LACCASE AND CELLULASE TREATMENTS BOOST THE ANTIOXIDANT ACTIVITY OF MODIFIED LIGNIN

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ABSTRACT

Lignin utilization is gaining significance as it is the most prevalent polyphenol in the environment. Lignin includes numerous polyphenolic compounds, exhibiting potent antioxidant activity. Although the high number of phenolic hydroxyl groups (p\textsuperscript{H}-OH) and low molecular weight (M\textsubscript{w}) of lignin have promoted its applications as a natural antioxidant, the outcomes must be improved to suit diverse prospects. The present review describes in detail the boosting effects of enzymatic treatments with laccase and cellulase on the functional properties of modified lignin, particularly antioxidant potential under environmentally sound technologies. In addition, the mechanism of lignin-enzyme interaction (lignin with laccases and cellulases) is discussed and also described widely used the 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH\textsuperscript{•}) scavenging free radical method to measure the antioxidant activity. Understanding the effects of modified lignin structure on its antioxidant activity is important for developing more beneficial antioxidants and revealing their scavenging mechanisms to realize various applications.

Keywords: Antioxidant Activity, Cellulase, Environmentally Sound Technologies, Laccase, Modified Lignin.

INTRODUCTION

Natural antioxidants are becoming increasingly popular in the food, medical, and chemical sectors because of their ability to neutralize free radicals and prevent oil oxidation.\textsuperscript{1–3} Lignin a common aromatic natural biopolymer\textsuperscript{2} is derived from the wood industry, with annual production estimated at 100 million tons.\textsuperscript{4} As shown in Fig.-1,\textsuperscript{5,6} lignocellulose is a heterogeneous polymer composed primarily of lignin, cellulose, and hemicellulose. These elements act synergistically to generate a porous cell wall structure, which is in a metastable state.\textsuperscript{5} Lignin shows potential antioxidant abilities as a naturally occurring biopolymer with a polyphenol structure.\textsuperscript{7,8} Regular consumption of plant-based foods rich in phenolic compounds has been associated with the prevention of cancer, oxidative insults, depression, inflammation, and neurological and cardiovascular diseases.\textsuperscript{9–11} Common delignification techniques include acid, kraft, and alkali treatments\textsuperscript{12} following which, the antioxidant activity of lignin slightly increases because of its intrinsic recalcitrance and the condensation of its structure. However, these treatments compromise the environmentally sound and cost-effective properties of antioxidants.\textsuperscript{13} In another study, enzymatic treatments\textsuperscript{14} were validated to be suitable for the transformation of lignin into useful substances. Lignin modification requires the application of renewable and affordable biocatalysts.\textsuperscript{8} Some recent studies focused on lignocellulosic biomass treatments using enzymes\textsuperscript{15–17} for their potential industrial applications. Various enzymes, such as pectinase, cellulase, laccase, and glucanase, have been used to release polyphenolics from cell wall matrices and improve their bioaccessibility and bioavailability after consumption.\textsuperscript{18} The antioxidant capabilities of...
lignin are thought to originate from the scavenging action of its phenolic structures on reactive free radicals that contain oxygen. The antioxidant activity of phenolics, alkaloids, flavonoids, carotenoids, and ascorbic acid (vitamin C) has been implicated in oxidative stress defense through the neutralization of hydrogen donors, free radicals, chelating metal cations, and electrons. According to previous research, the ability of lignin's phenolic moieties to scavenge free radicals accounts for the majority of its antioxidant activity. Lignin derivatives possess antioxidant properties because of which their oxidation in the presence of phenol-oxidizing enzymes, such as laccase, yields water. Consequently, laccase enhances the antioxidant potential of lignin derivatives. Meanwhile, cellulase can disintegrate the cell wall matrix, thereby releasing phenolic compounds from their bound forms. Thus, cellulase has been widely utilized to increase the phenolic content of fruit pulps. To this end, the present review summarizes the boosting effects of laccase and cellulase on the antioxidant activity of modified lignin. A brief discussion on the development of lignin, isolation, and chemical modifications was also presented. Therefore, the purpose of this review is discussed as not only exploring the influence of enzymatic treatment on the antioxidant activity of lignin but also modifying lignin with high antioxidant activity, which could be useful in a variety of applications.

![Fig.-1: Composition and Structure of Lignocellulosic Biomass Cell Wall](Image)

**Lignin Structure and Properties**

When lignin was isolated from various plants using the same extraction technique, the physical and structural features varied greatly, explaining the effect of the source on the characteristics of the extracted lignin. As such, the purity level, chemical composition, polydispersity, and molecular weight of lignin vary depending on the source. Additionally, the amorphous heterogeneous structure and common polar molecules of lignin limit its miscibility and compatibility with other materials, including biopolymer composites. Therefore, in studies of lignin-based biopolymers, structural and physical properties, miscibility, and compatibility are important considerations. Lignin is a natural polymerized product of optically active p-hydroxy cinnamyl alcohol monomers and related monolignols generated via oxidative processes. These monolignols are composed of three phenyl propane alcohols, namely synapyl, coniferyl, and p-coumaryl alcohol. In soft-woods, the coniferyl alcohol reaches 95% of the total monolignols. Whereas in hardwoods, synapylic alcohol content is more significant than coniferylic alcohol by 50%. As shown in Fig.-2, lignin polymer is the product of various interunit linkages in the monomers and monolignols [e.g., α-O-4, β-O-4, β-1, β-5, β-β, biphenyl (5-5), and 4-O-5]. Although the structure of lignin varies by species, the major bond i.e. β-O-4 accounts for around 50-80% of total interunit linkages. According to numerous studies, the β-O-4 bond is easier to disintegrate than the C–C bond. Given the abundance of these linkages, various approaches to breaking down lignin have been explored. In addition, lignin possesses a complex chemical structure containing methoxy and hydroxyl functional groups that produce an aromatic ring. Moreover, lignin exhibits potent natural antioxidant properties because of its low molecular weight and abundant phenolic hydroxyl (pH-OH) groups. The emergence of functional groups
during hydrogen donation may prevent oxidation propagation. Diverse renewable sources, including trees and agricultural products, contain lignin. Lignin constitutes one-third of the plant cell wall and is one of the widely used natural polymers. In plants, lignin affects water transport, retains structural integrity, and provides protection from chemical and biological attacks. Therefore, lignin has been used in an array of cosmetic compositions, such as topical formulations. Plant-derived bioactive compounds have garnered much attention in the recent decade, particularly in terms of their benefits to human health. In addition to its conventional applications, lignin, a biopolymer common in nature and with a polyphenol structure, can be employed as an antioxidant. Lignin is an aromatic biomacromolecule constituting 20–35% of the lignocellulosic biomass and has a molecular weight of 10,000 Da. Moreover, it exhibits natural antioxidant activity and excellent thermostability (decomposition temperature > 300°C). As a free radical scavenger, it can control the activity of reactive oxygen species. The antioxidant activity of lignin and other phenolic compounds can be enhanced via the introduction of carboxyl, hydroxyl, and methoxy groups. The structural variability and molecular weight of lignin must be minimized to increase its value. Dissolving the internal ether linkages through depolymerization and oxidation is a strategy to disintegrate lignin complexes into minor aromatic or free phenolic compounds. However, standard lignin modification approaches are incompatible with the current trends toward green chemistry. Consequently, additional environmentally friendly lignin modification treatments are being explored, with a particular focus on unique solvents, microbes, and enzymes, such as ligninolytic oxidoreductases. Here, we review enzyme-mediated hydrolysis of lignin and its antioxidant activity.

