces, obtaining micro and nanometer order. Nanoparticle, nanofibres, and nanotubes are used as biosensing and biofuel transport materials (Uematsu et al., 2001). Biosensor is basically a biological probe having electric transducer which converts biochemical signal into electric signal which perceive, convey and trace the signal according to biochemical change. Biosensors are being used as detectors in environmental and clinical analysis (Haghighi et al., 2003).

Laccases can catalyse electron transfer reactions without the requirement of additional cofactors, their use has been studied in biosensors and biofuel cells.

Some of the major qualities of a good biosensing system are its specificity, sensitivity, reliability, portability, real-time analysis and operation simplicity (D'Souza, 2001). Thus laccases can be applied as biosensors or bioreporters (Kunamneni et al., 2008; Rodrıguez Couto and Toca Herrera, 2006; D'Souza et al., 2001; Rodríguez-Delgado et al., 2015). For the high-sensitivity diagnostic field, the bioreporter applications are of great interest.

In terms of biosensors, laccases reduce oxygen to water and the biosensor then records the oxygen consumption during analyte oxidation. Laccase-based biosensors have been used in the food industry for detecting polyphenols in fruit juices, wine and teas and for quantifying fungal contamination in grape musts (Zouari et al., 1988; Ghindilis et al., 1992; Cliffe et al., 1994; Di Fusco et al., 2010). Laccase-based biosensors have been developed for detection of morphine and codeine, catecholamines plant flavonoids and also for electroimmunoassay (Bauer et al., 1999; Lisdat et al., 1997; Leite et al., 2003; Ferry and Leech, 2005; Jarosz-Wilkołazka et al., 2004; Kuznetsov et al., 2001; Milligan and Ghindilis, 2002). Franzoi et al. (2009) reported laccase based biosensor for determination of rosmarinic acid in plant extracts.

Laccase bound covalently with biobinding molecules used as biosensor for histochemical assay, immunoassay, nucleic acid detection assay and cytochemical assay (Saito et al., 2004). Immobilization has an important effect on the biosensor sensitivity. Freire et al. (2001) immobilized fungal laccase on carbon-fibre electrodes using classical methods – physical adsorption and treatment with glutaraldehyde, carbodiimide and combination of carbodiimide/glutaraldehyde. The highest biosensor response was obtained with carbodiimide/glutaraldehyde for coupling laccase to carboxyl groups on the carbon fibres. The developed biosensor showed an optimum response at pH 5.0 and at an applied potential of -100 mV. The immobilized laccase showed good activity for more than 2 months.

Martele et al. (2003) found that micropatterning was an efficient method for the immobilization of laccases on a solid surface for developing a multi-functional biosensor. Roy et al. (2005) observed that cross-linked enzyme crystals of laccase from *Trametes versicolor* could be used in biosensor applications showing great advantage over the soluble enzyme. Cabrita et al. (2005) immobilized laccase from *Trametes versicolor* on N-hydroxy-succinimide-terminated self-assembled monolayers on gold. This method could be useful for further development of biosensors. Laccase from *T. versicolor* on glassy carbon electrodes and an enzyme electrode based on the co-immobilization of an osmium redox polymer was used for ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, achieving nanomolar detection limits (Ferry and Leech, 2005). Laccase can be also immobilized on the cathode of biofuel cells providing power, for small transmitter systems (Calabrese et al., 2002; Chen et al., 2001). Biofuel cells appear to be attractive from an environmental point of view because electrical energy is produced without combusting fuel, thus, providing a cleaner source of energy.

The development of implantable biofuel cells harvesting power from natural sources is of great interest in the area of nanotechnology (Mate and Alclade, 2016). MacVittie et al. (2015) published on enzyme biofuel cell operating in an orange in vivo. The biofuel cell was composed of catalytic electrodes with glucose and fructose dehydrogenase enzymes immobilized on the anode and with *T. versicolor* laccase on the cathode. The cathode/anode pair was implanted in orange pulp, extracting power from the glucose and fructose content in the juice. The power harvested from the orange was used to supply a wireless electronic system.

