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Ligninolytic enzymes: a biotechnological alternative for bioethanol production

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Abstract

Ligninolytic fungi and enzymes (i.e., laccase, manganese peroxidase, and lignin peroxidase) have been applied recently in the production of second-generation biofuels. This review contains the analysis of ligninolytic enzymes and their applications in second-generation biofuels. In here, each of the ligninolytic enzymes was described analyzing their structures, catalysis, and reaction mechanism. Additionally, delignification and detoxification, the two most important applications of ligninolytic enzymes, were reviewed and analyzed. The analysis includes an evaluation of the biochemical process, feedstocks, and the ethanol production. This review describes the current situation of the ligninolytic enzymes technology and its future applications in bioethanol industry.

Keywords: Ligninolytic fungi; Bioethanol; Laccases; Manganese peroxidase; Lignin peroxidase; Detoxification; Delignification

Review
Introduction

The United States regulation requires 36 billion gallons of biofuels, and 21 billion should be produced from lignocellulosic material or other new advanced fuels by 2020 [1]. Lignocellulose, the most abundant source of organic material in the world, is a polysaccharide combination of cellulose, hemicellulose, and lignin [2]. Bioethanol is one of the most essential biofuels produced from lignocellulosic material. In general, bioethanol production from lignocellulosic material includes three principal steps: 1) pretreatment, 2) saccharification, and 3) fermentation. The pretreatment process modifies the lignocellulose structure by removing the lignin and altering the cellulose and hemicellulose structure [3]. Saccharification is the enzymatic transformation of cellulose and hemicellulose into monosaccharides as glucose and xylose. Whereas, fermentation is the transformation of these monosaccharides into ethanol. The current pretreatment methodologies utilize energy-intensive processes (high pressures and temperatures) and harsh chemical compounds (NaOH, H_2SO_4). This combination generates undesirable compounds and process inefficiencies. To overcome these issues, biomass

pretreatment has explored more sustainable technologies such ligninolytic enzymes.

In nature, different organisms degrade lignin; however, the most effective ones are the fungi belonging to the white rot fungi class [4]. These microorganisms possess an enzymatic system to effectively degrade lignin. This system contains three principal enzymes laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) [5]. These microorganisms have been used to delignify different substrates such paper, animal feed, and biofuels [3,6–8]. In biofuel production, the ligninolytic enzymes have two principal purposes, delignification and detoxification. Delignification methods apply ligninolytic fungus and their enzymes to reduce the lignin content in several feedstocks [3,5–8]. Detoxification utilizes the ligninolytic enzymes to reduce the toxic compounds present in the biomass hydrolysates after chemical or physicochemical pretreatments [9–14].

The aim of this review is to describe the relevant aspects of the ligninolytic enzymes laccase, MnP, and LiP and to analyze the present and future application of this technology in the production of biofuels.

Ligninolytic enzymes
Laccases

Laccases (E.C. 1.10.3.2) also known as benzenediol: oxygen oxidoreductase or p-diphenol oxidase belong to the
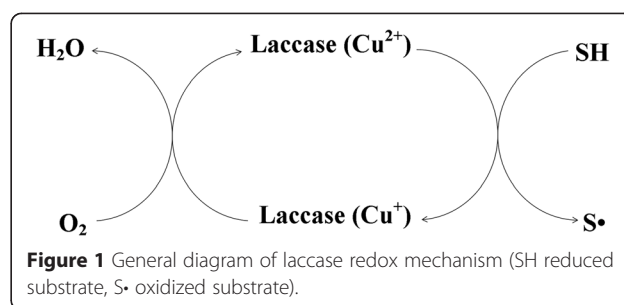
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oxidoreductase class. Yoshida, at the end of the nineteenth century, was the first to extract laccases from exudates of the Japanese tree *Rhus vernicifera*. Laccases are widely distributed in microorganisms, insects, and plants, showing a specific function in each of them. From this group, white rot fungi are the most studied laccases. These fungi use their enzymes to break the plant's lignocellulosic wall and obtain the host's nutrients. These fungal enzymes degrade the complex polyphenol structure that constitutes lignin, the principal recalcitrant component in the lignocellulosic wall. Besides their catalytic characteristics, these enzymes are attractive for biotechnological process, because they are extracellular and inducible, do not need a cofactor, and have low specificity. Laccase production is induced and increased by the addition of molecules as copper, dyes, or other recalcitrant compounds [15]. Laccase employs oxygen as an oxidizing agent and cofactor instead of other expensive cofactors such as pantothenic acid, thiamine, or biotin [16].

