Int J Biochem Mol Biol 2010;1(1):36-50
www.ijbmb.org /ISSN:2152-4114/IJBMB1004005

Review Article

Fungal biodegradation and enzymatic modification of lignin

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Received April 25, 2010; accepted May 21, 2010; Epub May 23, 2010; Published August 1, 2010

Abstract: Lignin, the most abundant aromatic biopolymer on Earth, is extremely recalcitrant to degradation. By linking to both hemicellulose and cellulose, it creates a barrier to any solutions or enzymes and prevents the penetration of lignocellulolytic enzymes into the interior lignocellulosic structure. Some basidiomycetes white-rot fungi are able to degrade lignin efficiently using a combination of extracellular ligninolytic enzymes, organic acids, mediators and accessory enzymes. This review describes ligninolytic enzyme families produced by these fungi that are involved in wood decay processes, their molecular structures, biochemical properties and the mechanisms of action which render them attractive candidates in biotechnological applications. These enzymes include phenol oxidase (laccase) and heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)]. Accessory enzymes such as H_2O_2-generating oxidases and degradation mechanisms of plant cell-wall components in a non-enzymatic manner by production of free hydroxyl radicals (•OH) are also discussed.

Keywords: Lignocellulose, bioconversion, fungi, lignin, ligninases

1. Lignocellulosic materials

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food. Such wastes are also present in municipal solid waste (MSW), and animal wastes [1-6]. These potentially valuable materials were treated as waste in many countries in the past, and still are today in some developing counties, which raises many environmental concerns [7,8].

Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues in varying degrees based on the source [9]. Cellulose, the major constituent of all plant material and the most abundant organic molecule on Earth, is a linear biopolymer consisting of anhydroglucopyranose-molecules (glucose) connected by β-1,4-glycosidic bonds. Coupling of adjacent cellulose chains via hydrogen bonds, hydrophobic interactions and Van der Waal's forces results in the parallel alignment of crystalline structures known as microfibrils [10]. Unlike cellulose, hemicelluloses are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. The highly variable composition of hemicelluloses is dependent on its plant source [11,12]. Lignin, the second-most abundant biopolymer on Earth and a heterogeneous polymer in lignocellulosic residues, is the only naturally synthesised polymer with an aromatic backbone. It generally contains three precursor aromatic alcohols including coniferyl alcohol, sinapyl and *p*-coumaryl [13]. These precursors form the guaiacyl- (G), syringyl- (S) and *p*-hydroxyphenyl (H) subunits in the lignin molecule, respectively [14]. The subunits ratio, and consequently, the lignin composition, varies

between different plant groups. Oxidative coupling of these lignin aromatic alcohol monomers creates a complex structure in lignin which is highly recalcitrant to degradation [15]. By linking to both hemicelluloses and cellulose, lignin acts as a barrier to any solutions or enzymes and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, of the components of lignocellulosic material, lignin is the most resistant to degradation [9,16]. Although lignin resists attack by most microorganisms, basidiomycetes white-rot fungi, are able to degrade lignin efficiently [15,17].

2. Lignocellulolytic enzyme-producing fungi

Lignocellulolytic enzymes-producing fungi are widespread, and include species from the ascomycetes (e.g. *Trichoderma reesei*) and basidiomycetes phyla such as white-rot (e.g. *Phanerochaete chrysosporium)* and brown-rot fungi (e.g. *Fomitopsis palustris*). In addition, a few anaerobic species (e.g. *Orpinomyces sp.*) are found to be able to degrade cellulose in the gastrointestinal tracts of ruminant animals [18,19]. Biomass degradation by these fungi is performed by complex mixtures of cellulases [20], hemicellulases [18] and ligninases [9,21], reflecting the complexity of the materials. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. Cellulases include endo-acting (endoglucanases) and exo-acting (cellobiohydrolases) enzymes, which behave in a synergistic manner in biomass-degrading microbes. Many microorganisms, including fungi and bacteria, have been found to be capable of degrading cellulose and other plant cell wall fibres. By 1976, over 14,000 fungal species expressing this ability had been isolated, but only a few of them were subjected to in-depth studies [22]. Most fungal strains produce and secrete various lignocellulolytic synergistically-acting enzymes into the environment, thus contributing significantly to the decay of lignocellulosic residues in nature. The breakdown of lignocellulosic biomass involves the formation of long-chain polysaccharides, mainly cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars. In biofuel production, these sugars can be further converted to bioethanol through fermentation processes [23,24].

