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Lignin biodegradation with laccase-mediator systems

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Lignin has a significant and largely unrealized potential as a source for the sustainable production of fuels and bulk high-value chemicals. It can replace fossil-based oil as a renewable feedstock that would bring about socio-economic and environmental benefits in our transition to a biobased economy. The efficient utilization of lignin however requires its depolymerization to low-molecular weight phenolics and aromatics that can then serve as the building blocks for chemical syntheses of high-value products. The ability of laccase to attack and degrade lignin in conjunction with laccase mediators is currently viewed as one of the potential "breakthrough" applications for lignin valorization. Here, we review the recent progress in lignin biodegradation with laccase-mediator systems, and research needs that need to be addressed in this field.

Keywords: lignin, biodegradation, laccase, mediator, laccase-mediator systems, lignin model compounds

INTRODUCTION

Lignocellulosic biomass is the single renewable resource on earth, reproduced at 60 billion tons as organically bound carbon per year, which has the potential to create a sustainable energy future. In their "billion ton vision," the U.S. Department of Energy (DOE) reported that nearly 1.3 billion dry tons of plant biomass could become available to produce biofuels and displace more than 30% of the nation's consumption of liquid transportation fuels (Perlack et al., 2005). Lignin removal from biomass helps enhance the efficiency of cellulose and hemicellulose hydrolysis, and therefore, facilitates the utilization of the carbohydrate portion of biomass in production of cellulosic ethanol and other biofuels (Siqueira et al., 2012). Annually, about 50–60 million tons of lignin are produced by the pulp and paper industry alone. The amount of available lignin is expected to further increase as a result of the recent biorefinery developments aimed at replacing fossil feedstocks with lignocellulosic biomass for biofuel production. A recent DOE report estimates that 0.225 billion tons of lignin (biorefinery lignin) could be produced from processing 750 million tons of biomass feedstock to biofuel (Bozell et al., 2007). However, the commercial use of lignin is limited to only 2% of its availability with the rest (Gosselink et al., 2004) usually burned to provide steam and process heat for the pulp and paper mills. It has been reported that lignin used as a low cost fuel has a value of only $0.18/kg whereas its value as a chemical feedstock is about sixfold higher (Macfarlane et al., 2009). Since existing lignin products are currently based primarily on low-value lignosulfonates (approximately 1 million tons) and kraft lignins (100,000 tons), lignin markets are stagnated at $300 million per year with very low growth rates (United Nations, 2012).

Therefore, new methods for lignin deconstruction and utilization for value-added products, other than just simply burning it as a solid fuel, are needed. Due to the complex cross-linked three-dimensional network structure, lignin is highly rigid and recalcitrant to degradation (Ruiz-Dueñas and Martínez, 2009). There are two alternative paths for the breakdown of the lignin polymer – chemical and biological. A distinct advantage of the biological approach is the lack of yield loss associated with the thermal decomposition of lignin, and the opportunity to "direct" lignin biodegradation utilizing selective lignolytic microorganisms and enzymes, thereby avoiding the formation of undesirable by-products. In addition, the biocatalytic process takes place under mild conditions that lowers the energy input and reduces the environmental impact. In nature, efficient and selective lignin biodegradation is mediated mainly by white-rot fungi and certain bacteria (Baldrian, 2006). The lignolytic enzymes are classified as peroxidases (lignin, manganese, and versatile peroxidase) and laccases. The broad substrate specificity of laccases and their ability to utilize atmospheric oxygen as electron donor instead of hydrogen peroxide used by peroxidases makes these enzymes a promising candidate for diverse industrial applications. These include use as a bleaching agent in pulp delignification, as a stabilizer in wine production, in detoxification of wastewaters and organic pollutants, in textile decolorization, biofuel cells and biosensors, manufacture of antibiotics and anti-cancer drugs, polymer and fiber surface modifications, etc. (Couto and Herrera, 2007; Medhavi and Lele, 2009). Among these applications, the ability of laccase to attack and degrade lignin in conjunction with laccase mediators is currently viewed as one of the potential "breakthrough" applications that are expected to offer great opportunities for lignin valorization. Here, we review the potential of laccase-mediator systems (LMS) for lignin biodegradation with related challenges and opportunities that currently exist.

LIGNIN

Lignin is most abundant naturally occurring aromatic polymer and following cellulose, the second most abundant organic

polymer on earth. It comprises 20–35% of the dry weight of plant cell wall, depending on the biomass source (Galbe and Zacchi, 2007). Softwoods generally contain more lignin (25–35%) than hardwoods (20–25%) (Faravelli et al., 2010). Lignin is predominantly concentrated in the middle lamella and primary cell wall. It surrounds and crosslinks the cellulose–hemicellulose matrix through lignin–carbohydrate network structures that provide stiffness to the cell walls and glue the cells together thereby shielding the polysaccharides against microbial degradation. As a hydrophobic polymer, lignin also serves as a barrier against water penetration. All these biological functions of lignin make this unique biopolymer one of the most recalcitrant naturally occurring compound (Ruiz-Dueñas and Martínez, 2009). The lignin macromolecule contains functional groups such as methoxyl, phenolic hydroxyl, alcoholic hydroxyl, and carbonyl groups that have a profound impact on its reactivity.

Lignin is composed of phenylpropanoid units, known as monolignols or lignin precursors, which are linked together through carbon–carbon and carbon–oxygen bonds with a varying degree of methoxylation (Adler, 1977; Karhunen et al., 1995) (**Figure 1**). The monolignols have been identified as *p*-coumaroyl, confineryl, and sinapyl alcohols, which are the respective precursors of *p*-hydrophenyl (H), guaiacyl (G), and syringyl (S) units in lignin (Zhang et al., 2003). Usually, plant cell wall lignification is accomplished through oxidative coupling and chemically controlled polymerization of the three lignin precursors in different proportions. For example, softwood lignin is built up mainly from G-units (up to 95%) with small amounts of H-units, whereas hardwood lignin has both S- and G-units in proportions from 1:1 to 1:3, with traces of H-units. All three lignin units are present in annual plants (Sjostrom, 1993; Brunow, 2001; Kang et al., 2012).

Lignin biosynthesis proceeds through oxidative coupling reactions of radicals generated by laccases and peroxidases that lead to formation of a growing polymer linked by carbon–carbon and ether bonds. The resonance-stabilized phenoxy radicals polymerize in a variety of monomer–oligomer and oligomer–oligomer coupling combinations through β-*O*-4, α-*O*-4, β-5, β-1, β-β, 5-5, dibenzodioxocin, and 4-*O*-5 linkages, some of them depicted in **Figure 2**. The most abundant structure in lignin is the arylglycerol-β-aryl ether structure with the β-*O*-4 linkages accounting for up to 60% of the total inter-unit bonds in lignin (Balakshin et al., 2009) whereas the carbon–carbon bonds in lignin are among the most resistant toward breakage (Capanema et al., 2004). However, it should be noted that despite the numerous studies on lignin that followed the pioneering work of Freudenberg and co-workers (Freudenberg and Hübner, 1952; Freudenberg, 1959, 1962, 1965; Freudenberg and Neish, 1968) more than six decades ago, its structure is still not completely understood.