Laccases have low substrate specificity; this characteristic allows the degradation of several compounds with a phenolic structure [17]. Therefore, laccases have been employed in several areas such as bioremediation of aromatic recalcitrant compounds [18], treatment of effluents polluted with lignin [19], chemical synthesis [20,21], degradation of a wide number of textile dyes [22–25], and biomass pretreatment for biofuel production [26–28].

Laccase structure and catalytic mechanism Laccases have a primary structure of approximately 500 amino acid residues organized in three consecutive domains, with a Greek key β barrel topology. The three domains are distributed in a first domain with 150 initial amino acids, a second domain between the 150 and 300 residue, and a third domain from the 300 to 500 amino acid. The structure is stabilized by two disulfide bridges localized between domains I and II and between domains I and III [16,29,30]. However, some laccases present three disulfide bridges. *Melanocarpus albomyces* has disulfide bridges inside domain I, another between domain I and domain III, and the last one between domain II and III [31].

Laccase belongs to multicopper oxidases (MCOs) and blue multicopper oxidases [21]. Laccases have four copper (Cu) molecules in their active site which participate in oxygen reduction and water production (Figure 1) [32]. The laccases' four copper atoms are disseminated in three types of cores or places: type 1 Cu (T1), type 2 Cu (T2), and type 3 Cu (T3). These cores are in two metallic active sites: the mononuclear location T1 and the trinuclear location T2/T3 [16,21,29,30]. Type 1 Cu (T1) or blue Cu is a paramagnetic copper with a strong absorption at 600 nm (blue coloration). This signal is generated by the covalent union between Cu-Cys in the mononuclear location [16,33]. In addition, T1 has the

Figure 1 General diagram of laccase redox mechanism (SH reduced substrate, S• oxidized substrate).

highest redox potential in the enzyme and participates in the enzymatic catalysis by oxidizing the substrate [16]. Laccase's oxidative power is affected by the amino acids surrounding T1 [16]. Similar to T1, T2 is a paramagnetic cooper; however, it lacks absorption in the visible region of the spectrum. This Cu exhibits a strong absorption under electron paramagnetic resonance (EPR); technique employed to identify this spot [16]. In addition, the T2/T3 core participates in the inhibition of the enzymatic activity by its interaction with anions such as fluoride or cyanide [34]. On the other hand, T3 Cu is a binuclear core formed by a Cu (II) dimer. This site presents absorption at 330 nm and a lack of EPR signal. Type 3 coppers are diamagnetic and have a Cu-Cu union which participates in the compound oxidation as an electron acceptor.

In laccases, the different types of coppers have variations associated with the coordination between them and the amino acids in the active site. T1 Cu exhibits a different triangular planar coordination similar to other MCOs. Laccases display two histidines (His) and one cysteine (Cys) as equatorial ligands. Meanwhile, the other MCOs have an additional axial ligand with a methionine. In contrast, laccases did not have this axial extra bond, they have a leucine (Leu) or a phenylalanine (Phe) substituting methionine [16,29–31]. On the other hand, T2 Cu exhibits coordination with two His and a water molecule; whereas, T3 Cu interacts with a water molecule and six His. In T3 Cu, the two Cu molecules share the water molecule and split the six His in two groups of three [16,29,30]. The two T3 Cu interact with the oxygen atom; however, the interaction between water and the two Cu at the same time only happens when the enzyme is in its oxidized form [16,29–31,33].

Laccase crystalline structures evidenced that T2 Cu (EPR active) could be any of the three Cu in the trinuclear site. In this site, two of the Cu reduce and become silent to EPR. Meanwhile, the other one remains active to EPR [35]. This interchange between T2 Cu and one of T3 coppers could be related to the reduction mechanism of the two oxygen molecules (cofactor), mechanism which is not clear for blue multicopper oxidases [35].

The enzymes' redox potential oscillates between 300 and 800 mV; these fluctuations depend on different factors

such Cu ligands, distance among Cu, and the second sphere of amino acids in the active site. The last factor is associated with the degradation of recalcitrant compounds as lignin or industrial dyes [16].

Another structural characteristic in laccases is the presence of ligands: monosaccharides (mannose, N-acetyl glucosamine), ions like Ca^{2+}, and organic molecules like glycerol [16,29–31,35]. The presence of these ligands produce significant differences between laccases, an example of that is the glycosylations exhibited in the isoenzymes from *Trametes versicolor* [30].

Heme-peroxidases

After laccases, lignin peroxidase (LiP) and manganese peroxidase (MnP) are the most significant ligninolytic enzymes. These enzymes belong to the heme-proteins because they have the protoporphyrin IX as a prosthetic group. Similar to other heme-peroxidases such cytochrome c peroxidase and horseradish peroxidase, the catalytic cycle of LiP and MnP have three reactions [32,36–39].

