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Bark Valorization for Bio-based Polyphenolic and Polyaromatic Compounds: A Review

Muhammad Ferhan

Abstract

Bark is considered as the outermost layers of stems and roots of woody plants existing outside of the vascular cambium. It mainly consists of the inner and the outer bark. The plants with bark include trees, shrubs, and woody vines. It makes up around 9-15% of the mill log by volume (1). Bark assumes a critical part in a living tree with a complex structure and molecular arrangement. Bark has three primary roles: (a) giving supplement transport from the leaves to the rest of the tree, (b) preventing the inward cambium from drying up, and (c) providing the essential resistance of the tree against out of control conflagration, mechanical wounds brought on by overwhelming wind, and assaults by phytopathogens, phytophagous bugs, bigger beasts, and so on (2). In the wood industry, bark is a residue in forest operations and it is mostly burned as a part of the hog fuel. This review will briefly cover the different aspects of valorization of bark biomass for bio-based Polyphenolic and Polyaromatic Compounds.

Key words: Bark valorization, biobased products, polyphenolics, polyaromatics

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Affiliation of author: *Centre for Biocomposites and Biomaterials Processing (CBBP), Faculty of Forestry, University of Toronto, 33-Willcocks Street, Toronto, ON, Canada, M5S 3B3 E.mail:* muhammad.ferhan@utoronto.ca

Chemical Composition of Bark

Bark has relatively similar chemical composition to wood, yet it contains more extractives, higher lignin content, and less holocellulose (Table 1.1). The bark extractives can be organized into polar and non-polar extractives. The quantity of polar extractives, containing flavonoids, phenolics, glycosides, tannins, sugars, and so on, is typically three to five times more abundant than the non-polar compounds, including waxes, resins, lipids, unsaturated fats, sterols, terpenes, and so forth. Chemical composition of bark can be divided into divisions with distinctive polarity through successive extractions utilizing a combination of organic solvents and hot water. To analyze lignin and polysaccharides, bark is usually first extracted using aqueous alkali solutions; followed by hydrolysis of the extractive-free bark by sulfuric acid to give Klason lignin and acid insoluble residue contents (Hon *et al*., 2000; Harkin *et al*., 1971)

Bark polyphenolics

Lignin

In bark, the polyphenolic part mainly consists of lignin, which is frequently depicted as a complex 3-D polymer having different types of linked phenylpropenoid units. In nature, it has an essential role to encase cellulose chains inside the ultra-structure of plant and wood fiber of the cell walls. Softwood and hardwood lignins may be distinguished by the presence of an extra methoxy group in the ortho-position of phenyl ring in hardwood lignins (Van Langenberg *et al*., 2010).

Mostly guaiacyl units are found in softwood lignin starting from the precursor, trans-coniferyl alcohol (Figure 1.1 (a), whereas hardwood lignin has combined guaiacyl and syringyl units originating from trans-coniferyl and trans-sinapyl alcohols (Figure 1.1 (b). Overall, hardwood bark lignins are mainly composed of syringyl, guaiacyl and small amounts of p-hydroxyphenyl units while softwood bark lignins have quite similar composition of syringyl-guaiacyl ratio but differ in higher proportion of p-hydroxyphenyl units, which come from trans-p-coumaryl alcohol (Figure 1.1 (c) (Hon *et al*., 2000).

. **Table 1.1.** General chemical configuration in wood and bark for hardwoods and softwoods (Harkin *et al*., 1971).

Weight (%)	Softwoods		Hardwoods	
	Wood	Bark	Wood	Bark
Lignin (%)*	25-30	40-55	18-25	40-50
Polysaccharides (%)*	66-72	30-48	74-80	32-45
Extractives (%)	2-9	2-25	2-5	5-10
Ash (%) *	0.2-0.6	Up to 20	0.2-0.6	Up to 20

Figure 1.1: Phenylpropenoid units in lignin precursors

Lignin valorization

Recently, there are significant efforts worldwide in finding higher valued application for lignin, especially lignin from the kraft pulping operation. Some research even focuses on finding favorable genetic variation in local populations of bioenergy crops and direct manipulation of biosynthesis pathways to create lignin feedstocks with favorable properties for recovery and downstream conversion. Refinement of biomass pretreatment advances has further encouraged lignin recovery. Coupled with biotechnology, there is a growing interests in uncovering new uses for this biopolymer, including carbon fibers, plastics and thermoplastic elastomers, polyurethane foams, biofuels, and biopolymers/chemicals as shown in (Figure 1.2) explained by Ragauskas *et al*., (2014).

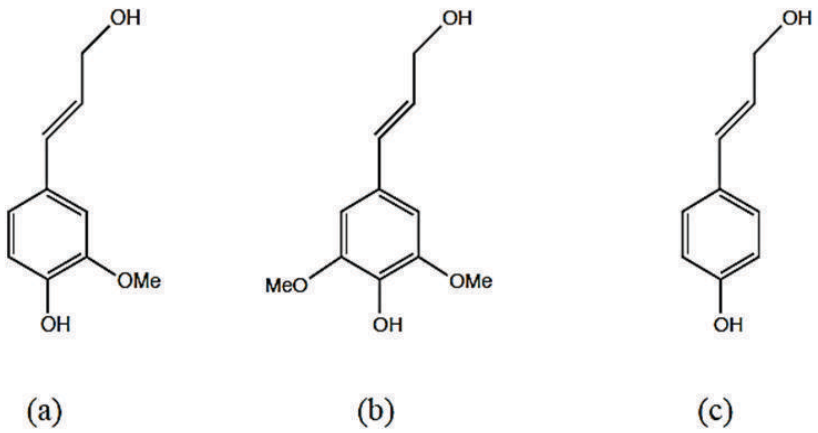
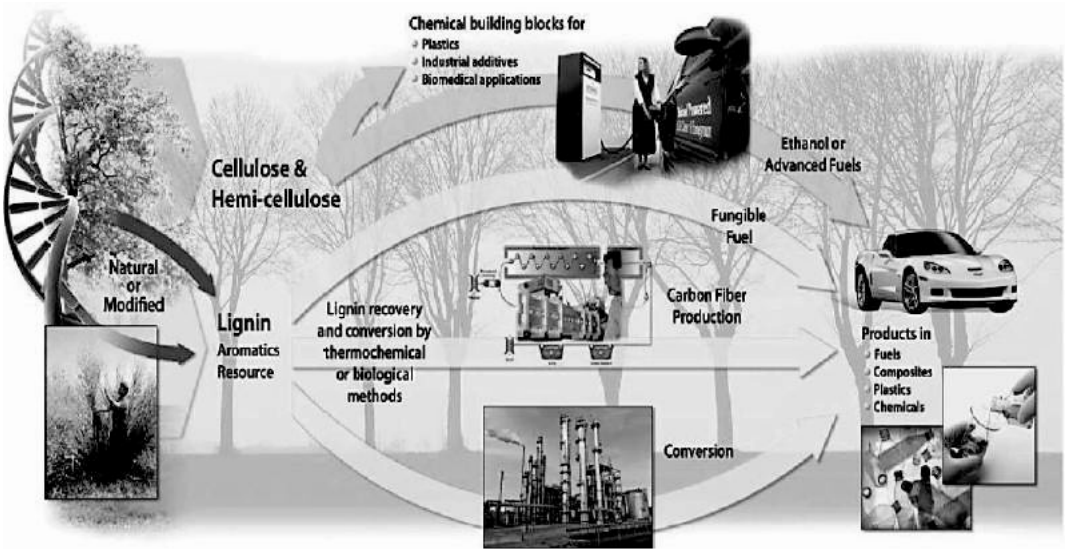