In nature, efficient lignin degradation during the process of wood decay became possible mainly by basidiomycetes white-rot fungi. Many white-rot fungi simultaneously attack lignin, hemicellulose and cellulose whereas some other white-rot fungi preferentially work on lignin in a selective manner. For example, while *Ceriporiopsis subvermispora* [25], *Phlebia spp*. [26,27], *Physisporinus rivulosus* [28] and *Dichomitus squalens* [27] selectively attack lignin, *Trametes versicolor* [29], *Heterobasidium annosum* [30] , *P. chrysosporium* [9] and *Irpex lacteus* [31] simultaneously degrade all cell wall components. Selective lignin degraders may have significant potential biotechnological applications when the removal of lignin is required to obtain intact cellulose such as in biopulping processes and also in procedures where the main objective is to provide an unprotected carbohydrate for subsequent use (e.g. animal feed and/or biofuel substrate) [32,33]. For example *C. subvermispora,* which lacks cellulase, has been selected for biopulping processes as a selective lignin degrader [32,34].

In contrast to white-rot fungi, brown-rot fungi, such as *Postia placenta*, *Laetiporus portentosus*, *Piptoporus betulinus* and *Gloeophyllum trabeum*, can degrade wood carbohydrates, but not oxidized lignin. As a result, brown-colored rot ensues [14,15]. Ascomycetes are mostly able to degrade cellulose and hemicellulose, while their ability to convert lignin is limited [14]. Plant pathogenic fungi such as *Fusarium solani* f. sp. *glycines* are able to degrade lignin by production of laccase and lignin peroxidase. Lignin degradation by the fungi is suggested to play a role in sudden death syndrome (SDS) in soybean [35].

2.1. Fungal extracellular ligninases

Fungi degrade lignin by secreting enzymes collectively termed “ligninases”. Ligninases can be classified as either phenol oxidases (laccase) or heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)] (**Table 1**) [14]. In general, laccases use molecular oxygen as electron acceptors while peroxidases use hydrogen peroxide as a co-substrate [33]. White-rot fungi variously secrete one or more of the lignin-modifying enzymes (LMEs) in addition to other compounds necessary for effective lignin degradation (discussed in section 2.2.) [36]. It has been

Table 1. The features of the main two groups of fungal ligninolytic enzymes

Type of enzymes	Reaction[1]	Cofactor	Metals or ions[2]	Mediators	Subunits & molecular mass (kDa)	Range of optimum temperature (°C)	Range of pH optimum	Localization	Glycosylation	References
Phenol oxidase (laccase)	4 benzenediol + O_2 = 4 benzosemiquinone + 2 H_2O	N/A	Ca^{2+} Cd^{2+} Cu^{2+} H_2O_2 Imidazole K^+ K_2SO_4 Mn^{2+} Na_2SO_4 $(NH_4)_2SO_4$	Phenols, aniline, 3-HAA, NHA, syringaldehyde, hydroxybenzotriazole and ABTS	Monomeric (43-100), dimeric, trimeric & oligomeric (e.g. tetramers with ~390 KDa)	20-80	2-10	Mostly extracellular[3]	Yes (*N*-glycosylated)[4]	[36,44,45,68,84,131]
Peroxidases:										
a) Lignin peroxidase (LiP)	1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 = 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O	Heme	Iron	Veratryl alcohol	Monomeric (37-50)	35-55	1-5	extracellular	Yes (*N*-glycosylated)[4]	[36,68,84,132]
b) Manganese peroxidase (MnP)	2Mn(II) + 2H^+ + H_2O_2 = 2Mn(III) + 2H_2O	Heme	Ca^{2+} Cd^{2+} Mn^{2+} Sm^{3+}	Organic acid as chelators, thiols, unsaturated fatty acids	Monomeric (32-62.5)	30-60	2.5-6.8	extracellular	Yes (*N*-glycosylated)[4]	[36,68,84,133,134]
c) Versatile peroxidase (VP)	donor + H_2O_2 = oxidized donor + 2H_2O (e.g. reactive black 5 + H_2O_2 = oxidized reactive black 5 + 2H_2O)	Heme	Mn^{2+} Ca^{2+} Cu^{2+} Iron	Veratryl alcohol, compounds similar to LiP and MnP mediators	Monomeric	Not known	3-5	extracellular	Yes	[68,101,135]