Fig. 2: (a) Structures of the Three Units of Lignin, Namely Syringyl Group (S-type), Guaiacyl Group (G-type), and p-Hydroxyphenyl Group (H-type). (b) Types of Inter-Lignin Bonds Comprising the Three Units.

THE ANTIOXIDANT ACTIVITY OF MODIFIED LIGNIN

W.W. Mar et al.
Lignin Isolation and Extraction Methods

To remove lignin, a wide range of plant fibers can be employed. Studies are ongoing worldwide to identify factors important for the optimization of the delignification process. Since the first application of the dioxane–water combination to separate lignin from lignocellulosic biomass, many methods for isolating or separating lignin with a less condensed structure and high purity have been developed. By degrading the lignin–carbohydrate complex (LCC) connections and enabling partial depolymerization, lignin can be separated from carbohydrates via the cleavage of ether links. The major components of lignocellulosic biomass, namely cellulose, lignin, and hemicellulose, can be fractionated through two alternative methods based on their solubility. In the first technique, lignin is retained as an insoluble byproduct after dissolving the carbohydrate components; the Klason method and enzymatic mild acidolysis are included in the first group. The second technique involves the extraction and dissolution of lignin, retaining the carbohydrate components as insoluble residues; lignosulfonate, ionic liquid, and oxidative alkaline wet pretreatments are included in the second group. The four primary commercial procedures used at present to manufacture pure lignin include soda, kraft, sulfite, and organosolv treatments. The process of extracting lignocellulosic biomass via pretreatment procedures is called lignocellulosic biomass extraction. The primary goal of this process is to reduce biomass recalcitrance for the manufacture of chemicals and fuels. A few biomass pretreatment methods using physicochemical, chemical, hybrid, and enzymatic extraction techniques have been developed, and research is underway on the development of additional procedures. These techniques must consider both economic and environmental factors in addition to considering purely technological aspects. The purity of lignin and generation of its constituents are affected by the biomass feedstock as well as the intensity and independence of the pretreatment processes. Even when the same biomass feedstock is utilized, the structure, purity, and efficiency of lignin may vary. To date, lignin has been isolated using diverse enzymatic, chemical, and mechanical approaches. The key steps in lignin isolation using enzymatic approaches are discussed in the following subsections. Enzymatic pretreatment methods for lignocellulosic biomass have attracted the attention of researchers due to their potential features. The major advantages of these techniques include their affordability, environmental friendliness, and high propensity to break down plant cellular components. For instance, cellulases and hemicellulases can be employed to break down polysaccharides into lignocellulose and simultaneously produce a solid lignin-rich residue. This residue contains 50–75% lignin as well as nitrogen, oligosaccharides, proteins, and ash. Furthermore, this type of lignin can be utilized in sorbents, resins, and polymeric compounds. In addition, ball milling, hot water treatment, mechanical refining, and acid and alkaline hydrolysis have been used as pretreatment methods. For instance, biomass ball milling combined with enzymatic digestion can produce cellulolytic enzyme lignin (CEL). In a recent study, CEL was obtained from raw and fungal-pretreated corncob; the authors discovered that lignin separation could be improved following synergistic fungal pretreatment. Pre-treated lignin showed a more linear structure with higher COOH contents, lower phenolic OH contents, higher S/G ratio, and fewer LCC linkages than raw lignin. The three widely used methods of residual lignin isolation and the physical structure, chemical reactivity, and structure of the isolated lignin are described below.