Figure 1.1: Phenylpropenoid units in lignin precursors

Figure 1.2: Conceptual theme of lignin valorization and application of this renewable resource for value-added products by converting into carbon fibers, biopolymers, biochemicals, and biofuels (Source: Oak Ridge National Laboratory, U.S. Department of Energy, Referred by: Ragauskas, Science Reviews, 2014 with permission.)



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Tannins

Tannins, with the molecular weights ranging from 500 to over 3000, are another important type of natural polyphenolic compounds present in a relatively large quantity in coniferous tree barks. The barks of some hardwood species, for example, Quercus, Eucalyptus, Acacia, and Salix also contain a lot of tannin extractives. Tannins can be structured into hydrolysable and condensed tannins based on their configuration and properties (Pizzi 1993; Van Langenberg *et al*., 2010).

Hydrolysable tannins

Polyesters originated from glucose can be considered as hydrolysable tannins, which could be sorted into: (1) gallotannins, which release gallic acid and its related products after acid hydrolysis. (2) ellagitannins, which liberate ellagic and valonic acids upon hydrolysis. Caustic hydrolysis of resorcinolic tannin has been reported to cleave the inter-flavonoid bond and open the etherocyclic ring joining the A and B rings of the flavonoid unit (Figure 1.3). Acid hydrolysis has been shown to easily open the heterocyclic ring of polyflavonoids with the formation of a carbocation, which is capable of reacting with another nucleophile present (Pizzi 1993; Van Langenberg *et al*., 2010).

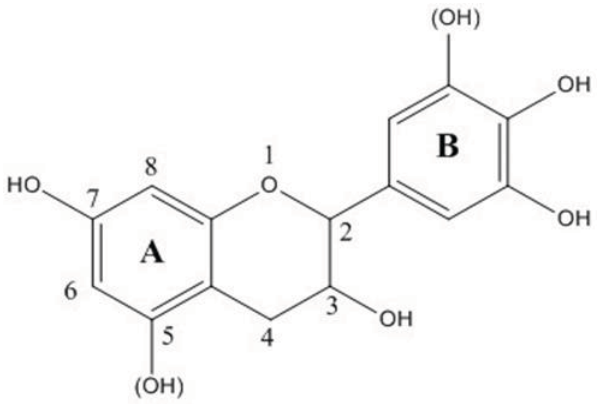


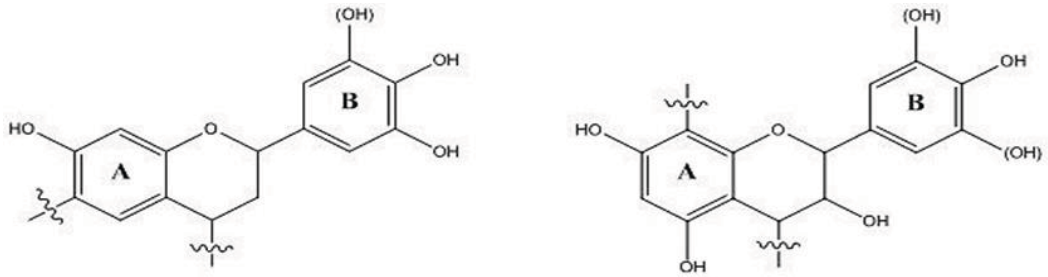
Fig. 1.3. Flavonoid unit

Condensed tannins

Catechins (flavan-3-ols) and leucoantocyanidins (flavan-3,4-diols) recognized as condensed tannins are comprised of flavonoid units. With the average degree of condensation ranging from 4 to 12 flavonoid units, it is commonly present as polymer and does not undergo hydrolysis. The condensed tannins constitute more than 90% of the total world production of commercial tannins.

The main structure of tannin extractives from quebracho, mimosa (black wattle), hemlock and Douglas-fir bark mainly composed of four to six linked flavonoid units where the A-ring is of resorcinol type and B-ring of pyrogallol type units (Figure 1.4), with a few flavonoid units entailing resorcinol A- and catechol B-ring.

In pine (taeda, aleppensis, patula, pinaster, radiata, eliotae, sylvestris, and so forth) species, the flavonoid units are of phloroglucinol A-ring and catechol B-ring (catechin group) mainly connected by four to eight bonds, with flavonoid units of phloroglucinol A-ring and phenol B-ring to a much lesser extent. The structures of the fundamental polymeric constituents of wattle and pine tannins are indicated in Figure 1.4 (Pizzi 1993; Van Langenberg *et al*., 2010).