6.9 PAINTS

Laccases can be used to replace toxic drying agents in paint (Mate and Alclade, 2016). Currently, water-based paints contain heavy metals that dry the alkyd resin films that are used as binding agents by catalyzing the oxidative cross-linking of unsaturated fatty acid moieties in the films. Alkyd resins are polyesters synthesized by the polymerization of polyalcohols, dicarboxylic acids or anhydrides and unsaturated fatty acids (Gooch, 2002). Chemical drying of these resins is based on heavy-metal catalyzed cross-linking of the unsaturated fatty acids. Heavy metals are often toxic, and the cobalt-based catalysts commonly used are found to be carcinogenic, and so alternative materials are being examined. Austrian Scientists have replaced the heavy metal catalysts with a laccase mediator-based, non-toxic biocatalyst. Laccases, can catalyze the oxidation of mainly phenolic substances, and are already used in other fields as described in the above sections.

Laccases could be an environmentally friendly alternative to the use of toxic heavy metal drying agents in paints.

By the use of mediators the substrate scope of laccase enzyme can be broadened. 1–HBT was found to be an effective mediator for laccase in the oxidation of the alkyd resin. The measurements of the oxygen consumption during the reaction showed that it proceeded by a two-phase radical mechanism, via peroxy-cross-linking (Greimel et al., 2013). Interestingly, the biocatalytic reaction was found to work both in aqueous media and in a solid film.

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Applications of laccases	
Bioremediation	29%
Biofuel cells and biosensors	22%
Textiles	20%
Pulp and Paper	11%
Food	10%
Organic synthesis	4%
Biofuel	2%
Fibreboards	0.7%
Cosmetic	0.7%
Paints	0.3%

Table 6.1: Application of laccases
Based on Mate and Alclade (2016)

Commercially available laccases	
Agaricus bisporus	ASA Spezialenzyme GmbH
Bacterial origin[a,b]	MetGen
Cerrena unicolour	Jena Bioscience
Trametes versicolor	ASA Spezialenzyme Gmb

Table 6.2: Few commercially available laccases
a) Name not specified
b) Laccase commercialized as thermoinactivated liquid crude cell lysate

Applications in forest industry
Pulping
Bleaching
Fibre modification
Pitch control
Deinking of recycled fibre
Treatment of mill process water and effluent
Biografting
Production of wood composite boards

Table 6.3: Applications of laccases in forest industry

Advantages
Reduced refining energy
Increased mill throughput in mechanical pulping
Enhanced paper strength properties
Alleviated pitch problems
Improved yield
Reduced environmental impact in mechanical and chemical pulping and papermaking

Table 6.4: Advantages of biopulping

Sequence	Pulp	Dosage of enzyme/ mediator (kg/TP)	Degree of delignification (%)	Max. brightness (% ISO)
L-E-Q-P	A	2/13	56.6	76.5
L-E-L-E-Q-P	A	2X2/2X8	50.6/67.7	82.7
L-E-Q-(P)	B	2/8	44.2	

Conditions:

Parameter	L stage	E stage	Q stage	P stage
Consistency (%)	10	10	5	10
Temperature (°C)	45	60	60	75
pH	4.5	11.5	5	11.2
Residence time (min)	120	60	30	210
Pressure (bar)	2	-	-	-
Dosage	Enzyme: 2kg/T Mediator: variable	NaOH	0.2% DTPA	3% peroxide

Table 6.5: Results from the pilot plant trial with laccase-mediator system
Based on data from Call and Mucke (1997)