Alkaline Pretreatment of Lignin

Numerous methods, including alcohol lysis, organic solvent fractionation extraction, alkaline extraction, and acid hydrotrropic fractionation, have been explored to enhance the antioxidant potential of lignin. In a previous study, hydrotrropic fractionation of switch grass using maleic acid was applied to obtain carboxylated dissolved lignin, which exerted antioxidant effects. Additionally, solvent extraction and alkaline depolymerization pretreatments have been applied to yield lignin with a lower molecular weight but a higher number of p-H-OH groups, leading to the improvement of its free radical scavenging ability relative to that of previously reported technical lignin. Some recent studies using alkali pretreatment focused on maximizing the alkali loading amount, pretreatment temperature, and pretreatment time and analyzed the structural changes in substrate constituents during pretreatment and their effects on the subsequent enzymatic hydrolysis. Alkali pretreatment produced a greater effect on enzymatic hydrolysis than acid pretreatment, because the former method eliminated the majority of the lignin from the substrate, thereby suppressing the principal inhibitor of hydrolysis. However, the alkali pretreatment reaction lasts.
longer and necessitates a neutralizing process. Alkali pretreatment with hydrogen peroxide is a practical method for achieving high lignin solubilization at ambient pressure and temperature. To reduce biomass recalcitrance, various alkali pretreatments have been developed, including dilute sodium hydroxide, green liquor, kraft pulping, ammonia hydroxide, and ethylenediamine pretreatments. Alkaline extraction with sodium hydroxide is the most popular method for isolating lignin and produces low-molecular-weight hydrophilic lignin. Lignin condensation occurs in an alkaline environment, leading to the formation of new, stable C–C bonds. Figure-3 illustrates the potential pathways of alkali-mediated lignin condensation in phenolic substructures. Nucleophilic assault by C1 or C5 of a higher phenoxide substructure on the α-carbon of quinone methide obtained from the release of hydroxyl ion from the α-position of the phenolic structure produces an α-5 or α-1 type of condensation product, respectively. Typically, the γ-hydroxymethyl group promotes the release of formaldehyde, which may then react with two phenolic guaiacyl units, producing diphenyl–methane complexes. These irreversible condensation reactions inevitably increase the molecular weight of lignin. Because of stronger hydrophobic interactions, high-molecular-weight lignin exhibits more unproductive adsorption with enzymes than low-molecular-weight lignin.

Acid Hydrolysis of Lignin
Extraction with acidic dioxane is a common chemical method of residual lignin isolation. Typically, kraft pulps are extracted with acetone before being subjected to acid hydrolysis. The extracted pulp is refluxed with 0.1 M hydrochloric acid in a 9:1 dioxane: water mixture (azeotrope boiling point = 88°C) in an inert environment, such as in the presence of nitrogen or argon. Lignin is then removed from the solution after solubilization. This process yields residual lignin devoid of impurities and carbohydrates. Contrary to enzymatic hydrolysis, acid hydrolysis is a rapid method to obtain pure residual lignin for further investigations. While acid hydrolysis yields high-purity residual lignin, low yields and the possibility of structural changes brought on by the acidic treatment place restrictions on it. The crude lignin was mixed in 100 mL of a 0.05 mol/L HCl solution in dioxane-water 85:15 v/v and refluxed for two hours under nitrogen after receiving mild enzymatic treatment (azeotrope boiling point 86°C). The lignin solution was recovered by filtering the mixture. Fresh dioxane was used to remove the solid residue until the filtrate was transparent. Solid sodium bicarbonate was used to neutralize the lignin solution, coupled with washing. From the neutralized solution, precipitated lignin was separated by centrifugation and freeze-drying in the indicated volume of acidified water (pH = 2). Dichloromethane was used to wash the lignin and isolate any remaining extractives (3 × 30 mL). Lignin isolation and separation from xylose were done by traditional acidification and hydrothermal acid hydrolysis using sulfuric acid from the pre-hydrolysis liquor (PHL) of kraft pulping. Traditional acidification could remove lignin of 26.8-32.7%, a much lower than hydrothermal acid hydrolysis of 57.1 %. Additionally, the hydrothermal acid hydrolysis also increased xylose content from 7.91 to 13.91-34.82 g/L, respectively. Meanwhile, organic acid hydrolysis is a
potential method for xylooligosaccharides (XOS) production from lignocelluloses.\textsuperscript{26} Theoretically, the breaking of covalent bonds between carbohydrates and lignin initiates the process of lignin removal. In the absence of an acid catalyst, acid hydrolysis could not yield any lignin in the solution, proving the speculated lignin–carbohydrate complex acid hydrolysis process. Certain structural modifications of lignin are expected following acid hydrolysis, such as the dissociation of several α-aryl and α-alkyl ethers as well as of β-aryl ethers in benzyl alcohol units.\textsuperscript{59} Therefore, a higher concentration of ether groups would be available in the wood or pulp as a result of the dissociation of aryl ethers, leading to the formation of p\textsuperscript{1H}-OH groups. Although theoretically possible, lignin condensation in an acidic environment is unlikely to occur during the separation process at least not in the case of residual lignin in kraft pulps.\textsuperscript{59,67}

**Enzymatic Hydrolysis of Lignin**

The enzymes used in this approach of enzymatic hydrolysis can hydrolyze nearly all pulp polysaccharides in a single enzyme treatment. The use of enzymatic hydrolysis to remove remaining lignin has several limitations.\textsuperscript{70} Following enzymatic hydrolysis, the insoluble lignin material is removed using 0.5 M sodium hydroxide, and the soluble part is precipitated using acid to extract the remaining lignin. Little to no water-soluble lignin is present in pulps with kappa values of 93–58; however, in pulps with lower kappa values, 20–60% lignin is dematerialized after enzymatic hydrolysis. Therefore, the residual lignin sample may contain 65–80% lignin and 7–8% carbohydrates as well as impurities, such as unresolved proteins, produced during the enzymatic treatment. Repeated enzymatic or purification treatment of milled wood lignin cannot eliminate the carbohydrates present in the isolated residual lignin. Meanwhile, enzymatic hydrolysis can recover a substantial amount of carbohydrates and proteins from residual lignin,\textsuperscript{71} perhaps because the enzyme fails to hydrolyze the carbohydrate bonds in lignin.\textsuperscript{72} Furthermore, samples of residual lignin produced using this approach contain protein impurities produced during enzymatic hydrolysis.\textsuperscript{26} Earlier, pretreatment of lignocellulosic biomass was essential to loosen its rigid structure and augment the yield of fermentable sugars with enzymatic hydrolysis. Low-moisture anhydrous ammonia (LMAA), a new improved pretreatment agent for corn stover, eliminated the water-intensive washing stage by substituting aqueous ammonia solution with anhydrous ammonia gas.\textsuperscript{73,74} LMAA pretreatment followed by enzymatic hydrolysis transforms the fermentable sugars from accessible carbohydrates in maize stover into a solid lignin-rich residue. Moreover, the LMAA pretreatment technique uses less severe conditions but a significantly longer pretreatment time, often around 24 h, than traditional pretreatment, which causes crystalline cellulose structure swelling and lignin modification.\textsuperscript{75} However, the produced lignin with a reduced molecular weight shows enhanced bioactivity and solubility. Furthermore, enzymatic hydrolysis requires a significantly longer duration than chemical separation methods, such as acid hydrolysis. Moreover, the generation of carbohydrate and protein impurities during enzymatic hydrolysis with currently used methods warrants further structural studies of lignin. Nonetheless, the residual lignin produced using this isolation approach is chemically intact, with substantial yield. In recent years, the economic value of lignin has grown, particularly for producing biofuels.\textsuperscript{51,59,75,76} One of the most significant applications of lignin is as an antioxidant. Lignin harbors ortho-methoxy groups, hydroxyl groups, non-etherified p\textsuperscript{1H}-OH groups, and double bonds between the outermost carbon atoms.\textsuperscript{19,77}