Wattle tannin unit Pine tannin unit

Figure 1.4: Wattle and pine tannin unit

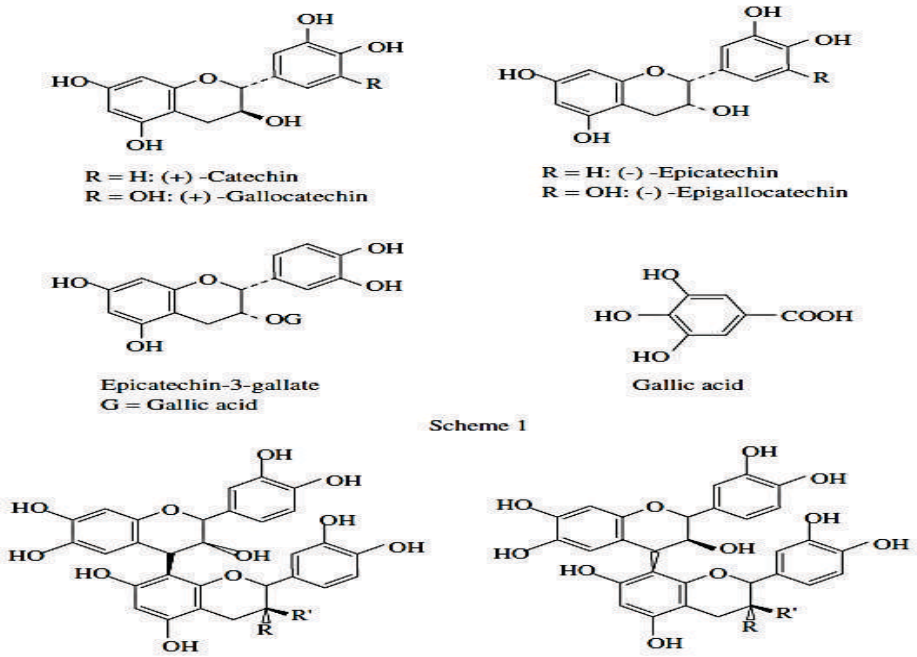
Besides the flavonoid units, non-tannins including carbohydrates, hydrocolloid gums, amino and imino acid fractions also exist in the bark tannin extractives. The hydrocolloid gums with hydrophilicity varying in concentration from 3 to 6% contribute significantly to the viscosity of the extractives despite their low concentrations (Pizzi 1993; Van Langenberg *et al*., 2010).

Challenges in extraction of bark polyphenolics

Polyphenolic substances include many classes of compounds ranging from phenolic acids, colored anthocyanins, simple and complex flavonoids. Similarly, pine bark has low levels of monomers (Shi *et al*., 2005). During extraction, a solvent is blended with the plant material (pine bark). Extraction might be accomplished by the evaporation of a solvent and the solvent could be easily removed either by drying, or ultrafiltration (Shi *et al*., 2005). After any of these methods, the concentrate must be dehydrated to get a powder form and quite a considerable amount of organic solvents is required. Separation of polyphenols by the membrane method is considered more efficient than the organic solvent extraction method. It is important to make effective and productive extraction methods to ensure clean polyphenol product (Shi *et al*., 2005).

Chemical and physical properties of polyphenolics

Two or more monomers are synthetically reinforced to make oligomeric proanthocyanidins. The two proanthocyanidin monomers are catechin and epicatechin. A couple of procyanidins are shown in (Figure 1.5) with the structures of catechins [illegible] molecular structures. Catechin and epicatechin can combine to produce esters, for instance, catechin/epicatechin gallate, comparably, like the bonds between sugars and proteins to make glycosidic and polyphenolic proteins. Around 162 dimers, including, gallic acid and glucose esters that could be made are reported by Bagchi (1999).



Scheme 1

Procyanidin B1: R'=OH, R=H

Procyanidin B2: R'=H, R=OH

Procyanidin B3: R'=OH, R=H

Procyanidin B4: R'=H, R=OH

Fig. 1.5. Structures of major identified polyphenols in bark (Source: Shi, J. *et al*., Food Rev Int, 2005 with permission.)

Polyphenol–protein interactions

Polyphenols interact with protein molecules through hydrophobic or hydrophilic interactions. These interactions lead to formation of soluble or insoluble aggregates that depend on different factors such as pH, temperature and ionic strength. The formations of these aggregates are involved in hydrophobic stacking of aromatic groups of protein and polyphenols, or the interaction between hydroxyl groups of polyphenols with protein chains. Proteins are recognized to play a key role in many physiological activities owing to their stable 3-D structure. Afterwards, unfolding of protein chains, upon binding with polyphenols, is assumed to affect the physiological activity of protein molecules (Bennick, 2002; Naczk *et al*. 2006; Liang *et al*. 2008).

Proteins (dry weight ≥ 33%), linked by hydrogen bonding, can be involved in a variety of supramolecular interactions (Loomis, 1969). Other than hydrogen bonding there are different stable bonds that indicate spatial arrangement of polypeptide′s backbone and its subunits. Distinctive substances react non-enzymatically with compounds, such as o-quinones. The materials having amino, thiol, and activated methylene groups could be polymerized, diminished, or maintained by nucleophilic attack (Pierpoint, 1970). Proteins and polyphenolic compounds in this mode have similar bonding characteristics as the quinine and hydrogen bonding reactions.

Polyphenols are known to form complexes with proteins leading to modifications in the structural, functional and nutritional properties of both compounds. The different parameters such as, temperature, pH, protein type, protein concentration, and the structure of phenolic compounds can affect the protein–phenolic interactions (Ozdal *et al*., 2013). To measure proteins, as, for instance, the haemoglobin, gelatin, and BSA assays, this property of polyphenolic compounds might be utilized.

Several analytical techniques have been developed to characterize the polyphenol–protein complex formation such as fluorescence, circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), Fourier transform infra-red (FTIR) spectroscopy, isothermal titration calorimetry (ITC), and nuclear magnetic resonance (NMR) and mass spectroscopy (ESI-MS) reported by Bandyopadhyay *et al*. (2012).

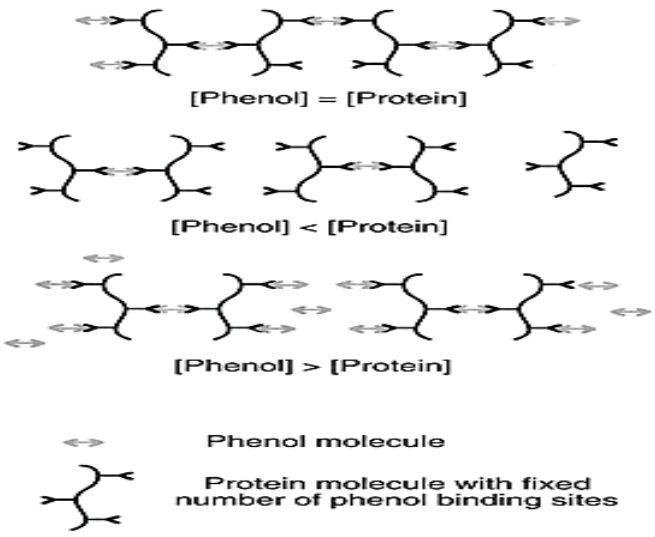


Fig.1.6. A model for protein-polyphenol interactions that elucidates having two sides that can join to protein. Proteins are defined as having a fixed number of polyphenol binding sites (Source: Siebert, K.J *et al*., J. Agric. Food Chem, 1996 with permission.)