Hydrogen peroxide causes the enzyme oxidation to produce the compound I and water:

Reduced peroxidase + $H_2O_2 \rightarrow$ Compound I + H_2O

The modified enzyme (compound I) catalyzes the production of a free radical (S•) and a second modified form of the enzyme (compound II) by an electron transfer from the substrate (SH: reduced substrate):

Compound I + SH $\rightarrow$ Compound II + S•
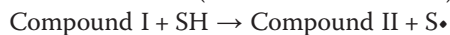
Finally, compound II reacts with a second substrate molecule to produce another free radical and water; meanwhile, the enzyme reduces to its original form.

Compound II + SH $\rightarrow$ Reduced peroxidase + S• + H_2O
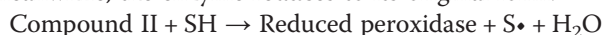
Lignin peroxidase (LiP) LiPs (E.C. 1.11.1.14) were originally discovered in nitrogen- and carbon-limited cultures of *Phanerochaete chrysosporium* [32]. LiP possess high redox potential (700 to 1,400 mV), low optimum pH 3 to 4.5, and the ability to catalyze the degradation of a wide number of aromatic structures such veratryl alcohol (3,4-dimethoxybenzyl) and methoxybenzenes [32,36,38]. LiP oxidizes aromatic rings moderately activated by electron donating substitutes; in contrast, common peroxidases participate in the catalysis of aromatic substrates highly activated (ammine, hydroxyl, etc.). An explanation for this type of catalysis is the production of veratryl alcohol radicals. These radicals have higher redox potential than LiP's compounds I and II and can participate in the degradation of compounds with high redox potential [40].

LiPs are monomeric glycosylated enzymes of 40 kDa, with 343 amino acids residues, 370 water molecules, a heme group, four carbohydrates, and two calcium ions [39]. Their secondary structure is principally helicoidal. It contains eight major helixes, eight minor helixes, and two anti-parallel beta sheets. LiPs contain two domains

at both sides of the heminic group. This group is inlaid in the protein but accessible to solvents via two small channels [39]. The heminic cavity includes 40 residues, and it bonds to the protein via hydrogen bridges. Additionally, the heminic iron (Fe) coordinates with a His and a molecule of water. This His is associated with the high redox potential of LiP. The enzyme's redox potential rises when the His has a reduced imidazol character [39]. In addition, a greater distance between the His and the heminic group increases the redox potential of the enzyme. This increment in the redox potential is a response to the electronic deficiency in the Fe of the porphyrin ring. In fact, this distance causes most differences among enzymes with similar porphyrin cores [39,41].

Another characteristic related with LiP's high redox potential is the invariant presence of a tryptophan residue (Trp171) in the enzymes' surface. Trp171 seems to facilitate electronic transference to the enzyme from substrates that cannot access into the heminic oxidative group [41]. Additionally, Trp171 participates with the catalysis of veratryl alcohol, a metabolite produced by some ligninolytic fungi. Veratryl alcohol participates in the oxidation of different aromatic molecules. Some researchers conceptualized that this alcohol protects the enzyme from the action of H_2O_2 and participate as a redox mediator between the enzyme and substrates which cannot get inside the heminic center [38,39].

Manganese peroxidase (MnP) Kuwahara in 1984 found the first MnP (E.C. 1.11.1.13) in batch cultures of *Phanerochaete chrysosporium* [32]. They are glycoproteins with a molecular weight between 38 and 62.5 kDa [42], approximately 350 amino acid residues, and a 43% of identity with LiP sequence. MnP structure has two domains with the heminic group in the middle, ten major helixes, a minor helix, and five disulfide bridges. One of those bridges participates in the manganese (Mn) bonding site. This site is a characteristic that distinguishes MnP from other peroxidases [37].

The enzyme's catalytic cycle starts with the transference of two electrons from the heminic group to H_2O_2; it produces compound I and water. After that, compound I catalyzes the oxidation of one substrate molecule with the production of a free radical and compound II. Compound II oxidizes Mn^{2+} to produce Mn^{3+}, the cation responsible to oxidize aromatic compounds. It is important to keep in mind that compound II demands Mn^{2+} presence for its reaction; in contrast, compound I can oxidize Mn^{2+} or the substrate. After Mn^{3+} is stabilized by organic acids, it reacts non-specifically with organic molecules by removing an electron and a proton from the substrates [42]. Mn^{3+} is a small size compound with high redox potential, which diffuses easily in the lignified cell wall. Therefore, Mn^{3+} starts the attack inside

the plant cell wall which facilitates the penetration and action of the other enzymes [38,41].