**Laccase Sources and Diversity in the Natural Environment**

Copper-containing oxidoreductases, called laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), show broad substrate precision, conferring biological activity. Laccase is present in vascular plants, most fungi, bacteria, and even insects.\textsuperscript{78–80} A Wide range of laccases play different functions depending on the organism and environmental condition.\textsuperscript{81} In addition, laccases play vital roles in a range of metabolic processes, including plant lignification, fungal pigmentation, humus turnover, lignin biodegradation, and cuticle selenization, and their substrates are low-molecular-weight phenolic compounds and natural fiber polymers.\textsuperscript{82,83} Bacterial laccases are involved in copper ion homeostasis, morphogenesis, brown spore pigmentation, and oxidative damage protection (induced by hydrogen peroxide and ultraviolet radiation) of the spore coat.\textsuperscript{78} Bacterial laccases are present in monomeric (Bacillus subtilis), homotrimeric (Streptomyces griseus), and multimeric (Azospirillum lipoferum) forms, with the molecular weight ranging from 50 to 180 kDa: monomeric (Bacillus subtilis), homotrimeric (Streptomyces griseus), and multimeric (Azospirillum lipoferum).\textsuperscript{84} Furthermore, the information related to enzyme source, their production
condition, and characteristics of bacterial laccase from different microorganisms are summarized in Table-1. One of the most important biopolymers found in plant cell walls, lignin, is polymerized in large part by plant laccases. Overall, plant laccase is made up of between 22 and 45 % monomeric protein and sugar. Plant laccases and peroxidases participate naturally in a complex multi-enzyme system, which is involved in the synthesis of lignin polymers. Monolignols are homomolecularly dimerized by plant laccases, producing homomolecular dimers with C–O or C–C bonding. Plant laccases are essential for the formation of oligomers and polymers because they bind the dimers together. Furthermore, deuteromycetes, ascomycetes, and basidiomycetes produce laccase. In fungi, laccases are involved in spore resistance, pigmentation, and detoxification. White rot fungi are effective lignin degraders and produce the majority of the fungal laccases identified thus far. Pleurotus ostreatus (Jacq.) P. Kumm., Pleurotus pulmonarius (Fr.) Quel., Ganoderma lucidum (Curtis) P. Karst., Botrytis cinerea Pers., Coprinus cinereus (Schaeff.) Gray, Rigidosporus lignosus, Rigidosporus lignosus (Klotzsch) Imazeki, and Trametes versicolor (L.) Lloyd produces laccases. In a previous study, laccase from Pleurotus salmonostreates (Lcc2) was successfully fused with the carbohydrate-binding module (Cbm36) of Geobacillus thermoleovorans IT-08. Further, heterologous expression of the Cbm36–Lcc2 fusion protein was successfully achieved in Escherichia coli BL21 and Saccharomyces cerevisiae BJ1824 host cells. Cbm36–Lcc2 showed increased stability and activity compared with Lcc2 alone. In another study, laccase from oil palm empty fruit bunches (OPEFBs) delignified the surface of biomass from corn cob and rice straw agricultural byproducts. Physical analysis of agricultural waste following fungal laccase treatment indicated damaged and hollow surface profiles (Fig.-4). Delignification of corn cob and rice straw was examined using scanning electron microscopy (SEM), which revealed morphological changes of lignocellulose in the waste samples.

![Fig.-4: Delignification in Control and Laccase-Treated Samples of Corncob (×1000 magnification) (a, b) and Rice Straw (×2000 Magnification) (c, d), as Observed Using Scanning Electron Microscopy. Red Boxes Indicate Holes in Corn Cob and Rice Straw Fibers After Laccase Treatment (size = 10 and 18 µm, respectively)](image)

Table-1: Production Conditions and Characteristics of Bacterial Laccase from Different Microorganisms

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>Carbon source/fermentation conditions</th>
<th>Substrate used in enzyme assay</th>
<th>Optimum Temp. (°C) of activity</th>
<th>Temp. stability</th>
<th>Optimum pH of activity</th>
<th>pH stability</th>
<th>The molecular weight of protein (kDa)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquisalibacillus elongatus</td>
<td>LB/37°C/SF/1 50 rpm/72 h</td>
<td>DMP</td>
<td>40</td>
<td>&gt;80%/ 25-55°C/ 6h</td>
<td>8.0</td>
<td>&gt;40%/ pH 5.0- 10.0/6h</td>
<td>69</td>
<td>90</td>
</tr>
<tr>
<td>Bacillus subtilis MTCC 1039</td>
<td>NB/37°C/SF/ 180 rpm/60h</td>
<td>Guaiacol</td>
<td>30</td>
<td>100%/ 30°C/60 h</td>
<td>5.0</td>
<td>100%/pH 5.0</td>
<td>NR</td>
<td>91</td>
</tr>
<tr>
<td>Bacillus sp. WT</td>
<td>SWNmnedim/3 5°C/SF/150</td>
<td>ABTS</td>
<td>37</td>
<td>&gt;100%/ 70°C/90 min</td>
<td>5.0</td>
<td>NR</td>
<td>180</td>
<td>92</td>
</tr>
</tbody>
</table>
**Structure and Catalytic Mechanism of Laccase**