[1]General reactions. [2]Differnet enzymes from different species need different metal(s) or ion(s). [3]Fungal laccases are mostly extracellular enzymes but cytoplacmic or intracellular laccases were also found especially in plants and bacteria [45]. [4]Glycosylation degree varies between different fungal ligninolytic enzymes [68]. N/A: not applicable; 3-HAA: 3-hydroxyanthranilic acid; NHA: *N*-hydroxyacetanilide; ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate).

shown that *P. chrysosporium* produces several LiP and MnP isoenzymes but no laccase [37]. Correspondingly, the genome of *P. chrysosporium* contains ten LiP and five MnP genes [38]. In addition, H_2O_2-generating enzyme, glyoxal oxidase (GLOX) has been found in *P. chrysosporium* cultures [39]. White-rot basidiomycetes, such as *Coriolus versicolor* [40], *P. chrysosporium* and *T. versicolor* [41], have been found to be the most efficient lignin-degrading microorganisms studied. Although LiP is able to oxidize the non-phenolic part of lignin (which forms 80-90% of lignin composition), it is absent from many lignin degrading fungi [40]. In addition, electron microscopy studies of the early stages of the fungal degradation of wood have shown that oxidative ligninolytic enzymes are too large to penetrate into the wood cell wall micropores [42]. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds must initiate changes to the lignin structure (as discussed below) [42,43]. **Figure 1** summarized the major steps and enzymes involved in lignin degradation by basidiomycetes white-rot fungi.

2.1.1. Phenol oxidases (laccases) (benzenediol:oxygen oxidoreductases, EC 1.10.3.2)

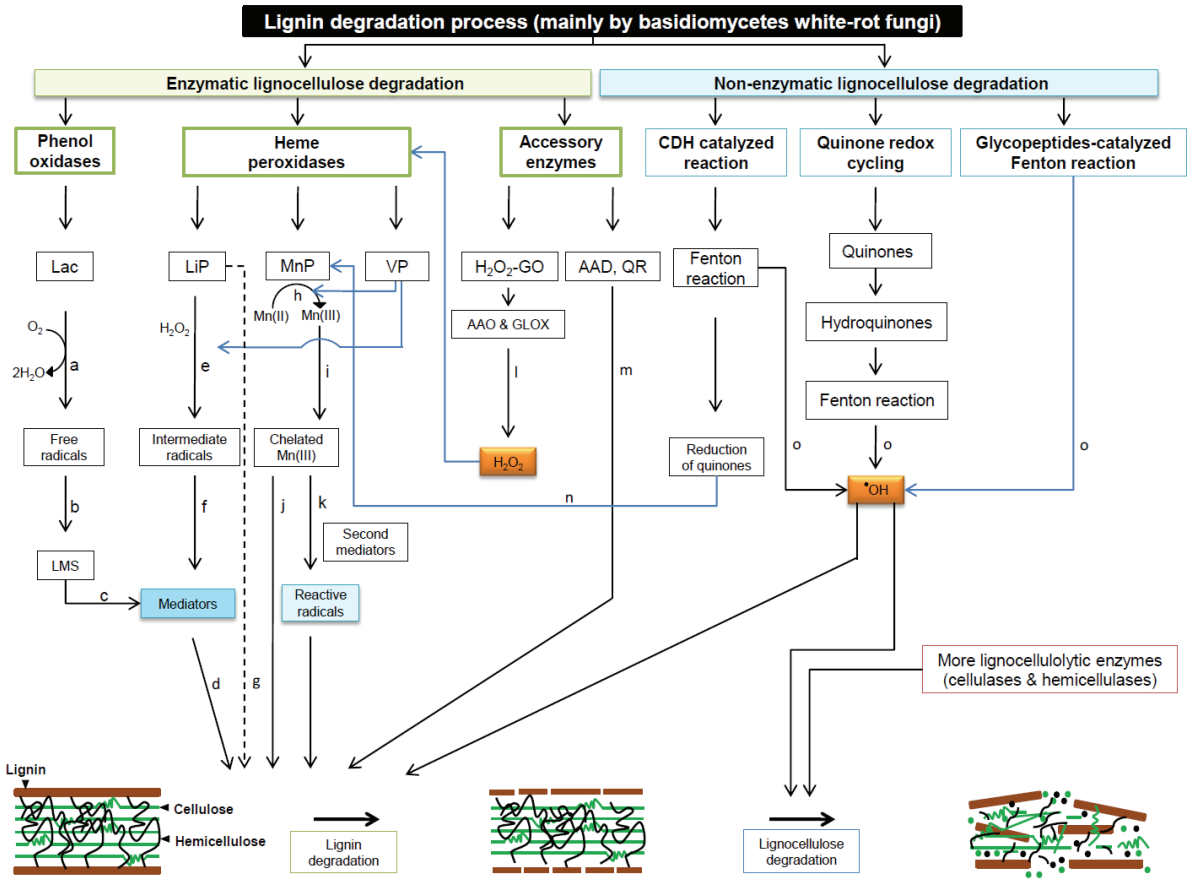
Laccases are glycosylated blue multi-copper oxidoreductases (BMCO) that use molecular oxygen to oxidize various aromatic and non-aromatic compounds through a radical-catalyzed reaction mechanism (**Table 1**) [44,45]. Laccases couple the electron reduction of dioxygen into two molecules of water with the oxidation of a vast variety of substrates, such as phenols, arylamines, anilines, thiols and lignins (**Figure 1, a**) [46]. Four copper ions in their catalytic center mediate the redox process. These are classified as being type-1 (T1), type-2 (T2) and two type-3 (T3 and T3'), based on the copper's coordination and spectroscopic properties [47]. The oxidation reactions catalyzed by laccases lead to the formation of free radicals which act as intermediate substrates for the enzymes (**Figure 1, b**) [48]. These mediators can leave the enzyme site and react with a broad range of high-redox potential substrates and thus create non enzymatic routes of oxidative polymerizing or depolymerizing reactions (**Figure 1, c**). Ultimately, laccase-mediator system (LMS) becomes involved in a range of physiological functions such as lignolysis (**Figure 1, d**), lignin synthesis, morphogenesis, pathogenesis and detoxification [49].