A hypothetical model as shown in Figure 1.6 shows that proteins have several sites where polyphenol can bind. In this situation, each polyphenolic molecule should have binding sites where two proteins may attach. However, it is unlikely that there will be enough additional polyphenolic molecules to bridge many of these "sandwiches" or "protein dimers" together. Structure of proteins and polyphenols play a significant role to determine accumulated amount between them.

With an extensive abundance of protein in respect to polyphenol, every polyphenol particle ought to have the capacity to extend between two protein molecules; however it would be unlikely that these proteins would be further connected to others. This would come about for the most part in protein dimers. With excess polyphenol relative to protein, all of the protein binding sites would be occupied, but probably that bridging would occur between polyhydroxyproline contents. Polypeptides with higher percentages of proline tend to form more haze. The amount of haze formed depends both on the concentrations of protein and polyphenol present in most beverage samples (Siebert K.J *et al*., 1996).

Currently, the chemical and biological aspects of proteins and polyphenols are challenging because of their applications in food, agriculture and their potential health benefits.

Interaction of low molecular weight (LMW) phenolics

There have generally been few studies conducted using protein and LMW-polyphenol interactions. Certain protein concentrates from sunflower seeds can create an unwanted brown color because of protein binding with the oxidation products of low molecular weight phenolic compounds, such as, chlorogenic acid (Sastry and Rao 1990). Currently, BSA was examined for its interaction with low molecular weight phenols (Bartolomé *et al*., 2000).

Protocatechuic and caffeic acid showed the most elevated binding for the protein, while p-hydroxybenzoic acid displayed the least binding limit; though, p-coumaric acid and (+)-catechin showed an irrelevant substance for protein- held phenols. The pH and the temperature play important roles in protein-polyphenolics interactions. Phenolic acids with single aromatic rings demonstrated much more significant interaction than multi-aromatic ring isoflavone. BSA-phenolic acid indicated substantial contrast in electrophoretic movement, and displayed total protein when contrasted with BSA (Bartolomé *et al*., 2000).

Biopulping

Biopulping is an industrial biotechnology process which utilizes different microorganisms, especially lignin-degrading fungi and enzymes (ligninases and xylanases) for converting wood chips into paper pulp. Biopulping provides an alternative solution to chemical and mechanical pulping. Ligninolytic enzymes attack lignin and decompose it, while xylanases degrade hemicelluloses and make the pulp more penetrable for the removal of remaining lignin (Ali and Sreekrishnan, 2001). Named 'biopulping', this

methodology displaces lignin as well as a portion of the wood extractives, while reducing the pitch content and effluent toxicity (Ali and Sreekrishnan 2001).

Biotechnological tools are gradually used to replace chemical processes in a wide range of industries. No pilot-scale biopulping plants are in operation right now as this procedure is still in its earliest stages (Ali *et al*., 2001). Mixed hydrolytic and oxidative enzymes have been well documented regarding biopulping and bio-modification of lignin, and thus provide an example which is related to effectiveness, despite the susceptibility of xylanases to deactivate by laccase-generated oxidants (Woolridge, E.M. 2014).

Role of ligninases in pulp delignification

LiP (lignin peroxidase, EC 1.11.1.7) and MnP (manganese peroxidase, EC 1.11.1.7) are Fe+2- containing glycoproteins which necessitate H2O2 as an oxidant. The fungal growth releases a few isoenzymes into their development medium, although the enzymes may also be cell wall bound (Lackner *et al*., 1991). Non-phenolic lignin substructures oxidize LiP to a radical cation followed by a proton loss (Higuchi, 1989).

LiP catalyzes a large variety of reactions e.g., cleavage of -O-4 ether bonds and C -Cβ bonds in dimeric lignin model compounds (Higuchi, 1989). LiP is released during secondary metabolism as response to nitrogen limitation. It is considered as strong oxidizer capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons (PAHs).

However, the degree of lignin biodegradation mainly depends on the environmental conditions and the fungal species involved (Archibald 1992). Previous studies mainly emphasized the mechanism of fungal degradation of lignin.

Oxidation of PAHs by synthetic LMS

The oxidation of polycyclic aromatic compounds (PAHs) was studied in laccase producing fungi *Trametes versicolor* and synthetic mediators were examined by Johannes *et al*. (2000). Enzymatic oxidation of acenaphthene, acenaphthylene, anthracene, and fluorene was mediated by different laccase substrates such as phenols and substituted amines or compounds produced and secreted by white rot fungi. There is an option of using wood-decay fungi able to produce hydrogen peroxide in presence of multi-enzyme systems or as mixed fungal cultures to improve decolorization.

The best natural mediators, such as phenol, aniline, 4-hydroxybenzoic acid, and 4-hydroxybenzyl alcohol were as effective as synthetic compounds e.g., ABTS [2,2⊠azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] and 1-hydroxybenzotriazole. Natural compounds, like methionine, cysteine, glutathione that contain sulfhydryl groups, were also observed as mediator compounds.

Fungal extracellular ligninases

Extracellular ligninases may be categorized as either phenol oxidases (laccase) or heme containing peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)). Commonly, laccases use molecular oxygen as electron acceptors whereas; peroxidases use H2O2 as a co-substrate. White-rot fungi secrete one or more of the lignin-modifying enzymes (LMEs) as well as other compounds required for efficient lignin degradation (Mai *et al*., 2004).

Non-phenolic aromatic substrates have high redox potential ability to catalytically oxidize and cleave C–C bonds and ether (C–O–C) linkages. Manganese peroxidases (MnP) oxidize Mn(II) to Mn(III), to cleave non-phenolic compounds. Combination of LiP and MnP with a bifunctional part was protected by versatile peroxidases (VP) reported by Wong (2009).