LACCASE-MEDIATOR SYSTEMS

LACCASE

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing phenol oxidase, which can oxidize electron-rich substrates of phenolic and non-phenolic origin with

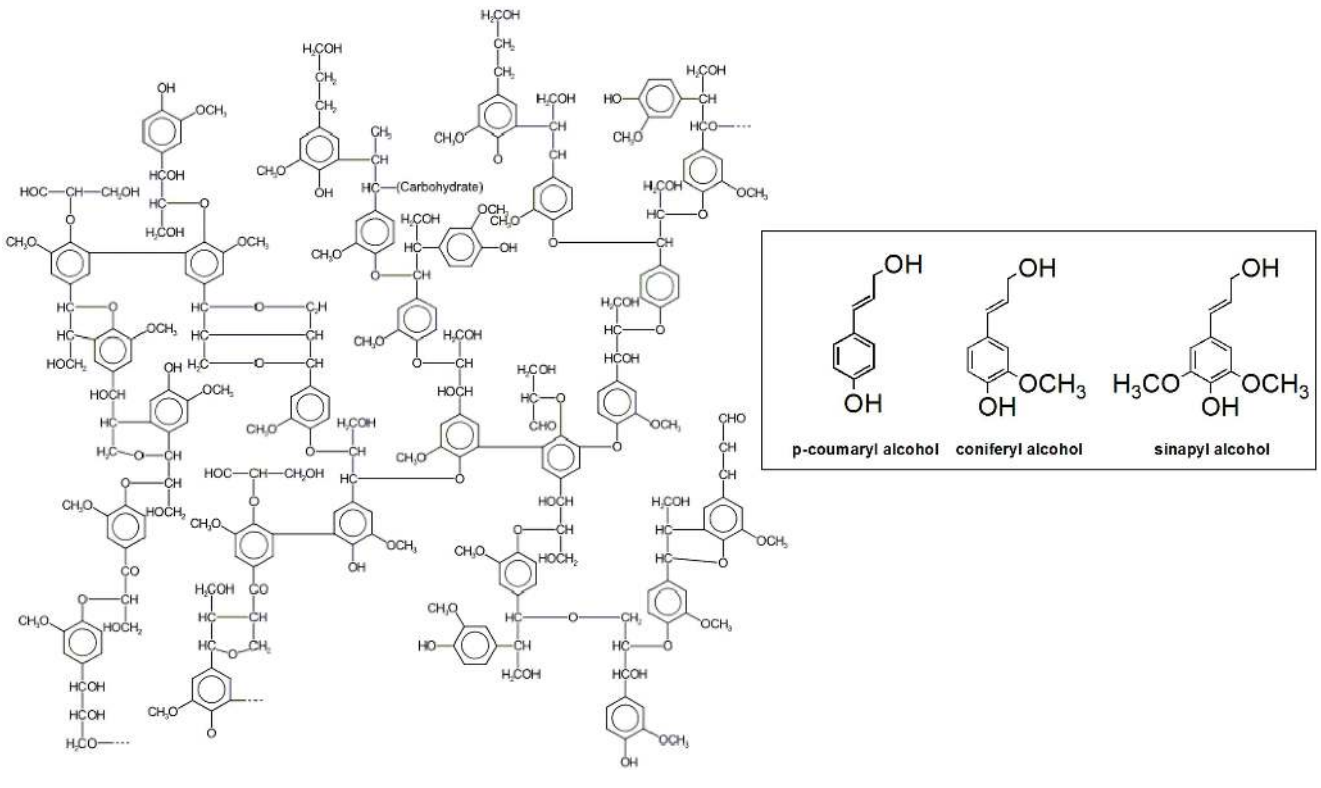

FIGURE 1 | Structure of lignin and lignin precursors of H-, G-, and S-units in lignin.

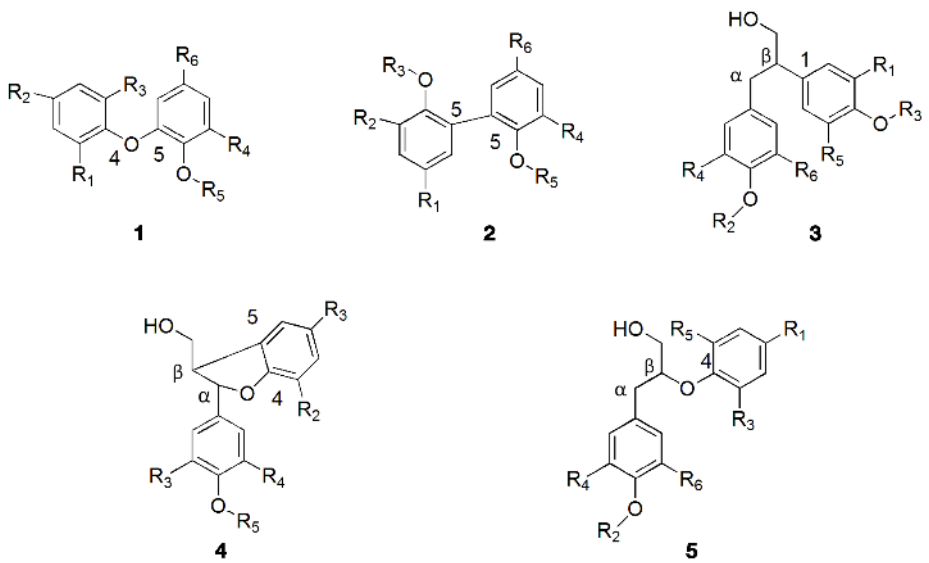

FIGURE 2 | Commonly found linkages in lignin: (1) 4-*O*-5; (2) 5-5; (3) β-1; (4) β-5; (5) β-*O*-4.