Initially discovered in the Japanese lacquer tree *Rhus vernicifera* [50], laccases have since been found in many other plants and insects [44]. However, for the most part, laccases have been found and studied in white-rot fungi, such as *Lentinus tigrinus* [48], *Pleurotus ostreatus* D1 [51], *Cerrena unicolor* strain 137 [52], *T. versicolor* [53], *Trametes* sp. strain AH28-2 [54], *Trametes pubescens* [55] and *Cyathus bulleri* [56]. Laccase production using a liquid culture has also been reported in brown-rot fungi, including *Coniophora puteana* [57]. Also, ascomycetes such as *Melanocarpus albomyces* [58], *Chaetomium thermophile* [59], *Magnaporthe grisea* [60], *Myrothecium verrucaria* 24G-4 [61] and *Neurospora crassa* [62] are be able to produce laccases. In addition by application of gene-specific PCR primers, laccase genes were detected in a few different fungal species including *Pycnoporus cinnabarinus*, *Pycnoporus coccineus*, *Pycnoporus sanguineus*, *Cyathus* sp. and also in xylariaceous ascomycetes *Xylaria* sp. and *Hypoxylon* sp. [63]. Interestingly, laccases were also detected in some bacteria such as *Bacillus subtilis* [64], while other bacteria, like *Streptomyces griseus* [65], produce a laccase-like phenol oxidase. Also, laccase genes were detected in bacteria such as *Bacillus licheniformis* [66] and *B. subtilis* [67]. For extensive information please refer to the following website http://www.brenda-enzymes.org [68].

As laccases work efficiently on a broad range of substrates without cofactors, they may have significant value in many biotechnological applications, such as pulp bio-bleaching [69], biosensors [70], food industries [71], textile industries [72], soil bioremediation [73] and in the production of complex polymers in synthetic chemistry [74]. However, commercial application of laccases face major obstacles, such as the lack of sufficient enzyme stocks and the cost of redox mediators [75]. Heterologous expression of the enzymes with protein engineering allows for the cost-effective creation of more robust and active enzymes. In addition, an improvement in immobilization methods would result in greater stability of laccases with long life times [76].