Laccases are copper-containing enzymes that oxidize a range of compounds, including diphenols, aromatic and aliphatic amines, methoxy-substituted monophenols, and molecular oxygen, reducing these to water. Four electrons are required for the reduction of molecular oxygen to water by laccase activity, and only one electron is required for the oxidation of four hydrogen donor substrates. Four copper atoms—two type 3 (T3) and two type 2 (T2) are clustered to form the active site of the enzyme. The characteristics of the copper ions' electron paramagnetic resonance (EPR) and their spectroscopic features aid in classifying them. Laccases are classified based on the electron paramagnetic resonance (EPR) of copper ions and their spectroscopic features. Significant electron uptake at the covalent copper–cysteine bonds of type 1 (T1) copper ions leads to monoelectronic oxidation of the substrate, conferring the enzyme with its characteristic blue color. Two equatorial histidine residues and one cysteine residue create trigonal interaction with T1 copper. Additionally, the enzyme bears an axial ligand, which is either leucine or phenylalanine in fungal laccases and methionine in bacterial and plant laccases. T1 copper atoms exhibit strong absorption at 600 nm and distinct EPR signals as a result of charge transfer from cysteine sulfur to copper atom. The blue color of the enzyme is a result of this charge transfer. In the trinuclear cluster created by T2 and T3 copper atoms, electron exchange reduces molecular oxygen to water. Two histidine residues coordinate T2 copper atoms. Regardless of the weak light absorption, T2 copper atoms exhibit paramagnetic characteristics based on their EPR spectra. When two T3 copper ions at the active center of laccase are oxidized, they unite to produce a binuclear center without EPR signals and with absorption at 330 nm. The three histidine ligands on each of the T3 copper atoms are connected by a hydroxide bridge, and the distance between each T3 and T2 copper ion is four residues. The T2/T3 trinuclear cluster is located in 12 amino acid residues from the T1 mononuclear site. Four molecules of the appropriate substrate are oxidized to generate four radicals, whereas one molecule of oxygen is reduced to produce two molecules of water, as illustrated in Fig.-5. The catalytic cycle starts with the monoelectronic reduction of a substrate at the T1 site. Consequently, the substrate is oxidized, and the T1 Cu(II) is reduced to Cu(I). Then, electrons are internally transported to the trinuclear T2/T3 copper cluster, where molecular oxygen is transformed to water through the Cys–His route. The reactive radicals can undergo non-enzymatic reactions, such as covalent pairing to form dimers, oligomers, and polymers via C–C, C–O, and C–N bonding, disintegration of larger molecules via the breakdown of covalent bonds, particularly alkyl–aryl bonds (occasionally in the presence of mediators), to release monomers; and breakdown of aromatic compound rings.

**Interaction Mechanisms of Lignin and Laccase**

The fundamental mechanism of the interaction between lignin and laccase remains unknown, and it may vary depending on the experimental conditions as well as the laccase and lignin sources used throughout the enzymatic treatment process. However, molecular docking and molecular dynamic simulation have...
recently been utilized to understand how laccase interacts with lignin compounds due to reliable methods for exploring protein-ligand interactions at the atomic and molecular levels for a variety of applications. Consequently, enzymatic treatment is frequently used to separate lignin fractions with significant antioxidant activity.

Fig.-5: Simplified Reaction Mechanism of the Oxidation of a Suitable Substrate by Laccase Activity; Coniferyl Alcohol is Used as an Example

In a previous study, laccase was used to modify lignin through alkaline hydrolysis; the modified lignin showed a lower molecular weight and higher number of $p^\text{H}$-OH groups than the original lignin. Laccase is a phenol-oxidizing metalloenzyme that is ubiquitous in nature, and it is one of the oxidative enzymes with immense potential for lignin valorization through polymerization or grafting processes. Laccases are particularly significant for lignin valorization because of their specific characteristics and ability to act on polymeric and phenolic aromatic compounds. Depending on the substrate, radical reaction, and mediator conditions, laccases can function as biocatalysts to polymerize or depolymerize lignin and phenols. Laccase activity may break the C–O links of methoxyl groups in lignin, leading to the demethylation of methoxyl groups in lignin units and the formation of a phenol hydroxyl-rich product with a high ortho-hydroxy substitution. Further breakdown of the β-O-4 bonds, the most prevalent type of interunit linkages in lignin, may result in the depolymerization of lignin, as shown in Fig.-6 (Inclusion of laccase in an enzymatic reaction leads to the breakage of β-O-4 bonds. Therefore, demethylation and depolymerization are associated with the number of $p^\text{H}$-OH groups and the molecular weight of lignin. Overall, the source, molecular weight, molecular weight dispersion, and number of $p^\text{H}$-OH groups of lignin determine its antioxidant activity. Low molecular weight and a limited range of molecular weight since the ether linkages in lignin are disrupted, additional phenolic hydroxyl groups are present. The phenolic hydroxyl group of lignin has been discovered to be advantageous, but the carbonyl group is harmful.

Laccase's main advantages are its low environmental impact and diverse variety of substrates. Laccases use $O_2$ as an electron acceptor and only release water as a byproduct, which means they do not put pressure on
the environment, which is crucial to developing a green economy.\textsuperscript{109} Laccase can be employed in a variety of applications thanks to its wide range of potential substrates. However, its activity can be reduced by a limited pH range and the presence of inhibitors. Laccase is not stable in the absence of acidic environments, limiting its use in various foods. Furthermore, Fe\textsuperscript{3+}, Ag\textsuperscript{+}, and Al\textsuperscript{3+} inhibit laccase activity, most possibly by altering its structure. Laccase's catalytic activity is also inhibited by anions such as Cl\textsuperscript{-} and NO\textsuperscript{3-}, but their effects are reversible.\textsuperscript{101}