a concomitant reduction of oxygen to water through a radical-catalyzed reaction mechanism (Bourbonnais et al., 1997). The functions of laccases are diverse. They are involved in both lignin biosynthesis and lignin degradation, pigment formation in fungal spores, plant pathogenesis, and as fungal virulence factors, in iron metabolism and kernel browning processes in plants (Hood et al., 2003; Higuchi, 2004; Hoopes and Dean, 2004; Zhu and Williamson, 2004). Laccase was first discovered in the Japanese lacquer tree *Rhus vernicifera* in the nineteenth century (Yoshida, 1883). Although laccases are present in higher plants, fungi, bacteria, and insects, the most studied group of enzymes to date is from fungal origin, including the genera of Ascomycetes, Deuteromycetes, Basidiomycetes, and cellulolytic fungi (Hatakka, 1994; Schneider et al., 1999; Pandey et al., 2001; Baldrian, 2006; Sharma et al., 2007). Among these, laccases from the white-rot basidiomycetes (white-rot fungi) such as *Trametes (Coriolus) versicolor*, *T. hirsuta*, *T. ochracea*, *T. villosa*, *T. gallica*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus edodes*, *Pleurotus ostreatus*, *Pycnoporus cinnabarinus*, *Coprinus cinereus*, etc., are most frequently described and reported (Baldrian, 2006). The first characterized ascomycete enzyme was from *Monocillium indicum* (Thakker et al., 1992). Fungal laccases are responsible for detoxification, fructification, sporulation, phytopathogenicity, and lignin degradation (Widsten and Kandelbauer, 2008). White-rot fungi have a strong ability to degrade lignin due to the high laccase activity they produce, and to their well developed hyphal organization that can efficiently penetrate plant cell walls (Grove and Bracker, 1970).

Compared to fungal laccases, bacterial laccases are generally more stable at high pH and temperatures (**Table 1**). Whereas fungal laccases can be both intra- and extra-cellular, bacterial laccases are predominantly intracellular, such as *Azospirillum lipoferum*, *Marinomonas mediterranea*, and *Bacillus subtilis* (Rosconi et al., 2005; McMahon et al., 2007). To date, only three bacterial laccases have been completely purified and characterized (as opposed to more than 100 fungal laccases): (1) from the rhizospheric bacterium *A. lipoferum* (Givaudan et al., 1993); (2) from a melanogenic marine bacterium *M. mediterranea* (Solano et al., 1997); and (3) from the endospore coat component CotA of *B. subtilis* (Kim et al., 2006). Laccases from *Streptomyces lavendulae* (Nandan and Nampoothiri, 2014) and *S. cyaneus* (Teeradakorn et al., 1998) have also been reported. Bacterial laccases normally have a higher pH optimum than fungal laccases (Margot et al., 2013), with optimum pH being acidic for the latter (**Table 1**) (Dhakar and Pandey, 2013). Due to the intracellular physiological properties of plant laccases, their optimal pH is in the neutral range (Dwivedi et al., 2011; Pezzella et al., 2013). The isoelectric point of plant laccases (pI 9) is also higher than that of fungal laccases (pI 3–7). The optimal temperature for most laccases is between 50 and 70°C (Chefetz et al., 1998). Their thermal stability depends on the microbial source. For example, bacterial laccases are more thermostable than fungal laccases (Hildén et al., 2007). Plant laccases, on the other hand, are more glycosylated and have a greater molecular weight than fungal and bacterial laccases (**Table 1**). Laccase is a secondary metabolite produced under growth-limiting conditions (limited nitrogen in particular), which however has a negative impact on the enzyme yields (Gianfreda et al., 1999). Due to the low laccase activities in most native fungi and bacteria that preclude industrial uses, improved productivity through cloning of laccase genes and their heterologous expressing has been targeted. Bacterial laccases from *B. subtilis*, *S. lavendulae*, and *Thermus thermophiles* have been expressed in *Escherichia coli* (Martins et al., 2002). Hosts for the heterologous expression of fungal laccases include yeasts such as *Saccharomyces cerevisiae* (Bulter et al., 2003), fungi such as *Trichoderma reesei* (Kiiskinen et al., 2004) and *Aspergillus oryzae* (Sigoillot et al., 2004), and plants such as tobacco (LaFayette et al., 1999) and maize (Bailey et al., 2004). Laccases can be produced in both liquid and solid state fermentation (Mazumder et al., 2009). Copper (Palmieri et al., 2000), ethanol

Table 1 | Properties of some bacterial, fungal, and plant laccases.

Laccase	T_{opt} (°C)	pH_{opt}	Mw (kDa)	Glycosylation (%)	Reference
BACTERIAL					
Bacillus subtilis	37	7.6	57.2	ND	Phelan et al. (2013)
Bacillus licheniformis	40	7.0	64	ND	Salkinoja-Salonen et al. (1999)
Streptomyces griseus	40	6.5	209	ND	Leigh (1997)
FUNGAL					
Agaricus blazei	20	5.5	66	ND	Ullrich et al. (2005)
Basidiomycota sp.	80	4.5	64	6.5	He and Li (2013)
Melanocarpus albomyces	60–70	6.0	80	ND	Berdy (2005)
Trametes hirsuta	45	4.5	70	12	Shleev et al. (2004)
Trametes versicolor	50	3.0	67	10–12	Solomon et al. (1996)
Trichophyton rubrum	20	5.5	65	ND	Yang et al. (2007)
PLANT					
Acer pseudoplatanus	15.5	6.6	97	40–45	Sterjiades et al. (1996)
Chaetomiaceae sp.	60	7.0	77	ND	Jiao et al. (2006)
Rhus vernicifera	25	7.0	110	45	Messerschmidt and Huber (1990)

(Lee et al., 1999), and aromatic compounds such as ferulic acid, gallic acid, veratryl alcohol, anisidine, xylidine, syringaldazine, and lignosulfonates (Saraiva et al., 2012) serve as efficient inducers of laccase production.