2.1.2. Heme Peroxidases

2.1.2.1. Lignin peroxidases (LiP)(1,2-bis(3,4-



Lac: laccase, LMS: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and $^{\bullet}OH$: free hydroxyl radicals.

Figure 1. Schematic diagram of lignin degradation by basidiomycetes white-rot fungi: the major steps and enzymes involved (refer to text).

dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductases, EC 1.11.1.14)

LiPs are heme-containing glycoproteins and play a central role in the biodegradation of the cell wall constituent, lignin [77]. LiPs catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds (diarylpropane), β-O-4 non-phenolic lignin model compounds and a wide range of phenolic compounds (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acteosyringone) with redox potentials up to 1.4 V (**Table 1**) [15]. LiPs oxidize the substrates in multi-step electron transfers and form intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations (**Figure 1, e**). These intermediate radicals undergo non-enzymatic reactions such as radical coupling and polymerization, side-chain cleavage, demethylation and intramolecular addition and rearrangement (**Figure 1, f**) [15]. Unlike the other peroxidises, like MnP, LiP is able to oxidize non-phenolic aromatic substrates and does not require the participation of mediators due to its unusually high redox potential (**Figure 1, g**) [15,40]. The crystal structure of the first LiP has

shown that the heme group is buried in the interior of the protein and has access to the outer medium through a channel. Although the size of the channel is not sufficient to allow the large polymer lignin to access the heme group, small molecule substrates can find a suitable binding site [77].

Since the discovery of LiP in *P. chrysosporium* [78] in 1983, more LiPs have been found in different *P. chrysosporium* strains [79,80] and other white-rot fungi, such as *T. versicolor* [81]. In addition, LiP genes were detected in a few different fungal species including *Panus* sp., *P. coccineus*, *P. sanguineus* and *Perenniporia medulla-panis* [63]. LiP was also detected in some bacteria, such as *Acinetobacter calcoaceticus* NCIM 2890 [82] and *Streptomyces viridosporus* T7A [83].

2.1.2.2. Manganese peroxidases (MnP)(Mn (II):hydrogen-peroxide oxidoreductases, EC 1.11.1.13)

MnPs are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme as iron protoporphyrin IX [84]. MnP catalyzes the peroxide dependent oxidation of Mn(II) (as the reducing substrate) to Mn(III) (**Figure 1, h**), which is then released from the enzyme surface in complex with oxalate or with other chelators (**Figure 1, i**). Chelated Mn (III) complex acts as a reactive low molecular weight, diffusible redox-mediator (**Figure 1, j**) of phenolic substrates including simple phenols, amines, dyes, phenolic lignin substructures and dimers (**Table 1**) [15,36,84]. The oxidation potential of Mn(III) chelator is only limited to phenolic lignin structures. However, for the oxidation of non-phenolic substrates by Mn(III), reactive radicals must be formed in the presence of a second mediator (**Figure 1, k**). Organic acids, such as oxalate and malonate, are the primary compounds that act as second mediators in the production of reactive radicals like carbon-centered radicals (acetic acid radicals, $COOH\text{-}C^{\bullet}H_2$), peroxyl radicals ($COOH\text{-}CH_2OO^{\bullet}$), superoxide ($O_2^{\bullet-}$) and formate radicals ($CO2^{\bullet-}$) [15,36,84]. In the absence of H_2O_2 (e.g. in fungi lacking H_2O_2-generating oxidases), these radicals can be used by MnP as a source of peroxides and increase the lignin-degrading efficiency of the fungi [15,36,85].

Since the discovery of MnP in *P. chrysosporium* in 1985 [86], more MnPs have been found in other basidiomycetes, such as *Panus tigrinus* [87], *Lenzites betulinus* [88], *Phanerochaete flavido-alba* [89], *Agaricus bisporus* [90], *Bjerkandera* sp. [91] and *Nematoloma frowardii b19* [92].