**Cellulase Sources and Diversity in the Natural Environment**

Endoglucanases (EGs) hydrolyze glycosidic connections at random in the amorphous parts of cellulose, producing oligomers with various degrees of polymerization. Endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) comprise the enzymatic complex that hydrolyzes cellulose. Cellulases can be found in animals, plants, and microorganisms. Cellulolytic complexes can be produced by fungi and bacteria.\textsuperscript{110,111} Cellulase is commercially produced for various uses, such as for paper recycling and de-inking in the paper industry\textsuperscript{112}; for juice extraction and clarification or as a food additive (as thickeners) in the pulp industry; and for the decomposition of impurities to improve soil fertility\textsuperscript{113}, and production of cellulosic bioethanol.\textsuperscript{114,115} Industrial enzymes are commonly produced using microorganisms. Cellulase producers include aerobic bacteria, fungi, such as yeasts, and actinomycetes, which break down cellulose by hydrolyzing the 1,4-glycosidic bonds in it.\textsuperscript{116} In addition, cellulases can be produced by several microorganisms using a wide range of substrates. Several recent studies have successfully produced cellulase from lignocellulosic biomass, primarily agroindustrial byproducts or residues. Solid-state fermentation (SSF) or submerged fermentation (SmF) are commonly used in the fermentation process. Because lignocellulosic fibers are insoluble, SSF has been demonstrated as a usable bioprocess in cellulase production.\textsuperscript{117} It has generally been difficult to advance a bioprocess employing wild-type microorganisms, which do not produce normally the complete enzyme complex. Hence, genetically modified microorganism technology is widely used for producing cellulolytic enzymes. The enzyme activity was increased from 6.32 to 9.20 U/g for β-glucosidase, from 1.60 to 2.20 U/g for Fpase, and from 10.25 to 13.13 U/g for CMCase, implying that mutations could be an interesting technique for increasing cellulase production are shown in Table-2.\textsuperscript{111} Cellulases are classified according to the state of substrate depolymerization. Endoglucanases hydrolyze glycosidic linkages in the amorphous regions of cellulose at random, resulting in oligomers with varying degrees of polymerization. Exoglucanases hydrolyze the β-1,4-glycosidic bonds of the oligomers at the reducing (EC 3.2.1.176) and non-reducing ends (EC 3.2.1.91), producing cellobiose, which is degraded to glucose by β-glucosidases.\textsuperscript{118,119} Cellulases attack the substrate nucleophilically as hydrolytic enzymes by introducing a water molecule. An identical chemical reaction is used by the three different types of enzymes (i.e., EG, exoglucanase, and BG, β-glucosidase) to catalyze acid oxidation.\textsuperscript{111} For instance, aerobic bacteria and fungi produce cellulases that are released into the extracellular medium as free molecules.\textsuperscript{120} In particular, cellulases produced by aerobic bacteria possess a unique structure with two domains: one responsible for binding with cellulose and the other responsible for catalysis; these domains are then joined via a peptide\textsuperscript{121}, and the protein\textsuperscript{111,122} bears additional structures.

**Table-2: Several Cellulase Producing Microorganisms Using the Substrate of Lignocellulosic Biomass**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Biomass-Substrate</th>
<th>Fermentation Process</th>
<th>Activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em> NS-2</td>
<td>Delignified agricultural and kitchen wastes</td>
<td>SSF</td>
<td>Fpase 16 U/g, CMCase 145.7 U/g, β-glucosidase 19 U/g</td>
<td>117</td>
</tr>
<tr>
<td><em>Acremonium strictum</em></td>
<td>Steam-exploded sugarcane bagasse</td>
<td>SmF</td>
<td>Fpase 10.82 U/L, CMCase 139.42 U/L, β-glucosidase 3.48 U/L</td>
<td>123</td>
</tr>
<tr>
<td><em>Curvularia clavavta</em> NZ2</td>
<td>Palm oil empty fruit bunches</td>
<td>SmF</td>
<td>–, CMCase 20 U/L, β-glucosidase –</td>
<td>124</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> A12</td>
<td>Sugarcane bagasse</td>
<td>Sequential SSF and SmF</td>
<td>Fpase –, CMCase 1052 U/L, β-glucosidase –</td>
<td>125</td>
</tr>
</tbody>
</table>
**Structure and Catalytic Mechanism of Cellulase**

For enzymatic hydrolysis of lignocellulose, different cellulase types and compositions use distinct processes. Natural cellulose and lignocellulose structures are broken down and hydrolyzed by the multi-hydrolase enzyme called cellulase. Glycoside hydrolases use acid–base catalysis to dissolve glycosidic bonds, and the two catalytic byproducts of the enzymes, a general acid (proton donor) and a nucleophile/base, catalyze the hydrolysis reaction. Based on the spatial positioning of catalytic residues, hydrolysis occurs via the retention or inversion of the anomeric form. Anomeric C containing the target glycosidic bond maintains the same (substituent) structure to “retain” cellulase following double-displacement hydrolysis with two significant glycosylation/deglycosylation events. However, after single nucleophilic-displacement hydrolysis, “inverted” cellulase flips the structure of anomeric C (substituent) as shown in Fig.-7.\(^\text{110,131}\)

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| **Penicillium brasillianum KUEB15** | Corn stover | SmF | – | 1.180U/mL | – | 126 |
| **Penicillium oxalicum RE-10** | Delignified corn cob and wheat bran | SmF (fed-batch) | 12.69U/mL | 33.38 U/mL | – | 127 |
| **Trichoderma harzianum HZN11** | Sweet sorghum bagasse | SSF | 5.56 U/g | 30.32 U/g | 11.21U/g | 128 |
| **Aspergillus niger RCKH-3** | Wheat bran | SSF | 7.9 U/g | 16.5 U/g | 87.6 U/g | 129 |
| **Trichoderma reseei QM9414** | Pretreated pea hulls | SmF | 0.372 U/mL | – | – | 130 |

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Fig.-7: Cellulase 3D Structure Modelling (PDB 5I2U)