The oxidative enzymes prepared for modifying such complicated and mixed structures are required. It was also felt that these enzymes did not require any cofactors, for example, NADPH since extracellular unit of such cofactors seemed, by all reasons, to be inactive. In 1984, Kuwahara *et al*. demonstrated that *Phanerochaete chrysosporium* excreted NADP+ into growth media and hypothesized a reactant part for these cofactors play a main role in delignification.

Hall (1980) suggested that delignification can include non-enzymatic attack on the polymer by "activated oxygen" species; H2O2, superoxide, oxygen, or -OH radicals have been found in lignin degradation. Further studies including ligninases purification by use of different spectroscopy methods, and recombinant DNA molecular techniques, have improved our understanding at the molecular level of microbial delignification.

Relative molecular weight ranges between (41,000 to 42,000) had Fe+2-containing ligninases defined by Kirk and Tien (1983). The glycoprotein non stereospecific (42,000 Mw) was stated by Tien and Kirk as detached from C -Cβ side chain in lignin, which, further oxidized benzyl alcohol to aldehydes or ketones. Therefore, it separated aromatic ring, hydroxylated benzylic methylene groups, and further catalyzed to oxidative coupling of phenols.

Ligninases can similarly catalyze H2O2- related oxidation of heterocyclic sulphur containing compound, thianthrene, to thianthrene monosulfoxide. Kirk and Tien (1983), shown degradation of β-1 and β-O-4 containing model compounds from lignin. Ligninases oxidize an extensive variety of lignin model compounds. Kuwahara *et al*. (1984) additionally proved that molecular oxygen factors in the cleavage of the C -Cβ link of 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy-(4"-methoxyphenyl) propane by ligninolytic enzymes.

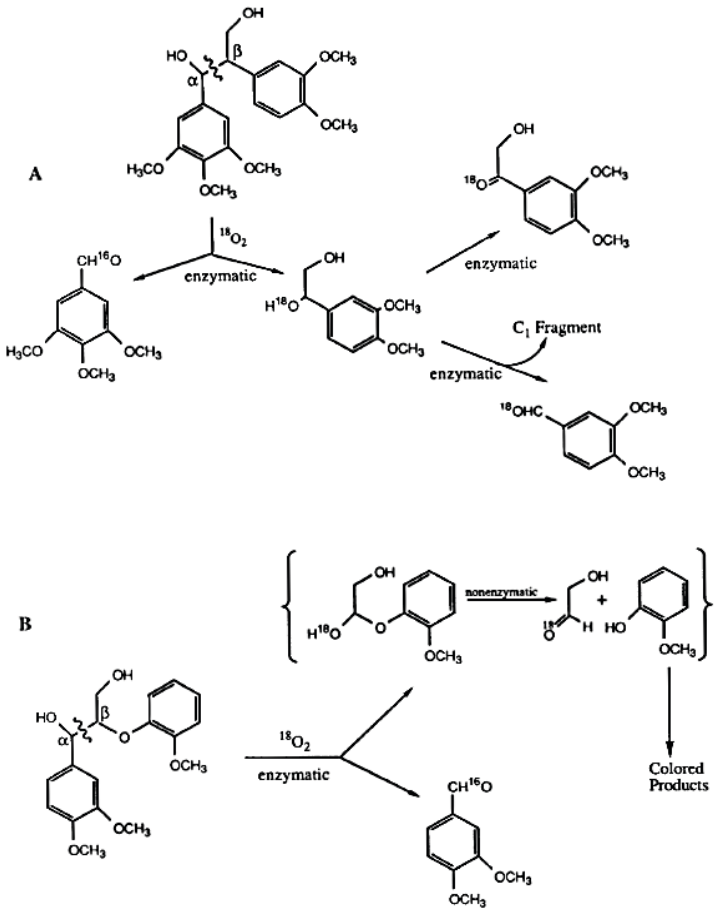


Figure 1.7: Degradation of oligomeric lignin model compounds by P. chrysosporium ligninases (A) β-1 and (B) β-O-4 (Proposed by Tien and Kirk 1984, Proc Narl Acad. Sci. USA., with author permission.)

Phlebia radiata produces lignin-modifying enzymes (LMEs), that showed to be an efficient oxidant producing carbon-carbon bond cleavage of a dimeric, non-phenolic lignin model compound (Lundell *et al*. 1993a). The antigens prepared from these three ligninases (I, II, III) with molecular weights of 42,000, 45,000, and 44,000 cross-reacted with each other. *Pleurotus sajor-caju* produces two flavins containing veratryl alcohol oxidases (Bourbonnais and Paice 1988). The role of the oxidases in biodegradation might be to produce H2O2 and possibly superoxide anion radical (O•2), during oxidation of lignin fragments (Leonowicz *et al*., 1999).

Enzymatic systems employed by microorganisms for oxidative transformation of various organic molecules include laccases, ligninases, tyrosinases, monooxygenases, and dioxygenases. The targeted enzymes are important to the carbon-cycle through either transformation or complete mineralization of organic molecules (Sariaslani, 1989).

Microbial enzymatic systems involved in the oxidation of organic molecules were reviewed. Enzymatic systems such as mono- and dioxygenases, with their inherent stereo- and regio specificities, provide powerful tools for generating chemicals that are difficult to synthesize through conventional chemical routes. Successful commercial applications of these catalysts will be possible through new systems, e.g., immobilization of either integral microorganisms or isolated enzyme preparations on various supports, utilization of organic solvents in the reaction mixtures, and genetic engineering technology (Sariaslani, 1989).

Laccase–Mediator Systems (LMS)

LMS perform as electron shuttles, offering oxidation of complex substrates (for example lignin polymers) where enzymes can't access the active sites due to steric interferences. Once it is oxidized and stabilized, the mediators are dispersed far from enzymatic compartment because of their high redox potential (Bourbonnais and Paice, 1990; Kawai *et al*., 1989). An ideal redox mediator would be a small-size compound which is able to produce stable radicals so it also works in recycling without disintegration (Canas *et al*., 2010).