Parameter	$D_OE_P*D_1D_2$	LEp**DOEp***D_1	$LE_P**AD_OEp***D_1$
Kappa factor	0.28	0.15	0.12
Reduction in ClO_2 Dose (%)	-	45.6	58.1
LEp□□ Brightness (%ISO)	-	46.4	-
LEp□□A Brightness (%ISO)	-	-	47.0
D_O Brightness (%ISO)	42.0	66.8	63.5
E_P□ Brightness (%ISO)	65.2	-	-
Ep□□□Brightness (%ISO)	-	79.4	78.9
D_1 Brightness (%ISO)	84.5	88.0	88.2
D_2 Brightness (%ISO)	87.8	-	-
Viscosity (cp)	7.2	7.1	6.9
Treatment conditions: L stage conditions: Laccase dose 60 U/g pulp, HBT dose 3%, pH 4.0, temp. 45 °C, consistency 15% and retention time 5 h A stage conditions: pH 2, temp. 90 °C, retention time 2 h, consistency 10% Ep□ stage conditions: NaOH dose 0.85%, H2O2 dose 0.5%, temp. 70 °C, consistency 10% and retention time 2 h Ep□□ stage conditions: NaOH dose 1.5%, H2O2 dose 0.5%, temp. 70 °C, consistency 10% and retention time 2 h Ep□□□ stage conditions: NaOH dose 0.8%, H2O2 dose 0.5%, temp. 70 °C, consistency 10% and retention time 2 h D_O stage conditions: pH 3.5, temp. 55 °C, retention time 30 min, consistency 10% D_1 stage conditions: ClO_2 dose 0.8%, pH 3.5, temp. 75 °C, retention time 3 h, consistency 10% D_2 stage conditions: ClO_2 dose 0.4%, pH 3.5, temp. 75 °C, retention time 3 h, consistency 10%			

Table 6.6: Effect of Laccase-Mediator System on ECF Bleaching
Based on Bajpai et al. (2006b)

Lipophilic wood extractives
Stearic acid (a fatty acid)
Abietic acid (a resin acid)
Sitosterol (a sterol)
Sitosteryl linoleate (a steryl ester)
Trilinolein (a triglyceride)

Table 6.7: Examples of prominent types of lipophilic wood extractives causing pitch problems

Benefits of recycled fibre
Uses less energy than virgin paper
Uses less water
Releases fewer pollution emissions to air and water
Requires lesser refining than virgin pulp and can be corefined with other pulps
Deinked pulp provides special properties (opacity, less curling tendency, less fuzziness and improved formation) to the finished papers in comparison with those made from wood pulp

Table 6.8: Benefits of using recycled fibre

Negative effects of DCS substances
Influence on the efficiency of papermaking chemicals
Scaling problems
Deposits of sticky material
Slime growth
Odours in the product
Corrosion

Table 6.9: Negative effects of DCS substances

Low-molecular weight compounds biografted to lignocellulosic materials with laccases
PAA
4-HBA
Vanillic acid
Syringic acid
Ferulic acid
Gallic acid
Guaiacol
Vanillin
Guaiacol sulfonate
3-hydroxytyramine hydrochloride
Tyrosine
4-hydroxy-3-methoxybenzylurea
Acrylamide

Table 6.10: Examples of low-molecular weight compounds biografted to lignocellulosic materials with laccase

Uses in food industry
Wine and beer stabilization
Baking
Fruit juice processing
Improvement of food sensory parameters
Sugar beet pectin gelation
Bioremediation of food industry waste water