Most laccases contain four copper atoms in their active site, which mediate the redox process and are classified in three groups according to their magnetic and spectroscopic properties (Messerschmidt and Huber, 1990). **Figure 3** shows the three types of copper coordination in laccases: type 1 or blue copper center, type 2 or normal copper, and type 3 or coupled binuclear copper centers (Wong, 2009). Type 1 copper, coordinated with one cysteine, one methionine, and two histidine molecules (Palmieri et al., 2003), contributes to the intense blue color of laccase with a strong electron adsorption at 600 nm. It is responsible for the substrate oxidation and redox potential of laccase. Type 2 copper coordinates with two histidines and a water molecule; it is colorless with no absorption in the visible spectrum (Piontek et al., 2002). There are three histidines as ligands to each type 3 copper atom, with anti-ferromagnetic coupling and a hydroxyl bridge between the copper pair that shows a weak UV absorbance at 330 nm (Piontek et al., 2002). The one type 2 and two type 3 copper atoms form a tri-nuclear center that catalyzes the fixation and reduction of oxygen to water. Enzyme stability is directly influenced by the hydrogen bonding and salt bridges that exist between the copper atoms (Hildén et al., 2009). All four copper atoms are fully oxidized (Cu^{2+}) in the native form of laccase. Because of that, laccase can decarboxylate, demethylate and demethoxylate phenolic, and methoxyphenolic acids, which are important initial steps in lignin degradation (d'Acunzo et al., 2002). Since laccase catalyzes one-electron oxidation of substrates, the transfer of four electrons from four laccase substrates via the type 1 copper to the tri-nuclear center with oxygen as the final electron acceptor represents one catalytic cycle of substrate oxidation and oxygen reduction. Based on the type 1–3 copper properties, laccases are categorized into enzymes with high (0.6–0.8 V) or low (0.4–0.6 V) redox potential (Gutiérrez et al., 2006). For example, the laccases secreted by the white-rot fungi *T. versicolor* and *Neurospora grassa* have a high redox potential of 0.78–0.80 V (Mikolasch and Schauer, 2009), whereas the redox potential of the laccases from *R. vernicifera* and *C. cinereus* are only 0.42 V (Reinhammar and Vänngård, 1971) and 0.55 V (Schneider et al., 1999). The catalytic efficiency of laccases appears to be directly proportional to the redox potential of type 1 copper, which explains the increased interest in laccases with high redox potential (Xu et al., 1996). Laccases occur as monomeric and polymeric glycoproteins, with most fungal laccases being reported as monomers, dimers, or tetramers. The first crystalline three-dimensional structure of a laccase from *T. versicolor* was published in 2002 (Piontek et al., 2002). The molecular mass of a monomeric laccase is typically in the range 60–110 kDa with 10–50% glycosylation (**Table 1**). The high carbohydrate content in the protein molecule is believed to provide thermostability to laccases of up to 70°C (Yaropolov et al., 1994). In addition, glycosylation impacts enzyme secretion and activity (Xu, 1999). Most white-rot fungi produce more than one laccase isozyme that differ in the degree of glycosylation, amino acid sequence, molecular weight, pI, and substrate specificity (Mansur et al., 2003).

It has been shown that small halide anions can inhibit the activity of laccase due to disturbance of the internal electron transfer of the type 2 and 3 copper atoms that coordinate these anions (Dwivedi et al., 2011). Some metal ions such as Hg^{2+}, Mg^{2+}, Ca^{2+}, and Zn^{2+} also inhibit laccase activity by modifying the amino acid residues or causing conformational changes in the laccase glycoprotein. Fatty acids, sulfhydryl reagents, hydroxyglycine, dithiothreitol, and glutathione have also been reported as laccase inhibitors as the copper type 2 atoms are chelated by these organic compounds (Blanquez et al., 2004).

Laccases are able to oxidize not only various aromatic compounds such as substituted phenols, aminophenols, polyphenols, *o*- and *p*-diphenols, polyamines, methoxy phenols, aryl diamines, aromatic amines, and thiols, but also some inorganic compounds such as iodine and ferrocyanide ions (Claus, 2003). The oxidation

of inorganic ions is accompanied by a simultaneous reduction of dioxygen to water without intermediate production of hydrogen peroxide (Morozova et al., 2007a). The laccase-mediated formation of phenoxyl radicals results in cleavage of carbon–carbon and β-aryl bonds as well as aromatic rings in lignin (Bourbonnais et al., 1995; Kawai et al., 1999). Due to the low redox potential of laccases (0.5–0.8 V), laccase alone can only oxidize phenolic lignin structures and not the non-phenolic aromatic structure, which comprises more than 80% of lignin (Wong, 2009). Only a small group of peroxidases secreted by lignolytic fungi such as lignin peroxidase with a redox potential of 1.15–1.25 V (Ward et al., 2003) can oxidize the non-phenolic groups of lignin directly (del Pilar Castillo et al., 1997). Furthermore, permeability studies have indicated that molecules larger than 2 kDa are unable to penetrate the pores in the plant cell walls (Srebotnik et al., 1988). However, in presence of low-molecular weight (LMW) chemical compounds – mediators, that normally have a redox potential higher than 0.9 V, the substrate range of laccase can be expanded to include oxidation of non-phenolic lignin. Therefore, the LMS play a key role in depolymerizing lignin (Schmidt, 2007).

MEDIATORS

A mediator is a small chemical compound that is continuously oxidized by the laccase enzyme and subsequently reduced by the substrate. As the substrate due to its size cannot enter the laccase active site, the mediator acts as a carrier of electrons between the enzyme and the substrate thereby overcoming the steric hindrances that exist between them (Li et al., 1999). The laccase reactivity decreases with the increase of the substrate size, therefore the limited substrate accessibility is overcome through the use of appropriate laccase mediators. In the initial reaction step, the mediator is oxidized to stable intermediates with high redox potential by laccase. Thereafter, following diffusion-controlled reaction kinetics, the oxidized mediator diffuses away from the enzyme, and due to its small size is able to penetrate the pores of the plant cell walls to reach the target substrate (**Figure 4**). As a result, the substrate (lignin, aromatic compounds, etc.) is oxidized by the intermediates that cannot be oxidized directly by laccase, while the oxidized mediator is reduced to its initial form (Call and Mücke, 1997). The ideal mediator should be non-toxic, economic, and efficient, with stable oxidized and reduced forms that do not inhibit the enzymatic reaction (Morozova et al., 2007b). Moreover, the redox mediator should be able to continuously maintain the cyclic redox conversion.

Bourbonnais and Paice (1990) first reported that in the presence 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), the laccases of *T. versicolor* can also oxidize non-phenolic lignin model compounds (LMCs) such as veratryl alcohol and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol to produce α-carbonyl derivatives. The laccase–ABTS system was also able to demethylate and delignify kraft pulp (Bourbonnais et al., 1997). ABTS is readily oxidized by free radicals, various peroxidases and laccase to the cation radical $ABTS^{+\bullet}$, and the concentration of the intensely colored, green–blue cation radical can be correlated to the enzyme activity. The cation radical can be oxidized further to the dication ($ABTS^{2+}$). **Figure 5** shows the laccase-aided modification of ABTS

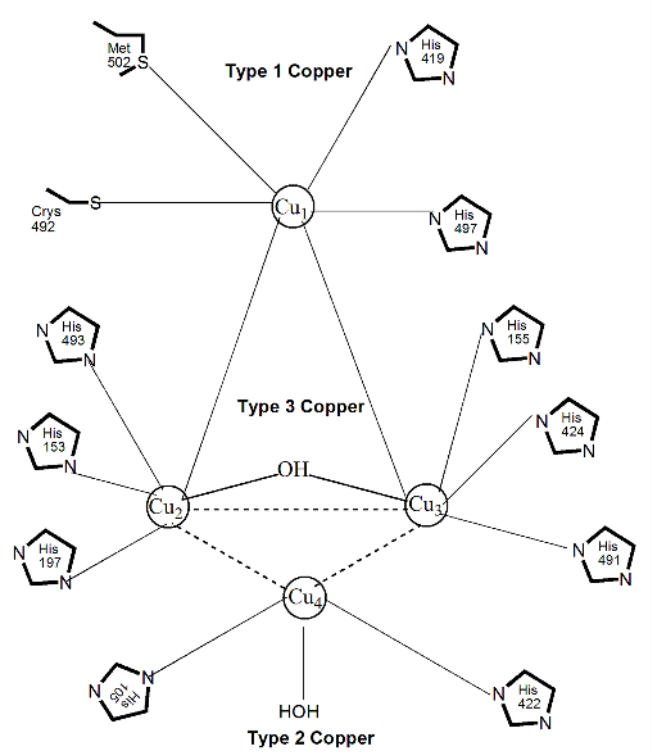

FIGURE 3 | Structure of the copper coordination network of the laccase molecule.