Previous studies proved that the *Trametes hirsute* laccase with 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS and Remazol blue used as mediators could oxidize nonphenolic lignin parts with high redox potential to produce veratryl alcohol and 1-(3,4-dimethoxy phenyl)-2-(2-methoxy phenoxy) propane-1,3-diol. Furthermore, laccase with ABTS can disintegrate a model lignin dimer, 1-(3,4-dimethoxyphenyl)-2-phenoxyethane-1,2-diol, to yield veratraldehyde and benzaldehyde. The oxidation of ABTS in presence of laccase is shown in Figure 1.7.

The presence of redox mediators is required for a number of biotechnological applications, to assist the oxidation of complex substrates not oxidized by the enzyme alone. However, it should not hinder the enzymatic response (Johannes *et al*., 2000). However, the oxidizing mediator could depend on the oxidation mechanism not on the enzyme, so it is possible to oxidize non-phenolic substrates (Fabbrini *et al*., 2002), subsequently shutting down the sequential catalytic cycle (Figure 1.8).

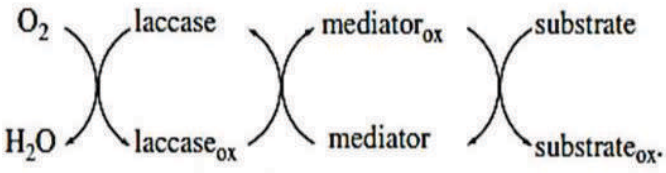


Fig.1.8. The sequential catalytic cycle incorporates a set of several compounds, to meet the condition of several reaction cycles in the redox process.

Distinctive structures of transition segments i.e. (K3[Mo(CN)8].H2O and K3[W(CN)8].H2O) FeII complex with o-phenanthroline and 4,4'-dimethylbipyridine, and some natural laccase substrates, e.g., ABTS and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TMPO). For stable nitroxyl radical, TMPO is different because it is oxidized by laccase and produced oxoammonium ion (oxidation to >N=O+ that correspond to high redox fungal laccase), which follows a non-radical-ionic-mechanism (Fabbrini *et al*., 2002). Conventional redox potentials have well defined transition metal complexes and can oxidize non-phenolic lignin structures for the most part at low concentrations in the reaction mixture.

ABTS is an organic compound suitable for the expression of "redox mediator". Oxidation of non-phenolic lignin structures offers a way to look at new laccase mediators. The use of eco-friendly mediators, can contribute to the industrial implementation of laccases and in the development of lignocellulosic biorefineries. The following N-OH laccase mediators and their structural formulas (A-E) are shown in Figure 1.9.

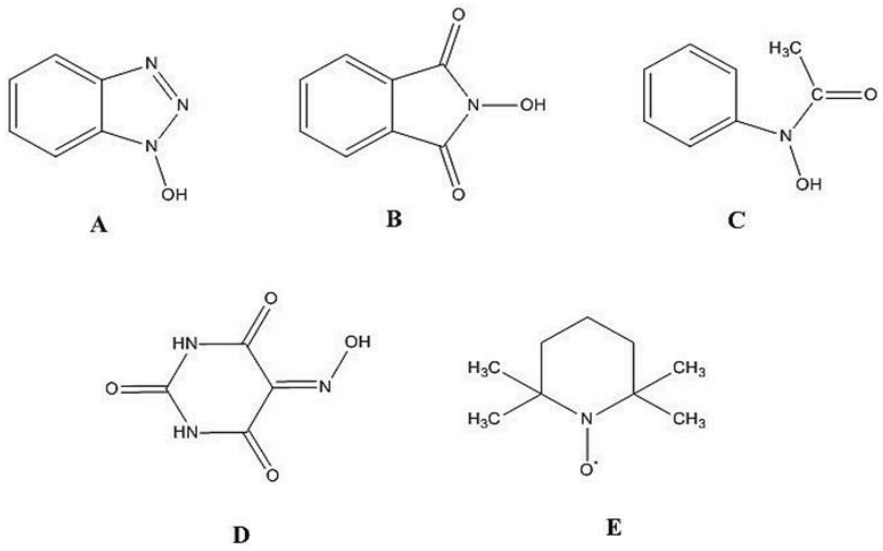


Figure 1.9: N–OH type laccase mediators and their chemical structures (A-E); A=1-Hydroxybenzotriazole (HBT); B= N-Hydroxyphthalimide (HPI); C=N-Hydroxyacetanilide (HAA); D= Violuric acid (VA); E= 2,2,6,6-Tetramethylpiperidin-1-oxyl (TMPO).

Natural mediators / phenolic enhancer

Natural mediators, in presence of laccases, expedite the oxidation of non-phenolics but it depends on the phenolic compound structure, as well as the reactivity vs. stability of phenoxy radicals produced (MS. > AS. > SA.) (Tania *et al*. 2012). Due to the complex nature of biomass, degrading enzymes, and their interactions, researchers are trying to profile radical-coupling routes involved in the development of several phenolic species that are classified as mediators. Moreover, there is a need to improve relationship between biomass structure, effectiveness of enzymatic hydrolysis and the biomass recalcitrance (Foston *et al*., 2012).

In presence of redox mediators, laccases enhance their substrate specificity. The enzymatic oxidation of syringyl-type phenolics identified as natural mediators, such as methyl syringate (MS), acetosyringone (AS) and syringaldehyde (SA), participate in phenolic oxidation depending on the negatively charged residues similar to a substrate binding site of the enzyme.

Biotransformation

Biotransformation is a series of biological modifications / conversions made by a living organism on a chemical compound. In the early 1990's, the oxidation of phenolics by ligninases was investigated for dimethoxylated compounds (veratryl alcohol and 3,4-dimethoxyphenyl acetic acid). In all examples, polyphenolic compounds were found to be exclusively oxidized in contrast with the dimethoxylated compounds. Ligninase catalyzes continuing delignification rather than further polymerization; a model to define the framework is displayed by Harvey *et al*. (1990).

Furthermore, the ligninases, lignin and phenolic lignin are broken down into small fragments and are connected to radical cations as mediators. If the free phenolic compounds are accumulated then eventually it causes inhibition of ligninases, which prevents lignin cleavage and produces more polyphenolics. Hence temporary inhibition of ligninases by phenols may play some role in metabolic regulation of lignin (Harvey *et al*., 1990).