Table 6.11: Uses of laccases in food industry

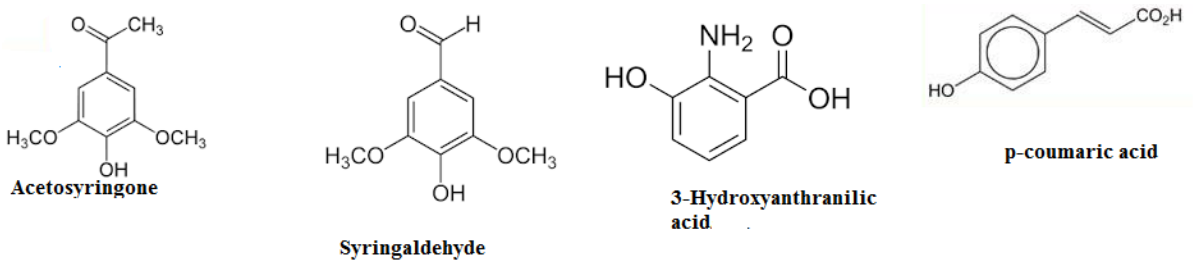

Figure 6.1: Chemical structures of most effective mediators

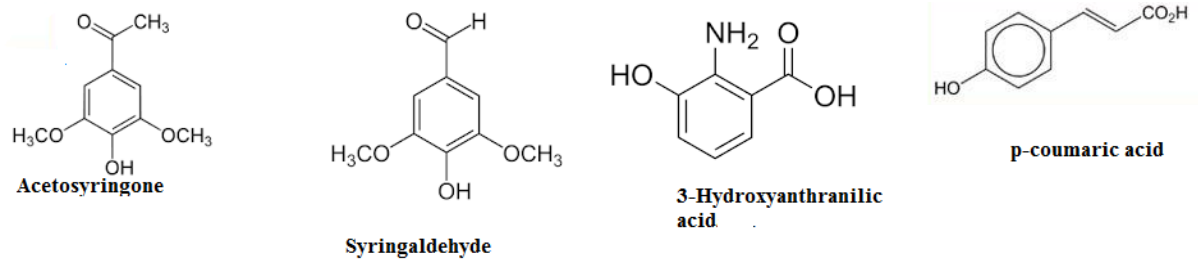

Figure 6.2: Chemical structures of natural mediators for laccases

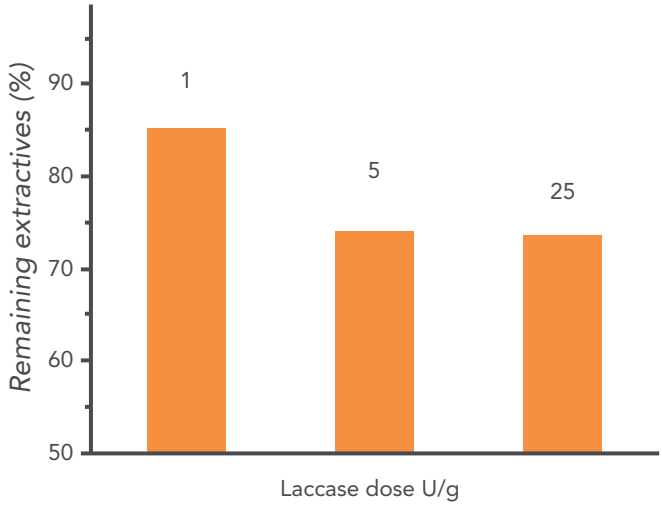

Figure 6.3: Effect of laccase treatment on removal of extractives from mechanical pulp; Based on Paice (2005)

