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Martina Andlar[1]
Tonči Rezić[1]
Nenad Marđetko[1]
Daniel Kracher[2]
Roland Ludwig[2]
Božidar Šantek[1]*

[1] Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia

[2] Department of Food Sciences and Technology, University of Natural Resources and Life Sciences, Vienna, Austria

Review

Lignocellulose degradation: An overview of fungi and fungal enzymes involved in lignocellulose degradation

This review aims to present current knowledge of the fungi involved in lignocellulose degradation with an overview of the various classes of lignocellulose-acting enzymes engaged in the pretreatment and saccharification step. Fungi have numerous applications and biotechnological potential for various industries including chemicals, fuel, pulp, and paper. The capability of fungi to degrade lignocellulose containing raw materials is due to their highly effective enzymatic system. Along with the hydrolytic enzymes consisting of cellulases and hemicellulases, responsible for polysaccharide degradation, they have a unique nonenzymatic oxidative system which together with ligninolytic enzymes is responsible for lignin modification and degradation. An overview of the enzymes classification is given by the Carbohydrate-Active enZymes (CAZy) database as the major database for the identification of the lignocellulolytic enzymes by their amino acid sequence similarity. Finally, the recently discovered novel class of recalcitrant polysaccharide degraders-lytic polysaccharide monooxygenases (LPMOs) are presented, because of these enzymes importance in the cellulose degradation process.

Keywords: Biological pretreatment / Carbohydrate active enzymes / Fungi and fungal enzymes / Lignocellulose degradation / Plant cell wall

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1 The composition of lignocellulose-containing raw materials

Cellulose, hemicellulose, and lignin are major constituents of lignocellulose-containing raw materials, but a small amount of pectin, nitrogen compounds, and mineral residues are also present in these feedstocks [1]. Depending on the origin, the amounts of the indicated constituents differ. The structure of the lignocellulose is compact with different bonding among cellulose, hemicellulose, and lignin that makes lignocellulose a very complex substrate for enzymes. Those constituents are mainly coupled by hydrogen bonds. Between hemicellulose and lignin are chemical bonds that primarily refer to the chemical bonds between galactose and arabinose residues and carbohydrates. Several factors responsible for lignocellulose-containing raw materials recalcitrance are: lignin content that protects cellulose; cellulose interweaving by hemicellulose; high crystallinity and degree of polymerization of cellulose and low accessible surface area of cellulose with strong fiber strength [2]. Cellulose is a linear homopolymer composed of D-glucospyranose subunits linked by β-1,4-glycosidic bonds. The repeating unit is the disaccharide, cellobiose, since the single glucose units are rotated 180° relative to each other [3]. The cellulose strands form micro-fibrils that are stabilized by intra- and intermolecular hydrogen bonds and van der Waals forces [4]. Hemicellulose is a polysaccharide formed from monomeric sugars and sugars acids: D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D–galacturonic, and D-glucuronic acids linked together by β-1,4- and β-1,3-glycosidic bonds. Xylan is the main carbohydrate in hemicellulose. Hemicellulose is characterized by branches with short lateral chains consisting of different sugars while cellulose by easily hydrolysable lower molecular

Correspondence: Prof. Tonči Rezić (trezic@pbf.hr), Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10000 Zagreb, Croatia

Abbreviations: AA, auxiliary activity; **CAZy**, carbohydrate active enzymes database; **CAZymes**, carbohydrate active enzymes; **CDH**, cellobiose dehydrogenase; **GH**, glycoside hydrolase; **LiP**, lignin peroxidases; **LPMO**, lytic polysaccharide monooxygenase; **MnP**, manganese-dependent peroxidases

*Additional correspondence: Prof. Božidar Šantek (E-mail: bsantek@pbf.hr), Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia

weight oligomers, respectively [4]. Lignin is a complex amorphous heteropolymer with a three-dimensional structure composed of phenylpropane derivates linked to each other by the irregular coupling of C–C and C–O bonds. It includes three basic structural monomers: p-phenyl monomer (H type) derived from coumaryl alcohol, guaiacyl monomer (G type) derived from coniferyl alcohol and syringyl monomer (S type) derived from sinapyl alcohol [5]. Lignin is linked to both hemicellulose and cellulose and it main function in the plant wall is to give structural support, impermeability, and resistance against microbial attack and oxidative stress [4].

2 Lignocellulose degrading fungi

Fungal strategies for lignocellulose depolymerization are substantially very complex, due to the high complexity of the raw materials. Recalcitrance to saccharification is a major limitation for the enzymatic conversion of lignocellulose-containing raw materials to get valuable end products. The combination of hemicellulose and lignin forms a protective barrier around the cellulose, which must be modified (or removed) before the hydrolysis of cellulose. But also crystalline structure of cellulose makes it insoluble and resistant for enzyme decomposition. Nevertheless, the removal of lignin is a key challenge to increase enzyme access to hemicellulose and cellulose [6].

Plant cell wall-degrading filamentous fungi have an important role in recycling nutrients in forest ecosystem. They are known to produce a broad variety of extracellular enzymes with diverse catalytic activities for the hydrolysis of renewable lignocellulose-containing raw materials.