The three structural components of cellulase include a non-catalytic domain/carbohydrate-binding module (CBM), a catalytic domain (CD,) and a linker (which connects to the CBM and CD). Depending on their source, cellulases present different physical and functional characteristics. EG, CBH, and BG are the different types of cellulases with various propensities for lignin. Specifically, exoglucanases release cellobiose or glucose via their processive action at the reducing or nonreducing terminals.\(^\text{119}\) The most extensively studied exoglucanase is CBH. Microorganisms synthesize these enzymes through diverse pathways EG and CBH are both disrupted by cellobiose. Cellobiose and soluble cellodextrins are disintegrated by glucoisidases (e.g., BG) into glucose. Through its active pocket, BG connects with non-reducing glucose units in cellobiose and initiates the hydrolysis reaction while producing a negligible impact on insoluble celluloses. The mechanism of enzymatic hydrolysis by cellulose activity is summarized in Fig.-8.\(^\text{118}\) As opposed to CBH, EG possesses a substrate tunnel and a shorter peptide loop. Consequently, CBH and EG form different lignin connections. In particular, lignin extracted from previously treated substrates has a strong adsorption affinity for CBH.\(^\text{132}\) Although lignin is bound to some cellulases, these enzymes can nevertheless hydrolyze cellulose. According to molecular docking studies, a hydrophobic cleft located across from the catalytic tunnel serves as the active site domain of CBH that is irreversibly adsorbed onto lignin. Except for a slight reduction in mobility, this form produces little effect on the catalytic activity of the enzyme.\(^\text{133}\) The active site of cellulase is far from the hydrophobic plaque of BG based on cellulase characteristics; this long-distance explains the enzymatic activity of BG adsorbed on lignin.\(^\text{134,135}\)
Interaction Mechanisms of Lignin and Cellulase

Nonproductive adsorption is a critical mechanism that modulates the interaction of lignin with enzymes. This consists of three types of interaction: (1). hydrophobic interactions, (2). electrostatic interactions, and (3). hydrogen bonding. Hydrophobic interaction is widely regarded as an essential factor in enzyme adsorption. Meanwhile, an in-depth study is focused on electrostatic interaction, while hydrogen bonding takes part in a minor role. The hydrophobic interaction occurs as a result of ring accumulation, that arises from the enzyme residues of aromatic amino acid and hydrophobic functional groups linked to the lignin surface. Lignin is highly hydrophobic compared to cellulose, therefore, cellulase is more likely to interact with lignin structures, according to the hydrophobic theory. An atomic molecular dynamics simulation containing lignin, cellulose, and cellulases was used to develop the mechanism for lignin inhibition by hydrolyzed cellulose.

Subsequently, TrCel7A comprises a natural cellulase multidomain structure with a large CD attached to a CBM through the flexible linker. The results demonstrated that the amorphous portion of cellulose can extensively cross-link with cellulase. As seen in Fig.-9, the presence of lignin prevents enzymes from entering the part of a hydrophobic chain of crystalline cellulose.

The inhibition activity of lignin on enzyme-mediated hydrolysis has been lessened by the addition of non-catalytic proteins and surfactants. Ionic and non-ionic surfactants, such as urea and polyethylene glycol, are mainly effective in boosting enzymatic hydrolysis efficiency. Surfactants improve cellulase accessibility and activity by binding to lignin or cellulase and inhibiting irreversible enzyme adsorption. The effects of different additives on molecules in various ways would be an interesting topic. Urea increases the effectiveness of enzymatic hydrolysis and decreases unproductive adsorption by severing the hydrogen bonding between lignin and the enzyme. Different chemicals can aid enzyme...
For instance, by lowering the ineffective adsorption of cellulase onto the lignin substrate and by forming lignin–metal complexes, metal ions enhance cellulose hydrolysis. Magnesium chloride can significantly aid the enzymatic hydrolysis of lignocellulose. As shown in Fig.-10, Mg$^{2+}$ ions can bind to the negatively charged lignin substrate, decreasing the electrostatic interaction of cellulase and substrate. Additionally, soy protein can increase the effectiveness of enzymatic hydrolysis by decreasing unproductive lignin adsorption onto cellulase.

![Mg$^{2+}$ blocking drives the lignin surface less negatively charged and debilitates the interaction between cellulase and lignin.](image)

Biotechnological conversion of cellulosic biomass may be a long-term strategy for developing novel bioprocesses and products. Cellulases are produced commercially by numerous companies worldwide and are widely utilized in food and animal feed production, fermentation, agriculture, pulp and paper industries, and textiles. Enzymatic conversion of renewable lignocellulosic materials into biofuels can offer a viable and environmentally sound alternative to fossil fuels. Nevertheless, the economic sustainability of this technique represents one of the greatest barriers to its deployment. Consequently, ongoing research about the bioconversion of lignocellulosic biomass into biofuels is focused on finding practical solutions that can compete with the current methods.

### Enzymatic Treatment of Lignin for Antioxidant Activity

Phenolic units of lignin are disintegrated into low-molecular-weight phenolic compounds under hydrothermal conditions. These phenolic compounds derived from the degradation of lignin possess significant antioxidant activity and can be exploited to produce commercial antioxidants. Therefore, lignin can be employed as a source for antioxidant synthesis. These lignin-derived compounds exhibit excellent antioxidant capability, implying their potential applications as commercial antioxidants. However, the precise antioxidant capacity of lignin cannot be quantified because of its extremely complex and irregular structure, which renders it impossible to estimate the contribution of each of the structural units to the overall activity. To improve the predictive power of these structure–activity correlations, theoretical chemistry data on various phenolic compounds can complement experimental findings.