Sources of ligninolytic enzymes

The ligninolytic enzymes are a ubiquitous group of enzymes found in different types of organisms as plants, bacteria, insects, and fungi. In plants, laccases are the most documented ligninolytic enzyme; these are extracellular glycoproteins composed by a monomeric protein with a sugar component. Laccases have been found in different types of plants: the Japanese lacquer tree, mango, mung bean, peach, tobacco, zea mays, etc. [43]. In plants, laccases realize different kind of functions such as lignin synthesis [43], iron oxidation from Fe(II) to Fe(III) [44], and regeneration of injured tissue [43]. In plants, lignin synthesis is laccases' most significant function. In these organisms, they catalyze the monolignol dimerization, the basic molecules of lignin polymers and oligomers [43].

In bacteria, lignin breakdown is exhibited by three groups: actinomycetes, α-proteobacteria, and γ-proteobacteria [45]. These three groups of bacteria were described in insects degrading wood microflora. These types of bacteria showed delignification in both *in vitro* and *in vivo* analyses [45]. *Streptomyces viridosporus*, actinomycetes species, and *Thermobifida fusca* exhibited LiP activities [45–47]. In contrast, *Azospirillum lipoferum*, *Thermus thermophilus*, *Marinomonas mediterranea*, *Bacillus subtilis*, and *Streptomyces cyaneus* showed laccase activity [48–51]. Bacterial laccases are intracellular enzymes with a monomeric, multimeric, or homotrimeric structure without carbohydrate moiety [43]. In nature, bacteria use laccases in spore protection and pigmentation. This type of enzymes exhibited resistance to high pH and temperatures [43,45,52].

In insects, the delignification process is done by ligninolytic enzymes produced by the insects or by the insects' microflora. The ligninolytic enzymes have been identified in different insects and different parts of them. An example of that are *Nephotettix cincticeps* (salivary glands) [53], *Manduca sexta* (Malpighian tubules, midgut, fat body, and epidermis) [54], *Reticulitermes flavipes* (gut) [55], and *Tribolium castaneum* (cuticles) [56]. The principal functions of insect's ligninolytic enzymes are cuticle sclerotization and pigmentation, toxic compound oxidation, and polymerization reactions [43,53].

The fungal ligninolytic enzymes are the most well-known enzymes, and they occur in ascomycetes, basidiomycetes, and deuteromycetes [57]. Inside these groups are several fungal species; however, the most studied fungi are: *Trametes versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Dichomitus squalens*, *Lentinula edodes*, *Irpex lacteus*, and *Cerrena maxima* [32,58]. The principal functions of fungal ligninolytic enzymes are lignin degradation, spore and fruiting body formation, and degradation of plant's toxic compounds [32,57].

Biofuel applications

In the last years, ligninolytic fungi and their enzymes have appeared as a new alternative for pretreatment processes. This technology has been useful principally in two processes, delignification and detoxification. In delignification, ligninolytic fungi or their enzymes have been used to reduce the lignin content in different types of feedstock [3,4,59]. Whereas, in detoxification, ligninolytic enzymes have been used in the removal of chemical compounds from sugar hydrolysates after traditional pretreatments.

Delignification

In delignification process, ligninolytic enzymes have been applied in four different methods: 1) fungal delignification, 2) enzymatic delignification, 3) laccase-mediator system (LMS), and 4) integrated fungal fermentation (IFF). Table 1 resumes the application of these four delignification technologies. Fungal delignification utilizes the complete ligninolytic microorganisms for biomass delignification. In those cases, the microorganisms grow with the target biomass in a submerged culture [8,60] or a solid state fermentation [3,7]. Both cultivations achieved good delignification percentages; however, these were not always related with high glucose yields. The work of Salvachúa et al. (2011) is an example of that; in that case, the fungi with higher glucose yield were different from the ones with the highest delignification percentage. However, the lack of relation between high glucose yield and high delignification percentage occurs in different pretreatments and not only in microbial delignification. In fact, the principal issue with fungal pretreatment is the large duration of the process compared with the other pretreatment technologies [5]. The length of time used by the microorganisms to obtain high delignification percentages is not less than 13 days and can be up to 40 or 50 days [4,8] However, this period will depend on the strain used. The effectiveness of microbial delignification has been improved by adding an alkali treatment previous the fungal pretreatment. The alkali pretreatment benefits were the reduction of the process duration and the increment in the glucose and ethanol yield [61,62].