MnPs may be capable of rivalling the potential applications of laccases in biotechnology. This is evident in studies which illustrate that the presence of MnP can increase the degree of dye decolorization. One study in particular found that MnP was the main enzyme involved in dye decolorization by *P. chrysosporium* [93]. Also, in another study, MnP produced by *P. chrysosporium* was used for the decolonization of sulfonphthalein (SP) dyes [94]. Complete decolorization took place at pH 4.0. In addition, MnP from white-rot fungi is considered the primary enzyme responsible for biobleaching of kraft pulps. The main drawback in commercial applications of MnP is the unavailability of the enzyme in large quantities; this can be resolved with the use of DNA recombinant technology [95]. For example, wild-type MnP from white-rot fungi [96] and recombinant MnP (rMnP) expressed in *Pichia pastoris* [97] have been used to remove lignin from cellulose fibers in pulp bleaching experiments [95]. The rMnP used in the study was found to be effective in lignin degradation and removal in both hardwood and softwood unbleached kraft pulps [95].

2.1.2.3. Versatile peroxidases (VP) (EC 1.11.1.16)

VPs are glycoproteins with hybrid properties capable of oxidizing typical substrates of other basidiomycetes peroxidases including Mn(II) and also veratryl alcohol (VA), MnP and the typical LiP substrate, respectively (**Figure 1**) [36,84,98]. VPs form an attractive ligninolytic enzyme group due to their dual oxidative ability to oxidize Mn(II) and also phenolic and nonphenolic aromatic compounds (**Table 1**) [36]. It has been found that VPs can also efficiently oxidize high redox-potential compounds such as dye Reactive Black 5 (RB5) as well as a wide variety of phenols, including hydroquinones [99,100]. It has been suggested that VPs can oxidize substrates spanning a wide range of potentials, including low- and high-redox potentials. This is a result of their hybrid molecular structures which provide multiple binding sites for the substrates [101]. This makes VPs superior to both

LiPs and MnPs, which are not able to efficiently oxidize phenolic compounds in the absence of VA or oxidize phenols in the absence of Mn(II), respectively [98]. Similar to the MnP mechanism, Mn(III) is released from VPs and acts as a diffusible oxidizer of phenolic lignin and free phenol substrates (**Figure 1, h, i** and **j**). Like other members of heme peroxidases, heme is buried in the interior of the protein and has access to the outer medium through two channels [100,101]. The function of the first channel is similar to that described for LiP and is conserved among all heme peroxidases. Conversely, the second channel is found to be specific to VP and MnP and is where the oxidation of Mn(II) to Mn(III) takes place [98].

Since the discovery of VP in 1999 in members of the genus *Pleurotus,* such as *P. eryngii* [101,102] and *P. ostreatus* [103], more VPs have been found in other basidiomycetes such as *Bjerkandera adusta* [100,104], *Bjerkandera* sp. strain BOS55 [105], *Bjerkandera* sp. (B33/3) [106], *Bjerkandera fumosa* [107] and *Pleurotus pulmonarius* [108]. Although *P. chrysosporium* did not show any VP activity, a putative extracellular peroxidase related to *Pleurotus* VP has been identified in its genome [109]. In addition, VP was detected in Polyporales basidiomycetes, including species from the genera *Panus* (e.g. *P. tigrinus* 8/18) [110].

Among basidiomycetes peroxidases, VPs have attracted the greatest biotechnological attention due to their catalytic versatility, which includes the degradation of compounds that other peroxidases are not able to oxidize directly. This unique feature allows VP to oxidize not only Mn (II) but also VA, phenolic, non-phenolic and high molecular weight compounds, including dyes in Mn-independent reactions [15,84]. Like MnP, VP's primary disadvantage in commercial applications is its limited availability in large quantities, which can be resolved with the use of DNA recombinant technology [95,98]. Efforts have been made to produce VP using heterologous expression systems. For example, in an experiment in which VP from *Pleurotus eryngii* was expressed under control of the alcohol dehydrogenase (alcA) promoter of *Aspergillus nidulans,* and lowering the growing temperature further improved the expression level [111]. Alternatively, other basidiomycete peroxidases such as LiP or MnP can be engineered to create peroxidases with new functions that emulate those of natural occurring VPs. For example, the Mn(II) binding site was introduced into a *P. chrysosporium* LiP by site-directed mutagenesis and the engineered enzyme also showed MnP activity while retaining its ability to oxidize VA (LiP activity) [112]. In another experiment, when a tryptophan residue analogous to the essential one in LiP was introduced to *P. chrysosporium* MnP by site-directed mutagenesis (single mutation, S168W), MnP with LiP activity was created while full MnP activity was maintained [113].