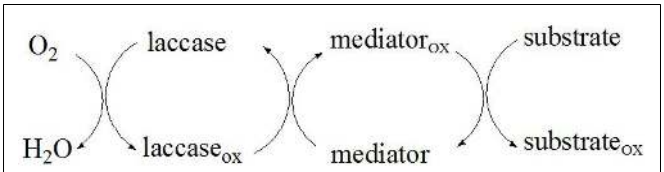

FIGURE 4 | Proposed mechanism of substrate oxidation by LMS.

during oxidization. The redox potential of the semi-oxidized and fully oxidized ABTS was measured as 0.68 and 1.09 V, respectively (Scott et al., 1993). 1-Hydroxybenzotriazole (HBT) was the second laccase mediator that was successfully used in lignin degradation and biobleaching of kraft pulps (Call, 1994). The search for new and efficient LMS has resulted in the chemicals synthesis and evaluation of a number of laccase mediators, including violuric acid (VIO), *N*-hydroxyacetanilide (NHA), *N*-hydroxyphthalimide (NHI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), etc. (Bourbonnais et al., 1997). The structural formulas of some synthetic mediators of the – *N*-OH-type are displayed in **Figure 6**(1). A method for selection of effective laccase mediators was proposed (Shleev et al., 2003) consisting of four steps: (1) select compounds that have heterocyclic atoms, OH-, and/or NH_2-groups in their structure; (2) test the electrochemical properties of these compounds with and without LMCs; (3) determine the optimum reaction conditions of these compounds during laccase-catalyzed degradation reactions of lignin

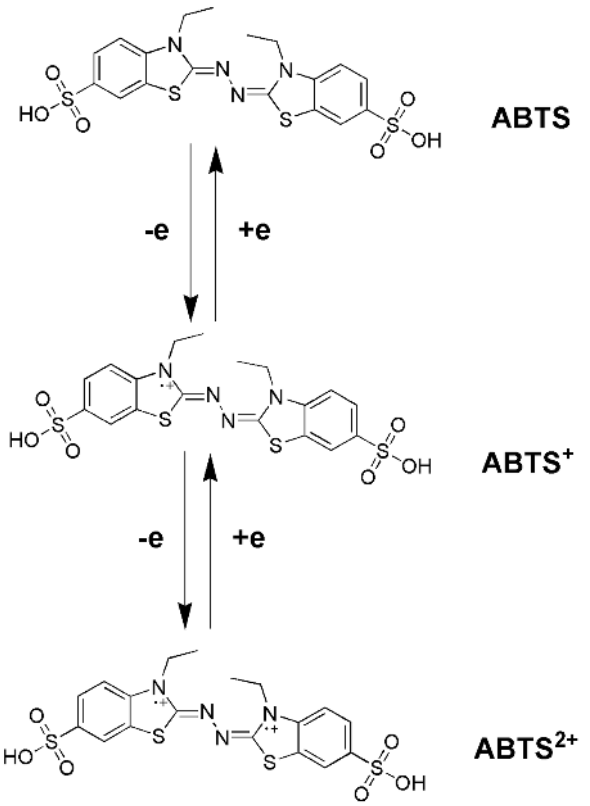

FIGURE 5 | Oxidation of ABTS in presence of laccase.

or LMCs; and (4) determine the products of lignin or LMCs degradation by LMS. Using this method, phenothiazine-type mediators have been selected [**Figure 6**(2)]. In another study, 20 heterocyclic compounds containing *N*-OH-groups and benzoic acid structures were screened as potential mediators for *T. hirsuta* laccase (Shumakovich et al., 2006). Derivatives of 1-phenyl-3-methyl pyrazolone were proposed as efficient mediators in LMS oxidation of veratryl alcohol [**Figure 6**(3)]. The major problems encountered during screening for synthetic mediators are associated with the instability of the mediator intermediates, which resulted in incomplete redox cycles or poor substrate oxidation. In addition, synthetic mediators are expensive, toxic, and difficult to reuse as they form by-products (Srebotnik and Hammel, 2000). Most of them are also inhibitive to laccase at concentrations higher than 1 mM. This prompted recent research efforts in search of naturally occurring mediators that may offer environmental and economic advantages.

Phenolic products generated during lignin degradation by white-rot fungi and fungal metabolites were evaluated as potential natural mediators (Eggert et al., 1996). Phenolic compounds have been reported to be able to mediate the oxidation of both non-phenolic part of lignin or non-phenolic model of lignin (Johannes and Majcherczyk, 2000; Astolfi et al., 2005). They can be obtained by extraction from pulp and paper effluents and plant materials at low cost (Camarero et al., 2007). Furthermore, laccases and their natural mediators can be applied in effluent bioremediation and lignocellulosic biorefineries (Cañas and Camarero, 2010). Example of natural phenolic compounds that can serve as laccase mediators in lignin degradation include acetosyringone, syringaldehyde, *p*-coumaric acid, vanillin, acetovanillone (Andreu and Vidal, 2011), 3-hydroxyanthranilic acid (3-HAA) (Eggert et al., 1996, 1997), 4-hydroxybenzoic acid and 4-hydroxybenzylic alcohol (Johannes and Majcherczyk, 2000), hydroquinone (Calcaterra et al., 2008), and phenolsulfonphthalein (d'Acunzo and Galli, 2003). **Figure 7** shows the chemical structure of some natural laccase mediators. LMS have gained increased attention in a number of potential applications such as detoxification of industrial effluents, textile dye bleaching, soil bioremediation, manufacture of pharmaceuticals, food and cosmetic products, etc. (Couto and Herrera, 2007). However, the focus of this review is on the potential of LMS to degrade lignin. The mechanism of lignin biodegradation was studied on LMCs, isolated lignins, and fiber-bound wood pulp lignins.