Enzymatic delignification is the application of enzymatic extracts and purified or semi-purified ligninolytic enzymes to realize the lignin degradation. Enzymatic delignification employs commercial or native ligninolytic enzymes [63]. Laccase is the most utilized enzyme followed by MnP and LiP; nevertheless, mixtures of two or three ligninolytic enzymes have been used for some delignification pretreatments. In those cases, the synergetic relationship between

Table 1 Delignification methods using ligninolytic fungi and ligninolytic enzymes

Feedstock	Fungal strain	Type of process	Mediator	Delignification %	Glucose yield	Ethanol yield	Process duration	Ref.
Wood pulp	*Trametes villosa*	*Enzymatic* Laccase	HBT HPA	50% 39%	-	-	15 h	[81]
Wood pulp	*Aspergillus fumigatus*	*Enzymatic* Laccase	HBT	14%	-	-	2 h	[59]
Hardwood chips	*Trametes versicolor*	*Enzymatic* Laccase	VA	54.2%,	-	-	2 h	[82]
Sugarcane bagasse	*Ceriporiopsis subvermispora*	*Fungal* MnP, LiP, Laccase	-	20%	-	-	30 d	[64]
Eucalyptus globulus	*Myceliophthora thermophila*	*Enzymatic* Laccase	MS	>25%	-	-	12 h	[19]
Paper pulp	*Pycnoporus cinnabarinus*	*Enzymatic* Laccase	AC SA CA HBT	21% 25% −9% 40%	-	-	4-24 h	[69]
Corn stover Switchgrass	*Ceriporiopsis subvermispora*	*Fungal* Laccase	-	>25% >25%	56% 37%	-	18 d	[4]
Ricinus communis	-	*Enzymatic* Laccase	-	85.69%	288.83 mg/g	-	4 h	[83]
Eucalyptus globulus	*Pycnoporus cinnabarinus*	*Enzymatic* Laccase	HBT	27%	-	-	2-4 h	[84]
Wheat straw	*Isolate Euc, Irpex lacteus*	*Fungal* Laccase	-	35% 35%	70% 70%	-	15-46 d	[2]
Wheat straw	*Ceriporiopsis subvermispora Irpex lacteus*	*Fungal* MnP, LiP, Laccase	-	34% 39%	69% 66%	90%	21 d	[3]
Corn stover	*Ceriporiopsis subvermispora1*	*Fungal* MnP Laccase	-	39.2%	66.9%	-	42 d	[7]
Corn stover (cs) Wheat straw (ws)	*Pycnoporus sanguineus H275*	*Fungal* Laccase	- - VA	27% (cs) 25% (ws) 71% (cs) 72% (ws)	-	-	21 d	[8]
Elephant grass (eg) Eucalypt wood (ew)	*Trametes villosa*	LMS	HBT	36% (eg) 58% (ew)	70.7% (eg) 57.8 (ew)	16.2% (eg) 12.3% (ew)	24 h	[72]
Corn stover	*Trametes versicolor*	LMS	HBT	-	31.7%	-	48 h	[70]
Wheat straw	*Sclerotium sp.*	LMS	ABTS	-	82.29%	-	48 h	[71]
Cotton gin trash	*Trametes versicolor*	LMS	PrimaGreen®	15%	23%	31.6%	96 h	[27]
Cotton gin trash	*Trametes versicolor*	LMS	PrimaGreen®	27%	41%	64%	96 h	[28]

Table 1 Delignification methods using ligninolytic fungi and ligninolytic enzymes *(Continued)*

Sugarcane bagasse	Pleurotus ostreatus IBL-02	Enzymatic MnP, LiP, Laccase	-	33.6%	72.9%	16.3 g/L	48 h	[67]
Wheat straw	T. versicolor I. lacteus	Fungal MnP LiP Laccase	-	-	68.33% 56.26%	-	21 d	[85]
Sugarcane bagasse	Phlebia sp MG-60	IFF		30%	-	45%	4 weeks	[86]
Sugarcane bagasse	Phanerochaete chrysosporium Lentinula edode Pleurotus ostreatus	Fungal MnP	-	64%	-	-	12 weeks	[87]
Oil palm empty fruit bunches	Pleurotus floridanus	Fungal		5%	15.4%	27.9%	4 weeks	[88]
Corn stover	Coprinus comatus	IFF		66.5%	82%		5-7 d	[76]
Wheat straw	Irpex lacteus	Fungal MnP Laccase	-	-	84%	74%	14-21 d	[62]
Corn stover	Phanerochete chrysosporium Coridus versicolor	Enzymatic MnP Laccase	-	-	323 mg/g	-	24 h	[66]
Bamboo culms	Punctularia sp.	Fungal		-	10%	-	12 weeks	[89]
Poplar wood	Trametes velutina	Fungal	-		43.6%	22.2%	8 weeks	[90]
Sugarcane bagasse	Pleurotus florida	Fungal MnP Laccase	-	7.91%	303.33 mg/g	-	25 d	[91]
Corn stover	Irpex lacteu	Fungal MnP		25.48%	308.98 mg/g	144.03 mg/g	28 d	[92]
Sugarcane Bagasse	Phlebia sp. MG-60	IFF	-	-	-	44%	4 weeks	[75]
Hardwood kraft pulp, Waste newspaper	Phlebia sp. MG-60	IFF	-	-	-	71.8% 51.1%	68 h	[73]
Sugarcane bagasse	Phlebia sp. MG-60	IFF	-	-	-	65.7%	20 d	[61]
Oak wood	Phlebia sp. MG-60	IFF	-	-	-	43.9%	56 d	[74]