A combination of experimental and theoretical approaches appears to be particularly well suited to solving lignin chemistry difficulties and developing lignin modification strategies for improving antioxidant activity. Lignin is resistant to oxidation and ultraviolet-induced damage because it comprises many $p^{	ext{II}}$-OH groups. Lignin is a safe, biodegradable, readily accessible, and cost-effective antioxidant compared with other antioxidants. However, lignin cannot be employed as an antioxidant in the polymer industry because of its convoluted history and unknown structure. In this context, lignin treatments using laccase and cellulase aimed at enhancing its antioxidant activity are discussed below. Enzymes that can disintegrate the cell wall matrix can release phenolic compounds from their bound forms, increasing their availability. Accordingly, cellulase is a well-known enzyme with cell-wall-degrading ability and has been employed to increase the phenolic content of pomace. In a previous study, the antioxidant properties of *Adenanthera pavonina* L. seeds were evaluated following enzymatic treatment with cellulase. By increasing the levels of phenolic compounds, specifically phenolic acids, enzymatic treatment with cellulase (1:1, v/v)
maximized the antioxidant activity of seeds. In another study, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays were performed to examine the antioxidant activity of phenolic extracts from oat bran. The total phenolic content, antioxidant potential, and protein oxidation prevention capacity of oat bran were significantly increased after cellulase treatment. Therefore, cellulase can contribute to the production of polyphenol-rich oat products with potent antioxidant activity. In another study, the antioxidant potential of lignin fractions was measured using in vitro DPPH radical scavenging assays, and the antioxidant activity of enzymatically treated lignin fractions and the corresponding untreated lignin was expressed in terms of ionization potential (IP, %). The IP (%) values of enzymatically treated lignin were higher than those of the corresponding native form, indicating that laccase treatment improved the antioxidant potential of lignin samples. According to the literature, the number of pH-OH groups and the molecular weight of lignin are jointly linked to its antioxidant activity. The hydrogen-donating capacity of lignin and the stability of free radical intermediates generated throughout the neutralization reaction may be the primary reasons for the free radical scavenging ability of lignin. The possible pathway for lignin polyphenols involves hydrogen donation from pH-OH to neutralize one DPPH molecule, followed by complexation with one aryl radical to generate an electron pair, as indicated in Fig.-11.

Applications of Modified Lignin as an Antioxidant

Antioxidants are compounds that absorb and neutralize free radicals to overcome the adverse effects of oxygen, and they are frequently used in the pharmaceutical, food, and chemical industries. The presence of abundant functional groups (carboxyl, carbonyl, methoxyl, and pH-OH groups), strong homogeneity, low molecular weight, and negligible carbohydrate impurities contribute to the potent antioxidant activity of lignin. By scavenging free radicals via the activity of its multifunctional side groups, lignin can act as a natural antioxidant. However, several variables, including extraction conditions, pretreatment, source, and chemical alterations, affect the antioxidant activity of lignin. This knowledge has been applied in certain attempts to produce lignin with strong antioxidant activity. For instance, a previous study demonstrated partial 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of lignin with an increase in the concentration of lignosulfonate-grafted copolymers. With the addition of lignosulfonates, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity increased from 0% to 31%, and phenolic content increased from 0 to 0.97 wt%. Hence, lignin has received significant interest because of its unadulterated nature, high safety, non-toxicity, easy availability, and high antioxidant potential. In another study a sustainable strategy based on enzymatic treatment with laccase was used to alter the molecular weight and pH-OH concentration of treated lignin, aimed at the potential improvement of the antioxidant activity of two lignin forms (alkaline lignin and hydrolysis lignin). Compared with untreated lignin, modified lignin showed considerably increased antioxidant activity. Enzymatically processed hydrolysis lignin with the highest pH-OH concentration and the lowest molecular weight showed the maximum antioxidant activity, superior to several commercial antioxidants. Furthermore, the
antioxidant ability of the phenolic extracts was evaluated by the scavenging of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical and protein oxidative damage protection assays after enzymatic treatment of cellulase from oat bran. In comparison to heating-only treatment, cellulase treatment significantly increased the total phenolic content, antioxidant capacity, and ability to protect the protein from oxidative degradation of the oat bran for a variety of applications. Overall, the phenolic reactivity of modified lignin is markedly enhanced, conferring it with antioxidant activity comparable to that of synthetic antioxidants produced from petrochemicals. The modified soda lignin was more effective than the reference synthetic antioxidant in preventing the thermo-oxidation of biopolymer composites. The antioxidant activity of soda lignin from ash trees, black alder, and flax has been investigated. Owing to their decreased heterogeneity and polydispersity and increased concentration of phenolic groups, oligomeric fractions of lignin are considered promising candidates for application as antioxidants. Because of the significant increase in their phenolic reactivity after enzymatic treatment, modified lignins can now compete with synthetic commercial antioxidants derived from fossil fuels in terms of antioxidant activity. Overall, modified lignins are exceptionally promising, environmentally friendly, and biodegradable antioxidants, as evidenced by the greater efficacy of modified lignins than that of synthetic antioxidants in preventing the thermo-oxidation of polyurethane films.

CONCLUSION

Enzyme technology has a lot of potential for modifying the properties of natural plant constituents such as proteins, polysaccharides, lignin, and polyphenols to improve their functionality. This review reveals that the extraction and isolation methods of lignin were thoroughly discussed, with particular attention paid to the use of enzymatic treatment such as laccase and cellulase to improve the functional properties of modified lignin for potential antioxidant activity in various applications. A deep understanding of the structure-antioxidant activity relationship will approve the development of high-antioxidant-capacity lignin, as well as its chemical modifications and broad applications. However, the vast structural heterogeneity, wide range of chemical compositions, and lack of documented structure–activity links, which would allow for the modulation of its antioxidant activity, represent the greatest barriers to the practical use of lignin as an antioxidant. Recent technological advances suggest that lignin molecules can be separated into fractions with low polydispersity and well-defined properties. For instance, solvent fractionation of technical lignin enables the selection of lignin antioxidants by adjusting their molecular mass, polarity, substrate compatibility, phenolic group concentration, and other features. However, commercial phenolic antioxidants can outperform lignin-derived antioxidants even though fractionation can increase their antioxidant reactivity.

ACKNOWLEDGMENTS

The authors express their special thanks to Indonesian Ministry of Education, Culture, Research and Technology and the Proteomic Laboratory, UCoE Research Center for Bio-Molecule Engineering, and Universitas Airlangga for supporting research activity.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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[RJC- 8412/2023]