BIODEGRADATION OF LIGNIN WITH LACCASE-MEDIATOR SYSTEMS

Lignin biodegradation with LMS is an oxidative process of enzyme-catalyzed and mediator-facilitated radical reactions of degradation of both phenolic and non-phenolic aromatic structures in lignin. It has been suggested that the initial oxidative attack is on the phenolic lignin moiety (<20% of total lignin), followed by the destruction of non-phenolic benzylic structures (Camarero et al., 1994). The preferential oxidation of phenolic lignin units results in the release of phenolic residues with oxidized side chains such as phenolic aldehydes, ketones, and acids. These LMW phenolic fragments are then able to penetrate the bulk lignin polymer and act as natural mediators to oxidize more recalcitrant non-phenolic lignin (Reddy et al., 2003). Lignin depolymerization with LMS has been further suggested to proceed by a C_α–C_β cleavage of non-phenolic sites in lignin with subsequent solubilization of lignin fragments by formation of hydrophilic lignin-mediator complexes (Srebotnik and Hammel, 2000). Oxidation of non-phenolic LMCs by LMS can follow three different oxidation mechanisms: (1) electron transfer; (2) radical hydrogen atom transfer; and (3) ionic mechanism (Fabbrini et al., 2002). **Figure 8** exemplifies the first two oxidation mechanisms. Evidence for the existence of these mechanisms has been provided by examining the product pattern of non-phenolic lignin degradation, and measuring the intra-molecular kinetic isotope effects (Barreca et al., 2004). In the oxidation of non-phenolic LMCs, the efficiency of LMS was reported to be independent of the enzymes properties (Rosado et al., 2012). Oxidation of non-phenolic LMCs by laccase–ABTS was reported to occur via the electron transfer route (Bourbonnais et al., 1998). The laccase–TEMPO acts according to the ionic mechanism, whereas the HBT- and HPI-mediated reactions favor the radical mechanism (Fabbrini et al., 2002). It has been proposed that the HBT radical forms a coupling intermediated product with lignin (Potthast et al., 1997). The intermediate product can subsequently degrade to release the reduced form of benzotriazole (BT), or form a stable complex that binds some of the HBT to lignin via covalent bonding. The laccase–HBT treatment of softwood lignin was reported to result in increased content of *p*-hydroxyphenyl

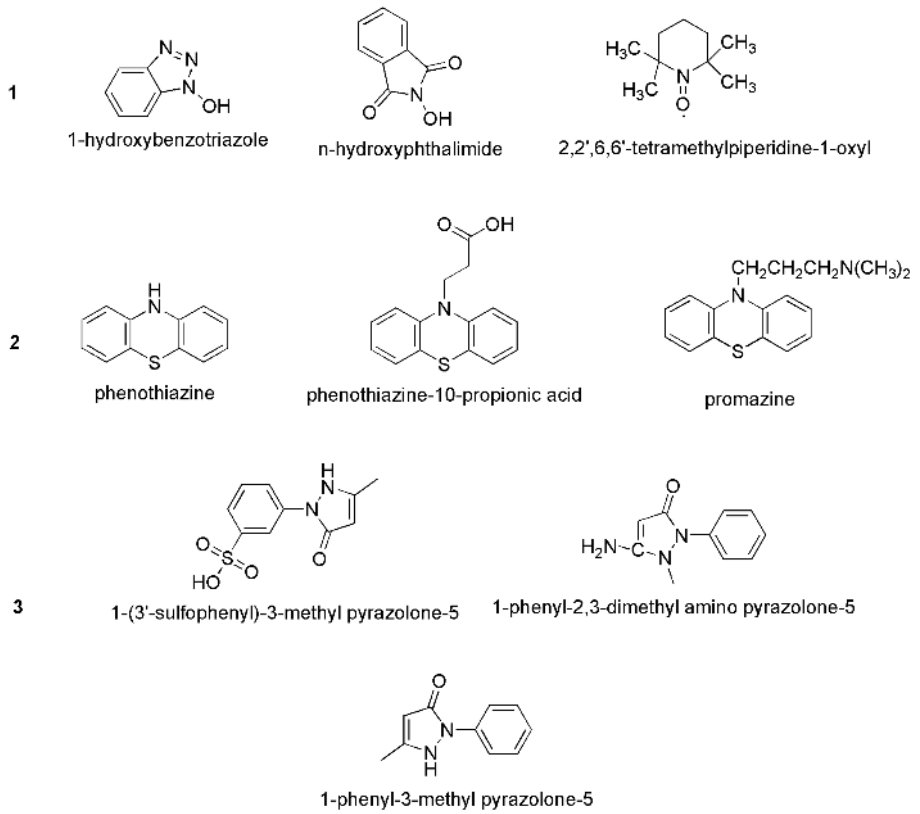

FIGURE 6 | Chemical structures of some synthetic laccase mediators: (1) >N–OH type; (2) phenothiazine-type; and (3) pyrazolone-type.

structures and reduced amount of G-units and aromatic lignin structures (Tamminen et al., 2003). Oxidation with laccase–HBT breaks the C_α–C_β link in LMCs such as hydrobenzoin dimers (Xu et al., 1997), and creates carboxylic acid groups in treated pulp lignins (Chakar and Ragauskas, 1999). **Figure 9** illustrates the oxidation of non-phenolic β-*O*-4-linked LMCs by laccase–HBT results in opening of the aromatic ring (π-electron oxidation), oxidation of C_α, and cleavage of β-ether and C_α–C_β bonds to generate aromatic carbonyl compounds and carboxylic acids (Kawai et al., 2002). In the oxidation reactions, the β-aryl radical cation or benzylic (C_α) radical intermediates are formed via the electron transfer mechanism. The β-ether cleavage of the β-*O*-4 lignin substructure is caused by reaction with the C_α-peroxy radical intermediate produced from the benzylic radical. When *T. villosa* laccase–HBT was applied in catalytic oxidation of phenolic and non-phenolic LMCs, their nominal molecular weight decreased 5- and 4-times, respectively. Cleavage of the C_α–C_β bond occurred in both LMCs thereby about 10% of their substructures were degraded (Srebotnik and Hammel, 2000). Typical products from lignin degradation with LMS include 2,6-dimethoxy-4-methylbenzaldehyde, 4-ethyl-2,6-dimethoxybenzaldehyde, and 2,6-dimethoxy-4-((*E*)-prop-1-enyl)benzaldehyde (Du et al., 2013). The molecular weight of lignin and its phenolic content are believed to influence the reaction pathways that are catalyzed by laccase and LMS (Niku-Paavola et al., 2002). It was suggested that parallel polymerization and depolymerization reactions compete during LMS treatment as phenolic groups in lignin (in particular LMW lignin) may serve as sites for lignin polymerization, which in turn would impede ligninolysis (Srebotnik and Hammel, 2000; Tamminen et al., 2003).