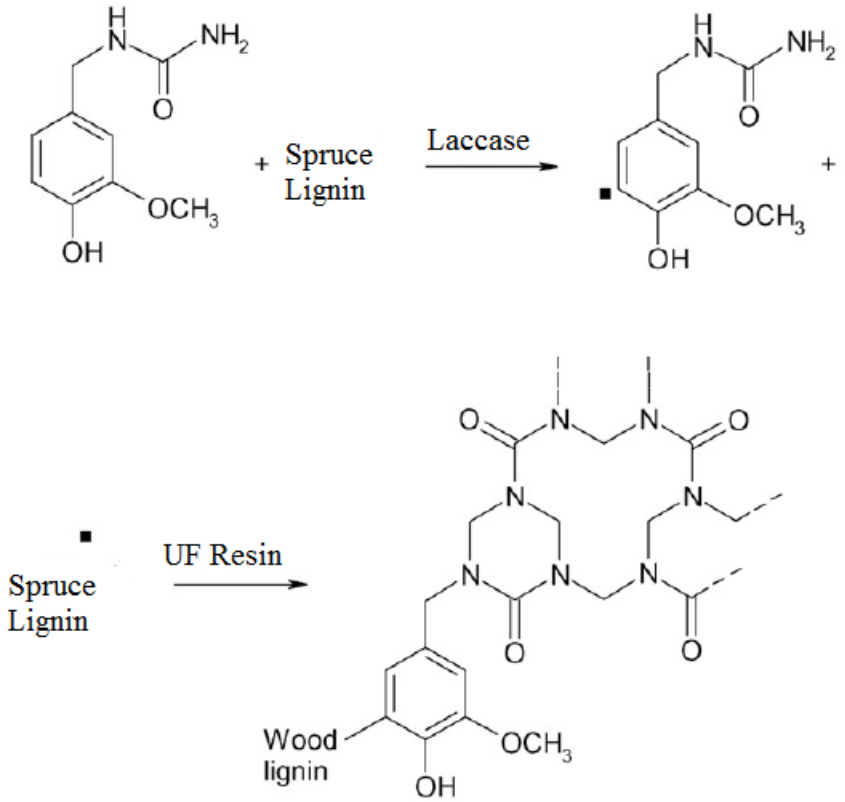

Figure 6.4: Laccase-assisted functionalization of spruce chips used for manufacturing particleboard with 4-hydroxy-3-methoxybenzylurea and cross-linking of functionalized lignin and UF-resin in particleboards. Based on Fackler et al. (2008)

7 FUTURE PERSPECTIVES

Laccase is currently considered a 'green enzyme' by many. An analysis of the scientific literature published in the last 10 years reveals a continuous growth of laccase application research in several industrial fields followed by the publication of a large number of patents. The patents concern methods of fermentation and optimization of laccase production, followed by inventions about enzyme modification aimed at its conjugation and/or immobilization for different uses. Analyzing the distribution of patent owners, Novozyme (Novo Nordisk, Denmark) owns the most of the patents (5%), followed by L'Oreal with 3%. The biggest share (78%) is occupied by companies or research centers possessing less than 1% of total number of published patents. Despite this large number of applications, laccase potential has not been fully exploited due to several issues. The most important obstacles to commercial application of laccases are the lack of sufficient enzyme stocks and the cost of redox mediators. Significant progress has been made over the last years to solve these problems. Thus, efforts have to be made for achieving inexpensive overproduction of this biocatalyst in heterologous hosts and also their modification by chemical means or protein engineering to achieve active and more robust enzymes. The development of an efficient system for immobilization of laccase also deserves great deal of attention. Immobilization could be performed by chemically modifying the substrates. Therefore, micropatterning, self-assembled monolayer method (SAMs) and layer-by-layer (*LbL*) techniques can be used to functionalise flat and curved surfaces for having specific adsorption. Laccase encapsulation using polyelectrolytes can be used as a microreactor for catalytic reactions by changing the permeability properties of the capsule wall. The general objective is to obtain stable catalysts with long life times and low cost; the combination of these techniques will improve the adsorption of laccase on a suitable substrate, the lifetime of the laccase activity and reuse of the substrate/laccase product.

Recent efforts to abundantly produce these enzymes, to improve enzyme activity and/or stability through immobilization and protein engineering, are boosting use of laccase at an industrial level.

Since the first commercial product based on laccase enzyme, launched in 1996 by Novozyme, the companies have been engaged in producing this enzyme in several formulations and for different purposes, mainly for the textile and food industries (Piscitelli et al., 2013). Less enzymatic formulations are available for the pulp and paper industries (Pezzella et al., 2015). Most of the new companies are located in Asia, where industry is expanding due to the less bureaucratic constraints for industrial production and lower salaries of employees in these regions in spite of the global crises (Piscitelli et al., 2013). Further, in Europe also, the increasing demand of specific and environment friendly alternative biocatalysts has nowadays induced the creation of new small companies offering customized formulations of laccases to target specific process conditions.