2.1.3. Other lignin degrading enzymes and accessory enzymes

In addition to ligninases, other fungal extracellular enzymes which act as accessory enzymes have been found to be involved in lignin degradation. These include oxidases generating H_2O_2, which provide the hydrogen peroxide required by peroxidases, and mycelium-associated dehydrogenases, which reduce lignin-derived compounds (**Figure 1, l**) [14]. Oxidases generating H_2O_2 include aryl-alcohol oxidase (AAO) (EC 1.1.3.7) found in various fungi, such as *P. eryngii,* and glyoxal oxidase (GLOX, a copper-radical protein) found in *P. chrysosporium* [39,114]. In addition, aryl-alcohol dehydrogenases (AAD) (a flavoprotein) and quinone reductases (QR) are also involved in lignin degradation by fungi (**Figure 1, m**) [115]. Moreover, it has been shown that cellobiose dehydrogenase (CDH), which is produced by many different fungi under cellulolytic conditions, is also involved in lignin degradation in the presence of H_2O_2 and chelated Fe ions [116]. It is proposed that the effect of CDH on lignin degradation is through the reduction of quinones, which can be used by ligninolytic enzymes or the support of a Mn-peroxidase reaction (**Figure 1, n**) (for detailed information please refer to the review by Henriksson et al. 2000 [117]).

2.2. Oxidative (non-lignocellulolytic) lignocellulose-degradation mechanisms in higher fungi

Over the last few decades, there has been emerging evidence in support of the involvement of non-enzymatic mechanisms in plant cell -wall polysaccharide degradation. These mechanisms are mostly assisted by oxidation through the production of free hydroxyl radicals (•OH). Many white and brown-rot fungi have been shown to produce hydrogen peroxide (H_2O_2) which enters the Fenton reaction and results in

Table 2. Mechanisms and enzymes involved in the production of ·OH in different fungi

Fungi	Mechanisms	Other enzymes involved/their function	References
White-rot fungi (e.g. *D. squalens*)	CDH catalyzed reaction	Oxalate decarboxylase/regulation of oxalate concentration	[136,137]
Brown and white-rot fungi (e.g. *C. puteana, P. chrysosporium*)	Quinone redox cycling	Benzoquinone reductases, CDH, sugar dehydrogenases/convert quinones to hydroquinones	[117,138]
Brown and white-rot fungi (e.g. *F. palustris, P. chrysosporium*)	Glycopeptides-catalyzed Fenton reaction	Cell wall-associated reductase/reduction of glycopeptides	[139]

release of ·OH (**Figure 1, o**) [114,118]. By attacking polysaccharides and lignin in plant cell walls in a non-specific manner, these radicals create a number of cleavages which facilitate the penetration of the cell wall by lignocellulolytic enzymes [119,120]. The pathways by which fungi generate free ·OH radicals are: cellobiose dehydrogenase (CDH) catalyzed reactions, low molecular weight peptides/quinone redox cycling and glycopeptide-catalyzed Fenton reactions (**Figure 1** and **Table 2**) [121].

CDH, an extracellular monomeric protein with some glycosylation, has been identified in a number of wood- and cellulose-degrading fungi, including basidiomycetes (mostly white-rot fungi) and ascomycetes, growing on cellulosic medium. This enzyme is able to oxidize cellobiose, higher cellodextrins and other disaccharides or oligosaccharides with β-1,4 linkages. In addition, CDH has been found with cellulose binding module (CBM) (in ascomycetes) and without CBM (in basidiomycetes). In the absence of CBM, CDH is able to bind to cellulose through hydrophobic interactions [122]. In some fungi, under cellulolytic conditions, CDH production increases, which in turn helps cellulases and hemicellulases [117,123]. It is now widely accepted that CDH is able to degrade and modify all three major components of the lignocellulosic residues (cellulose, hemicelluloses and lignin) by producing free hydroxyl radicals in a Fenton-type reaction (**Figure 1**) (for detailed information please refers to the review by Baldrin and Valaskova, 2008 [121]).