LMS: Laccase-Mediator System, IFF: Integrated fungal fermentation, HBT: 1-Hydroxybenzotriazole, HPA: N-Hydroxyphthalimide, MS: Methyl syringate, VA: Violuric acid, AC: Acetosyringone, SA: Syringaldehyde, CA: p-coumaric acid.

the ligninolytic enzymes improved biomass delignification [64,65]. In wheat straw, ligninolytic enzymes generated a reduction in the cellulose conversion during the saccharification process. To avoid cellulases' inhibition, ligninolytic enzymes were deactivated before the saccharification process [66]. Similar to fungal delignification, alkali pretreatments have been used in enzymatic delignification as an initial pretreatment to facilitate the delignification process [66]. Enzymatic processes achieved similar delignification percentages than microbial pretreatment. However, enzymatic processes employ less amount of time (between 24 and 96 h) to produce the same delignification achieved by fungal pretreatment [67]. Although this methodology reduces the delignification process duration, the current development of enzymatic delignification cannot compete with the conventional pretreatment technologies in terms of timespan and costs [5].

LMS utilize fungal laccases and redox mediators to achieve the delignification process. Redox mediators are chemical compounds, which act as electron carriers between the enzyme and the final substrate (Figure 2) [19]. Mediators' redox potential increases when the enzyme oxidizes them; after that, oxidized mediators react with the final substrate to recuperate the lost electrons. The increment in the redox potential allows the degradation of recalcitrant compounds such as lignin and different kinds of aromatic compounds that the enzyme by itself cannot break [19,68]. The mediator compounds most used are 1-hydroxybenzotriazole (HBT), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and some natural mediators as syringaldehyde or vanillin [69]. The principal applications of laccase-mediator system are bleaching and delignification in the paper industry. However, this system has been used for delignification of some biofuel feedstocks. In cotton gin trash, the delignification was performed by a commercial LMS from Genencor (Rochester, NY, USA). In that case, LMS was used after an ultrasonication and hot water pretreatments [27]. LMS was improved by the addition of alkali-ultrasonication process before the hot water and LMS pretreatment. Alkali-ultrasonication duplicated the ethanol and cellulose yield compared with the previous method [28].

LMS pretreatment was utilized over corn stover by using HBT as mediator [70]. In there, a correlation between laccase loading and enzymatic digestibility was found. Additionally, they found the importance of corn

stover previous silage to increase LMS effectiveness [70]. In wheat straw, LMS pretreatment was used after the steam explosion pretreatment and was performed before and simultaneously with the enzymatic saccharification. The results in both processes were similar. Thus, LMS can participate in a semi-consolidate process for delignification and saccharification [71]. The application of LMS in elephant grass and eucalypt wood is the first report of LMS achieving significant delignification without a previous pretreatment. This improvement was achieved by using four steps of LMS pretreatment and NaOH washing [72]. LMS is less used in biofuels than the paper industry. This lack of applications is related with the small development of specific mediators for feedstock delignification.

Integrated fungal fermentation (IFF) is a consolidated process where a fungus or a group of fungi transform biomass into ethanol without the participation of other treatments or microorganisms [73]. *Phlebia* sp. MG-60 is a white rot fungus with the ability of selectively transforming lignin under solid state fermentation and produce ethanol from delignified biomasses under semi-aerobic submerged fermentation [74]. *Phlebia* sp. MG-60 was selected from a group of other 12 fungi. From this group, *Phlebia* sp. MG-60 was the only one to exhibit the uncommon characteristic of degrade lignin and produce ethanol from cellulose [73]. This fungal strain has been evaluated in the production of ethanol from different feedstocks such as hardwood kraft pulp, waste newspaper, sugarcane bagasse, and hard wood [73–75]. The addition of basal media, organic compounds, and minerals to the culture media increased the ethanol yield and the delignification percentage [75]. *Phlebia* sp. MG-60 produced an ethanol yield between 30% and 70% depending of the type of biomass utilized [61]. The process duration is between 6 and 29 days and is associated with the type of biomass and the presence of pretreatment. The addition of alkali pretreatment generated a reduction in the process timespan from 21 days to 240 h [61]. On the other hand, fungi co-culture is an alternative to generated and integrated process for ethanol production. In this case, ethanol is produced by mixing two types of fungi to develop delignification, saccharification, and fermentation. *Coprinus comatus* and *Trametes reesei* were cultivated to produce ethanol from corn stover. In this case, co-culture achieved greater delignification and cellulose conversion than monoculture [76].