The LMS was first developed as an environmentally friendly bleaching agent for use in the pulp and paper industry (Bourbonnais and Paice, 1990). The system oxidizes and cleaves phenolic and non-phenolic lignin units that become depleted in bleached pulps following chemical bleaching resulting in lower kappa number and higher pulp brightness (Sealey and Ragauskas, 1998; Poppius-Levlin et al., 1999). This process is known as biobleaching and was first introduced as xylanase-aided bleaching of kraft pulps (Viikari et al., 1986). The biobleaching effect with xylanase is due to partial hydrolysis of xylan that is redeposited on the fiber surface during kraft pulping, thereby improving the access of bleaching chemicals residual lignin (Kantelinen et al., 1993). While xylanase assists in lignin removal from pulps indirectly, the LMS attacks lignin directly leading to formation of lower molecular weight oxidized lignin fragments that are susceptible to further degradation and solubilization in alkali (E) during chemical bleaching (Bourbonnais et al., 1997). The LMS-catalyzed lignin degradation and removal from bleached pulps enables the use of less hazardous chlorine-containing bleaching chemicals of environmental concern such as chlorine (C), chlorine dioxide (D), and sodium hypochlorite (H). For instance, biobleaching of industrial pulps with *T. hirsuta* laccase–HBT increased pulp brightness by up to

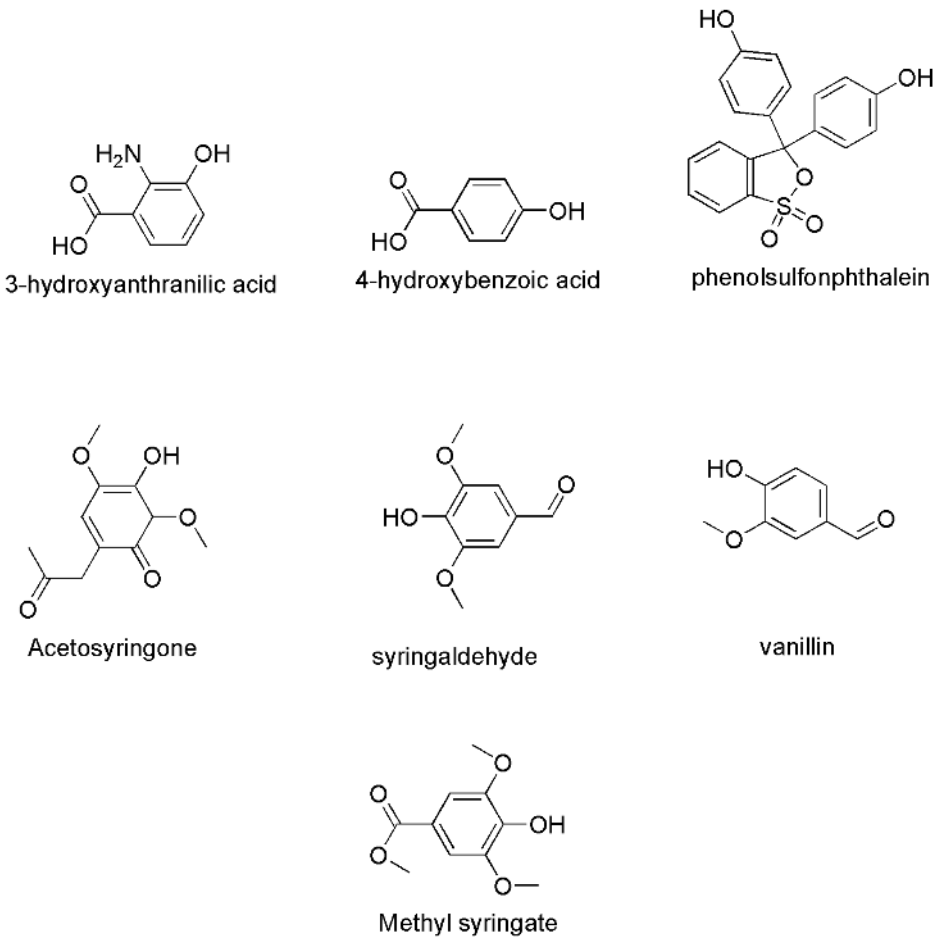

FIGURE 7 | Chemical structures of some naturally occurring laccase mediators.

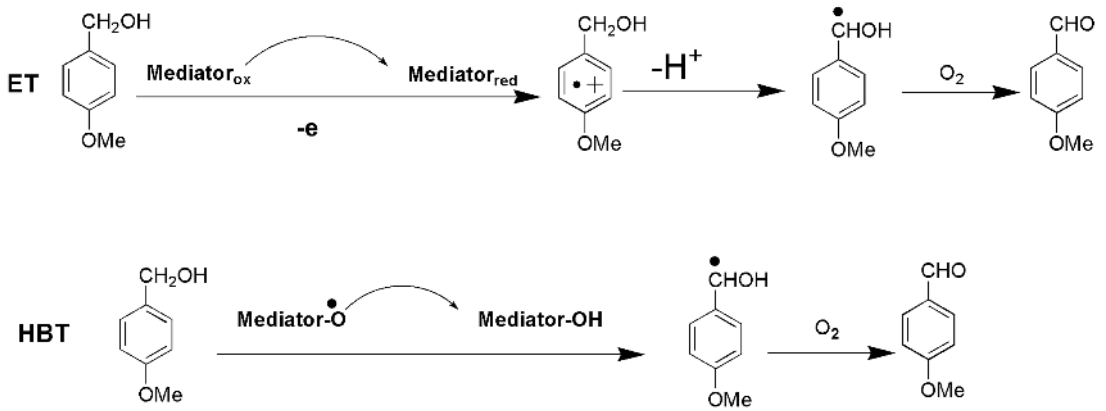

FIGURE 8 | Proposed mechanisms of LMS-catalyzed oxidation of 4-methoxybenzyl alcohol (non-phenolic LMC) to 4-methoxybenzaldehyde: (1) electron transfer (ET); (2) hydrogen atom transfer (HAT).

12.2 points in a DED pulp bleaching, alternatively, 40–60% chlorine dioxide could be used to reach brightness of control pulps (Kandioller and Christov, 2001). In a comparative study, kraft pulp was delignified with several fungal laccases in conjunction with HBT as mediator (Bourbonnais et al., 1997). The most efficient laccase was that of *Ganoderma colossum* with 40% lignin