It has been shown that white and brown-rot fungi produce low molecular weight chelators which are able to penetrate into the cell wall. For example, *G. trabeum* produces a low molecular weight peptide (known as short fiber generating factor, SFGF) which can degrade cellulose into short fibers by an oxidative reaction [120,124]. It has also been reported that some of these low molecular weight compounds are quinones, which must first be converted to hydroquinones by particular fungal enzymes (**Table 2**) before free hydroxyl radicals can be produced through the Fenton reaction (**Figure 1, o**) [40].

Glycopeptides of varying molecular weights (ranging from 1.5 to 12 kDa) have been found in many brown-rot fungi, such as *G. trabeum* [125] and white-rot fungi, such as *P. chrysosporium* [43,126]. Similar to other mechanisms, glycopeptides are able to catalyze redox reactions and thus produce free hydroxyl radicals (**Figure 1, o**).

3. Concluding remarks

Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as potential biomass degraders for large-scale applications due to their ability to produce vast amounts of extracellular lignocellulolytic enzymes. Lignin, the most recalcitrant component of lignocellulosic material, acts as a barrier for any solutions or enzymes by linking to both hemicelluloses and celluloses and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. It is primarily basidiomycetes white-rot fungi that are responsible for efficient lignin degradation in wood decay processes. Their production of extracellular enzymes known as lignin-modifying enzymes (LMEs) facilitate the degradation process. Based on their activity on lignin and other lignocellulosic materials, white-rot fungi are categorized into two groups, simultaneous and selective degraders. Selective lignin degraders white-

rot fungi are most attractive for their potential biotechnological applications in removing lignin, as in biopulping processes, and for providing an unprotected carbohydrate for subsequent use, as in animal feed and/or biofuel substrate. LMEs include mainly two ligninolytic enzyme families; i) phenol oxidase (laccase) and ii) heme peroxidases (LiP, MnP and VP). LMEs, especially VPs require more research to understand the efficiency of the enzymes on lignin oligomers and the mechanisms of their action. In addition, accessory enzymes, such as oxidases that generate the H_2O_2 required by peroxidases, have been found to be involved in lignin degradation. Hydrogen peroxide is used by many white and brown-rot fungi to produce $^{\bullet}OH$ in the Fenton reaction. Ultimately, these free radicals attack polysaccharides as well as lignin in plant cell walls in a non-specific manner creating some cleavage sites which allow for easier penetration by lignocellulolytic enzymes.

Genome sequencing projects of 62 fungal species, including six basidiomycetes and 27 ascomycetes, have been completed thus far [127]. Availability of the full genome sequence of white -rot fungi, such as that of *P. chrysosporium*, now allows for the creation of proteomic methods to identify all enzymes involved in lignin degradation. In turn, such methods may lead to the discovery of new enzymes involved in the degradation [17]. In order to achieve this, small sample sizes were used to identify the proteins released by *P. chrysosporium* grown in a solid-substrate culture (red oak wood chips). Traditional two-dimensional (2-D) gel electrophoresis was coupled to advanced instrumentation, such as matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) or capillary liquid chromatography-nanoelectrospray ionization-tandem MS (CapLC-ESI-MS/MS). Using this combination, 16 extracellular proteins were identified from over 40 proteins spotted on 2-D gel [17]. However, it was predicted that *P. chrysosporium*, which was found to have a 30-Mb genome with more than 10,000 gene models (based on computational analysis), could potentially release about 790 proteins into the culture medium [128]. The goal of this and other on-going projects is to identify the extracellular proteome (proteomic secretome) of *P. chrysosporium* or other basidiomycetes white-rot fungi when grown on a solid substrate. More recently, the first genome-level transcriptome study of *P. chrysosporium* was performed to determine all the gene products involved in wood degradation using red oak as a carbon source. The results have shown that in addition to other lignocellulolytic enzymes, lignin peroxidase and alcohol oxidase (H_2O_2-generating enzyme) are highly expressed during lignin degradation [129]. Further investigation is needed to identify the novel proteins involved in fungal lignin degradation and their mechanisms of action. In addition, the suppression of lignin biosynthesis enzymes via plant genetic engineering may be of potential use in overcoming some of the problems related to the recalcitrance of lignin [130].

Acknowledgements

This work was supported by a grant from OGS (Ontario Graduate Scholarship) to M.D. and NSERC-RCD and Ontario Research Chair funding to W.Q.

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