Coupling routes for polyphenolics oxidation

In the laccase mediator system (LMS), the catalytic cycle demonstrated an interchange of paths including phenoxy radical as an intermediate. When they are oxidized in an enzymatic system, a proton and an electron lead to discharge radicals and release phenolic substrates. In order to oxidize non-phenolic compounds by laccases, the phenoxy radicals are involved in three different

possible routes. Moreover, it can either be combined with radical recombination, cross-coupling or self-coupling to continue with or without release of substituent groups which further change into other coupling structures (Figure 1.10).

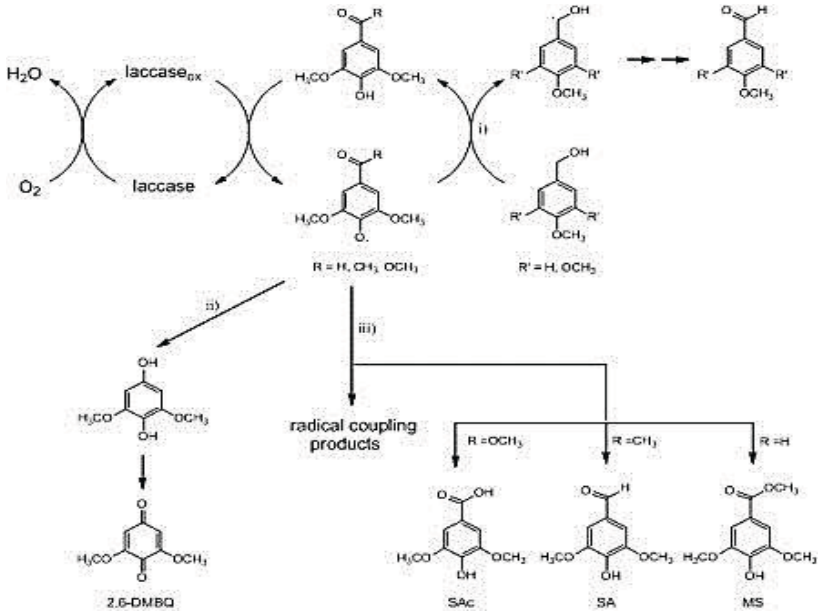


Fig. 1.10. The catalytic cycle of LMS showing different paths implicated in development of several phenolic species (Source: Rosado *et al*., Bioresource Technol, 2012 with permission.)

The newly identified radical coupling routes which involved several C−O coupling reactions, as well as, different dimeric and trimeric structures were proposed (Tania *et al*. 2012). The catalytic cycle supports the demand of added phenolic compounds in laccase mediator system which explains the role of phenolics as ‘‘mediators’’ rather than ‘‘enhancers’’ (Díaz-González *et al*., 2011).

Coupling routes for non-phenolics oxidation

For oxidation of recalcitrant aromatic compounds, the >N−OH mediators, for instance, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VA) or N-hydroxyacetanilide (NHA) (Xu *et al*. 2000) are the possible laccase mediators; their mechanism is shown in (Figure 1.11). Laccase produces an extremely reactive nitroxyl radical (> N−O•), owing to the enzymatic removal of an electron by release of a proton and an oxidation of this type of mediators. The target substrate oxidized nitroxyl radicals by hydrogen atom transfer (HAT) route (Xu *et al*., 2000).

A mediator-to-substrate molar ratio can affect the efficiency of oxidation procedure. The low redox potential substrates could be responsible for the oxidation of non-phenolic substrates under non-catalytic conditions (Cantarella *et al*., 2003).

Function of laccases with combination of natural mediators (NMs) for lignin biodegradation

The phenolic compounds related to phenolic structures in lignin are able to help oxidation of non-phenolic compounds by radical mechanism. This finding may support the role of laccases in lignin biodegradation with no other mediators. Therefore, oxidation of phenolic lignin units with laccases or ligninolytic peroxidases would release phenolic natural mediators as shown in (Figure 1.12) that could help in start oxidation of more recalcitrant non-phenolic lignin moiety (Canas *et al*., 2010).

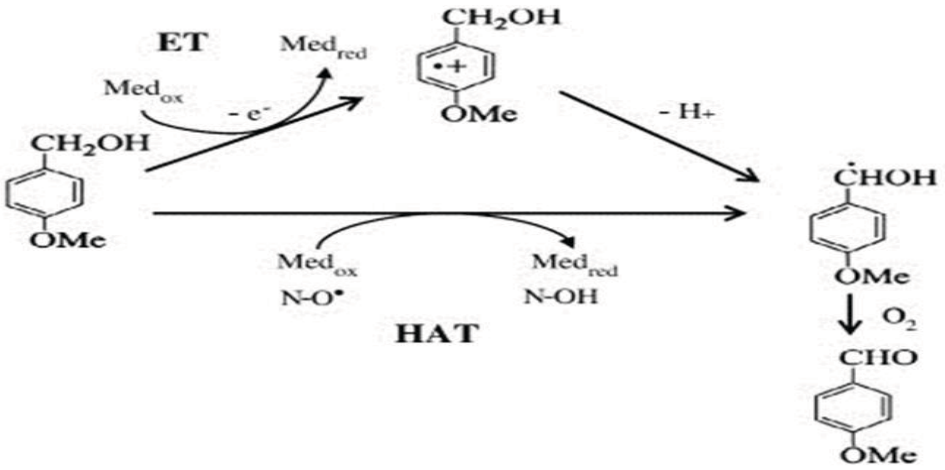


Fig. 1.11. Non-phenolic lignin model compound oxidation by laccase mediator system (LMS) ensuing by two different oxidation methods: ET (electron transfer) and HAT (hydrogen atom transfer) (Source: Fabbrini *et al*., J. Mol. Catal. B: Enzym, 2002 with permission.)

Lignin removal from biomass by using laccases might be achieved by various methods, depending on different types of lignocellulosic material and its further use. Alternatively, the phenolic residues in lignocellulosic material i.e., (p-hydroxycinnamic acids) highly abundant in herbaceous plants, could act as in situ laccase mediators which assist to remove recalcitrant lignin moiety as has already been indicated with model compounds (Cho *et al*., 2008; Nousiainen *et al*., 2009).