Delignification using ligninolytic enzymes can be upgraded by improving the enzymatic catalysis using protein engineering. This area utilizes three types of approaches to modify ligninolytic enzymes: rational approaches, semi-rational approaches, and directed evolution [77]. Rational approaches are the molecular modification (site direct mutation) of the ligninolytic enzyme sequence using prior structural information as foundation for the

Figure 2 Laccase-mediator system's cycle.
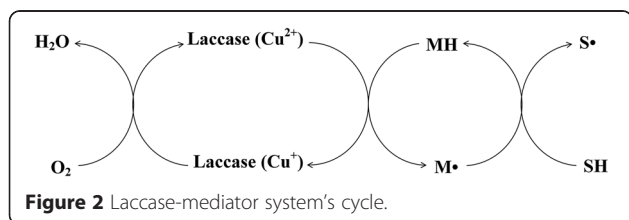
specific sequence modification. Rational approaches have been used to improve the laccase ability to degrade non-phenolic substrates and to enhance the capacity to oxidize bulky phenolic molecules [78,79]. Semi-rational approaches employ saturation mutagenesis to modify hotspot residues in the enzymes. Saturation mutagenesis replaces the selected amino acids by all the codons that can generate the additional 19 amino acids [77]. This approach exhibited the production of enzymes with three- to eightfold higher catalytic efficiencies [80]. Directed molecular evolution is an approach that utilizes random mutation, gene recombination, and selection as fundaments. This molecular approach allows realizing the enzymatic design in absence of structural information. Direct molecular evolution has been used to improve catalytic activity and solvent tolerance [77].

The four delignification processes are compared in Table 2. In enzymatic and microbial process, the pros observed are: positive environmental impact, high delignification and detoxification, low sugar losses, and the possibility of develop a consolidate process. On the other hand, microbial and enzymatic have different cons. The enzymatic processes cons are: high costs, low commercial availability, and mediator necessity. Meanwhile, in microbial processes, the principal limitation is the process duration. Besides the limitations and assets, ligninolytic fungi and their enzymes represent an interesting option to the conventional chemical and physical processes used in biofuel production.

Detoxification

Some of the conventional biomass pretreatment technologies produce toxic compounds after treat biomass. These toxic compounds are classified in four groups: 1) furan derivatives, 2) pentose and hexose degradation, 3) weak acids, and 4) phenolic compounds from lignin [81]. These compounds affect the fermentative microorganisms and the cellulolytic enzymes, which generate an overall ethanol yield reduction [26].

The detoxification process employs chemical, physical, and biological strategies. Chemical and physical techniques utilize technologies such as filtration, anion exchange chromatography, and NaOH precipitation; these techniques are expensive, produce sugar losses, and do not remove all the inhibitors in the hydrolysates [12]. The biological detoxification (Table 3) process employs ligninolytic fungi or their enzymes to reduce the concentration

of toxic compounds in feedstock hydrolysates [14]. The detoxification process is performed after or before the saccharification process and has a duration between 1 to 12 h [11,13,14,93]. Detoxification after or before saccharification is a controversial point. Jurado et al. [93] recommended enzymatic detoxification after cellulose hydrolysis because enzymatic detoxification leads to a reduction in the saccharification process [93]. In contrast, Moreno et al. [26] suggested enzymatic detoxification before saccharification, because it enhances ethanol yield, reduces microbes lag phase, and generates an increment in substrate loading. The authors considered these positive factors more important than the reduction in glucose recovery [26].

From the four types of toxic compounds produced in biomass hydrolysates, phenolic compounds are the most degraded type by ligninolytic enzymes [14,93]. Phenolic compounds inhibited fermentation at concentration of 1 ppm [98]. On the other hand, the other types of toxic compounds exhibit a variable behavior. These compound detoxification is affected by feedstock nature, enzyme characteristics, and pretreatment harshness [26]. Enzymatic detoxification has been applied in the reduction of toxicity after different pretreatments such as steam explosion, strong acids, organosolv, and hot liquid water. In addition, ligninolytic enzyme detoxification increase fermentation rates and ethanol yield [26]. As an example, in sugarcane bagasse, enzymatic detoxification generated an ethanol yield five times greater than ion exchange detoxification [14]. Lee et al. [96] improved the detoxification of rice straw hydrolysates by optimizing the temperature, enzyme concentration, and mediator compounds. The optimization increased the detoxification of phenolic compounds up to 92% by using HBT as mediator [96]. Ligninolytic enzyme detoxification is a future option for biofuel industries. However, this enzymatic option needs to improve their ability to degrade other types of toxic compounds from hydrolysates.