Many companies are offering laccases for pulp and paper, food, textile, pharma, cosmetic, paint or furniture industries (Pezzella et al., 2015; Mate and Alclade, 2016; Cannatelli and Ragauskas, 2017; Rodrıguez Couto and Toca Herrera, 2006). But for fully realizing the potential of laccases to compete in the biotechnology race, some obstacles must still be overcome. So, there is a need to produce laccases in industrially relevant hosts, particularly filamentous fungi like *Aspergillus sp*. and also at competitive prices and high titres. Some progress has been made in this regard through protein engineering (Mate and Alcalde, 2015). But, the use of laccases on a large industrial scale is still not the norm. Another major problem is the high cost of redox mediators and their inhibitory effect on laccase activity. In this regard, implementing LMS based on natural mediators obtained from lignin combustion is an area for in depth pursuing. Also, the design of laccases with customized features by the use of protein engineering will expand the portfolio of highly efficient enzyme variants and their use in various applications ranging from organic synthesis to the production of biofuels and beyond (Mate and Alcalade, 2016).

The use of laccases for the synthesis of fine chemicals has been a developing area of research over the past two decades in several laboratories (Cannatelli and Ragauskas, 2017). The types of chemical transformations that can be performed and the chemical structures that can be accessed are immense. By the use of LMS, these can be further broadened. Mekmouche et al. (2015) and Schneider et al. (2015) paired laccases with palladium and ruthenium transition metal complexes for the first time for the selective oxidation of benzylic alcohols and olefins, respectively.

There is a need to make more efforts towards using laccases to modify lignin for the fabrication of novel biomaterials (Hüttermann et al. 2001; Cannatelli et al., 2015; Stewart, 2008). Lund and Ragauskas (2001) successfully grafted water soluble phenols onto the surface of kraft lignin using laccases, rendering the lignin more water soluble. By copolymerizing lignin with phenolic monomers via laccase-assisted grafting, Aracri et al. (2014) were able to transform lignin into a useful adhesive for wood floor coverings.

There is a trend in industry towards biomass derived fuels and materials and away from those derived from petroleum for reducing carbon footprint (Cannatelli and Ragauskas, 2017). Therefore, research into the valorization of lignin will continue to be a promising area in the development of fully integrated biorefineries with laccases projected to perform multiple prominent roles (Ragauskas et al., 2006; Ragauskas et al., 2014; Roth et al., 2015). Laccases have been used with *Rhodococcus opacus* for conversion of lignin into liqid fuels. Fungal laccases actually catalyze the depolymerization of lignin, allowing for greater microbial lignin conversion, resulting in a significantly increased yield (Zhao et al., 2016).

Although the use of laccases for industrial purposes is promising, challenges facing the commercialization of laccase-mediated processes exist. These challenges are the lack of availability of affordable, highly active enzymes, and separation of enzyme from the reaction medium after completion of the process. To tackle these problems, advances in protein engineering have made it possible for producing thermostable laccases, tolerant to organic solvents and ionic liquids increasing their suitability for industrial applications (Rasekh et al., 2014; Dabrimanesh et al., 2015). Recently it has been found that by combining laccase with Au nanoparticles to formulate laccase-Au hybrids, the activity of laccase can be increased dramatically (Guo et al., 2015). Advances in enzyme immobilization technology, such as cross-linked enzyme aggregates and adsorption onto multi-walled carbon nanotubes provide improved storage and enzyme operational stabilities. Also it provides a means to separate and reuse the enzyme (Matijošytė et al., 2010; Piccinino et al., 2015; Durán et al., 2002; Fernández-Fernández et al., 2013). Furthermore, combination of laccases with sonochemistry has shown to reduce the chemicals and energy consumption, thus providing a cost effective means to upscale laccase-catalyzed processes to an industrial scale (Gonçalves and Silva, 2015).

Recently, new and existing laccases from different fungal species have shown medicinal application, with the ability to inhibit HIV–I reverse transcriptase and also show anti proliferative and cytotoxic effects on tumor cells (Wu et al., 2014; Rashid et al., 2015; Mizerska-Dudka et al., 2015; Sun et al., 2014). Researchers are discovering the innovative ways in which laccases can continue to advance sustainability in the chemical industry and beyond.

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