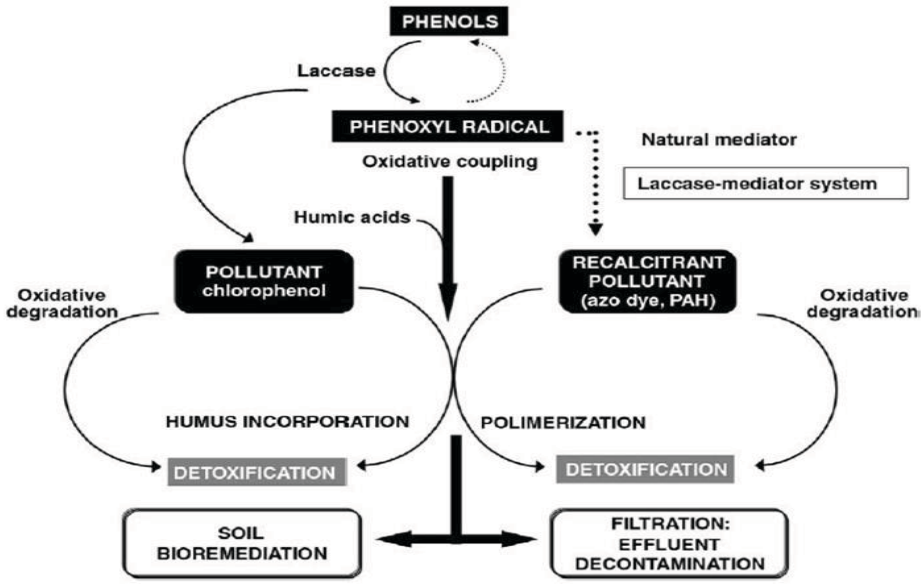


Fig. 1.12. Conceivable function of laccases with combination of phenolic natural mediators (NMs) in decontamination of industrial effluents and soil bioremediation (Source: Cañas *et al*., Biotechnol Adv, 2010 with permission.)

The utilization of laccases and their natural mediators represents a promising alternative for environmentally friendly delignification of paper pulp. These enzymatic frameworks could be incorporated at different phases of biopulping and paper production (Camarero *et al*., 2007). As by-products from pulp production, these natural phenolic mediators can be obtained. Eucalypt kraft pulping black liquor possesses a large amount of some of these phenolic compounds (e.g., acetosyringone and syringaldehyde) which may be sufficiently separated by little efforts from pulp and paper industrial effluents (Camarero *et al*., 2005).

Functionalization of wood and non-wood by laccases has not been completely explored (Chandra and Ragauskas, 2002). Figure 1.13 indicates biotechnological application of laccases (and natural mediators), which can contribute most significantly to develop Integrated Lignocellulosic Forest Products Biorefineries (ILFPB). Wood products (boards and panels) used in furniture manufacturing are bonded with high cost adhesives derived from petroleum. Most of these adhesives are formaldehyde-based resins, due to its harmful emissions to limit the reuse of fiberboards (Felby *et al*., 1997). Therefore, laccases can contribute to reduce (or eliminate) the use of these synthetic adhesives by catalyzing the cross-linking of polyphenolic residues in lignin based materials to produce medium density fiberboards (Canas *et al*., 2010).

Biografting of phenols onto different types of pulps can provide new physico-mechanical properties to paper such as improved strength due to promotion of new hydrogen bonding and crosslinking between phenoxyl groups on pulp surface (Chandra *et al*., 2004). Laccase-assisted grafting of phenols can also modify optical properties like color. Protection against antimicrobial activity can be obtained using an enzymatic method to make cellulosic products for food packaging or cleansing materials (Elegir *et al*., 2008).

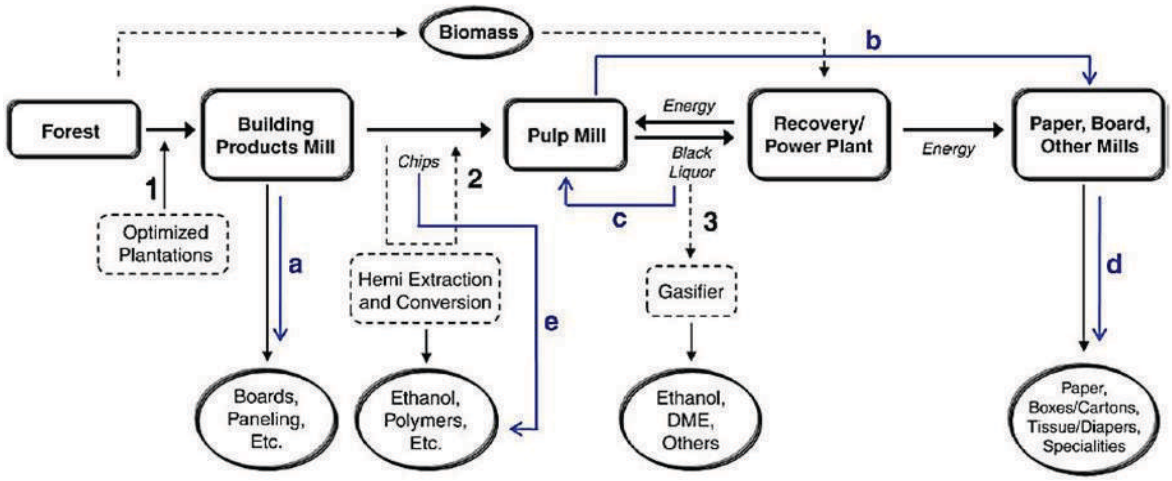


Fig. 1.13. Strategic plan of Integrated Lignocellulosic Forest Products Biorefinery (ILFPB): where, (1) different lignocellulosic reserves; (2) extraction of hemicelluloses to pulping; and (3) biomass gasification. The main features where enzyme technology may help twice as notably to this idea are also shown: (a) assembling of wood (or lignocellulose) products; (b) create of more proficient and environmentally friendly industrial procedures for pulp and paper production; (c) extraction of phenolic mediators and reusing of black liquors; (d) production of cellulosic products with modified characteristics; or (e) enhanced biofuel production (Source: Cañas *et al*., Biotechnol Adv, 2010 with permission.)

In nature, these phenolic compounds (released during lignin depolymerization or present as free acids in plants) perform as natural mediators. Moreover, by the application of these enzymes, sustainable industrial processes are able to move forward by the presence of these environmentally-friendly mediators, easily available from lignocellulosic biomass (Canas *et al*., 2010). Therefore, there are important economical, ecological and environmental reasons for achieving better understanding of fungal activities and their implementation in lignocellulosic green biotechnology (LGB) (Harms *et al*., 2011).

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