In recent years, enzymatic detoxification of furan derivatives has been evaluated. This type of detoxification is performed by aryl-alcohol oxidase (AAO) which is a less investigated group of ligninolytic enzymes. AAO have been assessed in the degradation of 5-hydroxymethylfurfural (HMF) and polyunsaturated alcohols [99–101]. Carro et al. [101] evaluated the transformation of HMF into 2,5-furandicarboxylic acid (FDCA) by AAO catalysis. AAO

Table 2 Delignification technique comparison

Delignification process	Delignification	Sugar losses	Process duration	Economic	Environmental impact
Enzymatic	High	Low	2 to 48 h	High costs	Positive
Fungal	High	Low	6 to 45 days	Low costs	Positive
LMS	High	Low	2 to 48 h	High costs	Depends of the mediator
IFF	Medium	Some	1 to 12 weeks	Low costs	Positive

Table 3 Feedstock hydrolysates detoxification

Feedstock	Microorganism	Enzyme	Pretreatment	Detoxification	Ethanol yield	Ref.
		Laccase		71%	0.55	
Wood	*Trametes versicolor*	LiP	Steam explosion, 205℃ for 6 min.	53%	0.40	[9]
		Laccase+LiP		66%	0.47	
Spruce chips	*Trametes reesei*	Laccase	H_2SO_4 (0.5%) Steam explosion, 222℃, 23 bar 7 min.	80%	0.47	[10]
				80%	0.43	
Wheat straw	*Trametes versicolor*	Laccase	Hot liquid water at 180℃, 15psi, 30 min.	65%	-	[11]
Sugarcane bagasse	*Trametes versicolor*	Laccase	Steam explosion, 215℃ during 10 min.	80%	0.43	[13]
Sugarcane bagasse	*Cyathus stercoreus*	Laccase	HCl (0.5–3.5% v/v),140℃ for 30 min.	77.5%	0.374	[14]
Wheat straw	*Coriolopsis rigida*	Laccase	H_2SO_4 (1% w/w), 190℃, 12 bars, 10 min.	75%	-	[93]
Wheat straw	*Trametes villosa*	Laccase	Steam explosion, 220℃, 2.5 min.	88-92%	-	[26]
Rice straw	*Coltricia perennis*	Laccase	H_2SO_4 (0.5% w/w), 121℃, 15 min	37-76%	-	[94]
Wheat straw	*Trametes versicolor*	Laccase	Ethanol organosolv process, 220℃ 120 min	20-90%	0.374g/g	[95]
Rice straw	*Yarrowia lipolytica*	Laccase	H_2SO_4 (0.5%, w/w) 5 h, steam explosion 121℃ 30 min	52%		[96]
		LMS		92%		
Wheat straw	*Pycnoporus cinnabarinus*	Laccase	Steam explosion 200℃, 10 min	-	-	[97]

(*Pleurotus eryngii*) transformed efficiently HMF into 2,5-formylfurancarboxylic acid (FFCA); however, it was not capable of transforming directly from HMF into FDCA. To complete the catalysis of HMF, a fungal heme peroxygenase (*Agrocybe aegerita*) was added to the reaction. This enzyme transforms FFCA into FDCA using the H_2O_2 previously generated by AAO [101]. Ferreira et al. [100] determined the ability of AAO to reduce polyunsaturated alcohols. They evaluated 12 different compounds; the best degradations were observed in benzyl alcohol, m-fluorobenzyl alcohol, and p-fluorobenzyl alcohol. The enzyme has the ability to catalyze 'non-activated' alcohols which is an important factor for future detoxification procedures [100]. Other enzymatic process to transform furfurals have been studied; however, these processes utilize non-ligninolytic enzymes from different types of bacteria. In the future, furfural detoxification using ligninolytic enzymes need to be tested in biomass hydrolysates to have a more accurate vision of their real efficiency and applicability.

Conclusions

Ligninolytic fungi and their enzymes exhibited several biophysics and biochemical characteristics that have been applied in the delignification and detoxification of biofuel feedstocks. Ligninolytic processes (enzymatic and fungal) produce ethanol yield, glucose conversion, and delignification and detoxification percentages similar or superior than conventional detoxification and delignification methodologies. Nevertheless, ligninolytic processes have low cellulose modification, the presence of mediators, and long duration process (fungal processes).

The transformation of ligninolytic enzymes as viable option for biofuel industry requires the improvements in the process duration, cost reduction, and more specialized mediators.

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