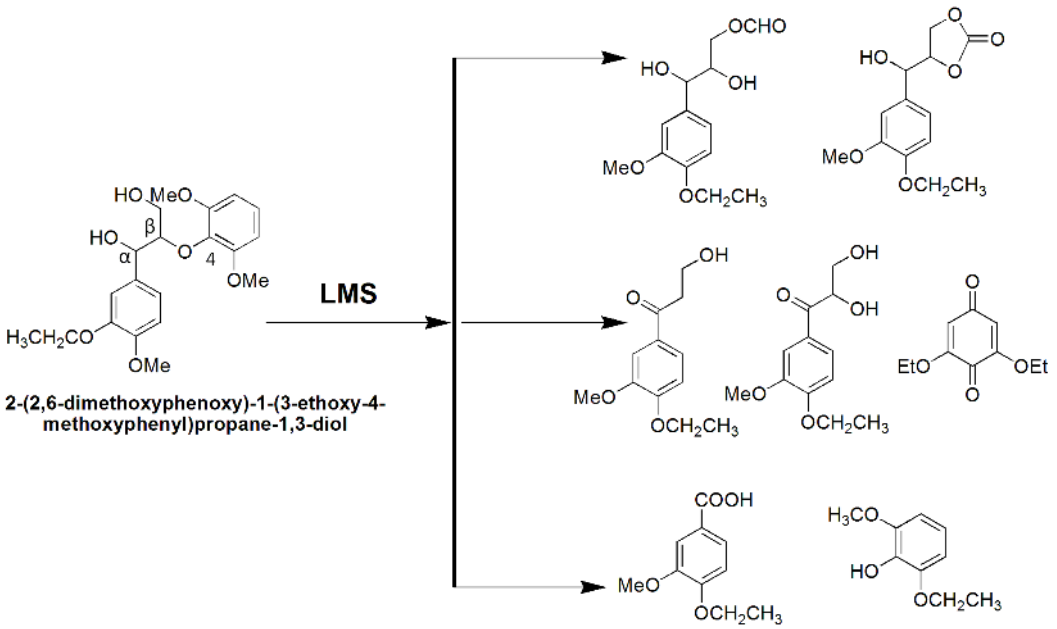

FIGURE 9 | Oxidation of a non-phenolic β-*O*-4 LMC by *Trametes versicolor* laccase–HBT system.

removal, followed by *T. versicolor* laccase (22%) and *P. ostreatus* laccase (13%). The *Fusarium proliferatum* laccase–ABTS system was able to oxidize and remove 19% from industrial kraft pine lignin (Indulin AT) predominsntly in the form of ketones (10%) and acids (2%), with significant reduction of G-units and methylated side-chain phenolic compounds in residual lignin (González Arzola et al., 2006). Two LMS, *P. cinnabarinus* laccase–HBT and *Myceliophthora thermophila* laccase–methyl syringate, were applied to delignify unbleached eucalyptus kraft pulp (Du et al., 2013). Pyrolysis-GC/MS and NMR analysis revealed that with both systems, C_α in lignin was oxidized whereas the C_α–C_β bond and aromatic ring were both cleaved, with accumulation of xylan–lignin fractions containing S and G uncondensed units. Biobleaching of eucalyptus kraft pulps was also studied using bacterial laccases from *S. cyaneus* (Arias et al., 2003) and *Pseudomonas stutzeri* (Sharma et al., 2007) with ABTS and HBT as mediators. Due to the high activity, alkali and thermal stability of these enzymes, the LMS proved as efficient lignin degraders (Moya et al., 2011). The extent of pulp delignification with LMS depends on the pulp and mediator type, source of pulp and laccase, and amount of enzyme and mediator used. For instance, pine kraft was delignified 19–40% depending on the laccase preparation (from several fungal strains) and mediator choice (HBT and ABTS) (Bourbonnais et al., 1997). It has been demonstrated that laccase in combination with HBT is effective on pulps derived from various fiber sources (hardwood, softwood, and bagasse) and produced with different pulping procedures (kraft of sulfite pulping), resulting in delignification up to 60%. In 1997, the first pilot plant trial with laccase–HBT (Lignozym®-process) on oxygen-delignified kraft pulps achieved 55% delignification (Call and Mücke, 1997). However, the LMS is still to be applied on a large scale. Major impediments are the mediator costs and environmental concerns about potential toxic effects of mediators (Ruiz-Dueñas and Martínez, 2009).

CONCLUDING REMARKS

Lignin presents an abundant component of lignocellulosic biomass, which is currently largely unutilized. It has the potential to replace fossil-based oil as a renewable feedstock for sustainable production of valuable chemicals. The efficient utilization of lignin however requires its depolymerization to LMW phenolics and aromatics that can then serve as the building blocks for chemical syntheses of high-value products. Lignin biodegradation with LMS presents a technological challenge and opportunity that needs further development and optimization.

The ideal laccase should have the following properties: (1) broad substrate specificity; (2) high redox potential; (3) high tolerance to inactivation by radicals, organic solvents, and shearing forces; (4) ability to work with a large number of mediators; (5) broad pH and temperature optima; (6) high enzyme activity and stability; and (7) low production costs. Unfortunately, there is no "universal" laccase that fulfills all above criteria at present, although novel enzymes with improved properties have been obtained. Strategies for further improvements of laccase may include generic engineering and cloning in suitable heterologous hosts for enzyme overproduction, protein engineering to enhance enzyme kinetics and substrate binding, and directed evolution to improve enzyme activity and stability, etc. One of the main targets for improvements is the type 1 copper site, which defines the redox potential of laccases. Modifications in the amino acid composition in the enzyme active site of *T. versicolor* laccase improved enzyme activity and affinity toward larger phenolic substrates (Galli et al., 2011). Substitution of the aromatic amino acids residues with non-aromatic resulted in increased resistance to inactivation by

free radicals (Li et al., 1999). Directed evolution was recently used to: (1) increase laccase activity 170- to 32,000-fold and improve its pH and temperature stability (Bulter et al., 2003; Maté et al., 2010); (2) increase the tolerance of laccase to ethanol and acetonitrile by 30 and 20%, respectively (Alcalde et al., 2005). Recent advancements in non-aqueous enzymology allows improved performance of solvent-tolerant laccases in lignin biodegradation due to the better accessibility to lignin in organic solvents such as dimethyl sulfoxide, cetyltrimethylammonium bromide, ionic liquids, etc. (Weihua and Hongzhang, 2008; Rehmann et al., 2014). In addition, development of effective laccase immobilization systems will decrease the cost of operations and create a more sustainable process (Couto and Herrera, 2007).

Challenges that need to be overcome in the development of laccase mediators include: (1) reduced production costs; (2) use of natural, non-toxic mediators present *in vitro* in lignocellulosic biomass; (3) improved recyclability; (4) high redox potential and ability to effectively oxidize both phenolic and non-phenolic lignin structures; (5) stability of mediator intermediates; (6) lack of mediator side-reactions and consumption in by-products; (7) no inhibitory effect on laccases; (8) ability to form highly efficient LMS with more than one laccase. Nowadays, the central role that LMS can play in lignin biodegradation at the future lignocellulosic biorefineries is increasingly recognized. The potential of LMS to remove lignin from plant biomass could be exploited as an enzymatic pretreatment method in cellulosic ethanol production. This would prevent the formation of inhibitory compounds such as furfural and phenols, which are typically generated during thermochemical pretreatment, and facilitate the enzymatic hydrolysis and fermentation steps of biomass conversion to bioethanol (Jurado et al., 2009).

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