Predominantly responsible for lignocellulose degradation are wood-degrading fungi. They decompose and assimilate the most recalcitrant organic polymers, which is mainly attributed to their highly adaptive lifestyles, reflected by a large phylogenetic and phenotypic diversity [7]. If they want to overcome the physical and chemical stability of lignocellulose, the fungi have to employ large sets of enzymes, which they release into the environment during their growth. Fungi have two types of degradation systems: intracellular, together with the outer cell envelope layer, and extracellular, important for polysaccharide degradation. Furthermore, the extracellular enzymatic system includes two types of enzymes: hydrolytic, responsible for polysaccharide degradation; and oxidative, which degrade lignin and open phenyl rings. Three groups of fungi, with different effects and degradation mechanisms onto the lignocellulose, have been described: soft-rot, brown-rot, and white-rot fungi [4].

Soft-rot fungi are mostly ascomycete fungi that can degrade polysaccharides in the surface layers of plants. Degradation leads to darkening and softening of the wood by the produced laccases and peroxidases involved in lignin modifications. These enzymes are unspecific and more limited in function then those isolated from white-rot and brown-rot fungi. The soft-rot fungi belong to genera *Aspergillus* and *Neurospora.* Still, little is known about the degradation mechanisms of lignocellulose by soft-rot fungi [8].

Brown-rot fungi are basidiomycetes that rapidly metabolize cellulose and hemicellulose while only slightly modifying lignin. They have no lignin degrading enzymes except small molecule reactive species to depolymerize lignin. At an advanced stage of degradation, wood residue exhibits cube-shape and has a brownish color due to the predominant presence of oxidized lignin. Disruption of the lignocellulose matrix by brown-rot fungi can be demonstrated using iron-dependent Fenton chemistry known as chelator-mediated Fenton system (CMF). The CMF system is a unique substrate deconstruction system based on oxygen radical chemistry that allows nonenzymatic deconstruction of the cellulose. Briefly, brown-rot fungal hyphae during the growth in the lumen area of plant cells produce oxalic acid, iron-reducing compounds (RC), and hydrogen peroxide (H_2O_2). The oxalic acid binds to a Fe^{3+} ion formatting the complex that diffuses into cell wall along with H_2O_2 and RC. With the pH change, RC sequesters Fe^{3+} from the Fe-oxalate complex and reduces it to Fe^{2+}. Fe^{2+} then reacts with H_2O_2 (Fenton reaction) and produces hydroxyl radicals (-OH). Upon attack of -OH radicals, lignocellulose matrix is disrupted. Models for the study of brown-rot fungi are *Gloephyllum trabeum, Coniophora puteana*, and *Postia placenta* [9].

White-rot fungi are able to decompose all lignocellulose constituents: lignin, cellulose, and hemicellulose. Degradation of lignin is more efficient than in the case of brown-rot and soft-rot fungi, because they possess an unique ability to its complete mineralization to CO_2 [10]. Therefore, white-rot basidiomycetes could be an interesting source of lignocellulose-active enzymes to supplement the commercial cocktails of hemicellulases and cellulases originated from ascomycetes such as *Aspergillus niger* or *Trichoderma reesei* [4]. Some of the white-rot fungi capable of causing selective delignification of wood are *Phanerochaete chrysosporium*, *Phanerochaete carnosa, Pleurotus ostreatus, Pycnoporus cinnabarinus, Botrytis cinerea, Stropharia coronilla,* and *Trametes versicolor.*

3 Degradation of lignocellulose-containing raw materials by fungal enzymes

Biological degradation of lignocellulose-containing raw materials employs fungi, mainly belonging to the group of white-rot and brown-rot basidiomycetes. It requires long application periods with the rate of fungal degradation that is too low for industrial use and consumes a fraction of the plant polysaccharides [11]. More convenient than fungal is enzymatic degradation, which is very selective and fast but also expensive at a large scale. The enzymatic degradation of lignocellulose-containing raw materials is achieved through the multiple carbohydrate-active enzymes, usually acting together with complementary, synergistic activities, and modes of action [12]. In the following sections, the main enzymes and enzymatic degradation processes of the main lignocellulose constituents are described.

3.1 Lignin degradation by ligninolytic enzymes system

Lignin degradation is an oxidative process mainly attributed to the secondary metabolism, or to restricted availability of carbon, nitrogen, or sulphur, and it is normally not degraded as sole carbon and energy sources [13]. In nature, it is generally

attributed to the metabolism of basidiomycetes white-rot fungi, since they degrade lignin more rapidly and extensively than other microorganisms [8]. Some white-rot fungi species that preferentially attack lignin more readily than hemicellulose and cellulose are *Ceriporiopsis subvermispora, Phellinus pini, Phlebia* sp., *Pleurotus* sp., *Phanerochaete chrysosporium, Trametes versicolor, Heterobasidion annosum,* and *Irpex lacteus.* These fungi produce a set of ligninolytic enzymes that catalyse the oxidation of an array of aromatic substrates, producing aromatic radicals and changing the structure of the lignocellulose-containing raw materials and lignin.

Lignin consumption is mainly accomplished by laccases, manganese-dependent peroxidases (MnP), lignin peroxidases (LiP), and versatile peroxidases (VP) that are the major groups of ligninolytic enzymes produced by the white-rot fungi [7].

Laccases (EC 1.10.3.2; CAZy AA1) are blue multicopper enzymes able to oxidize a variety of phenolic and nonphenolic compounds. From a molecular point of view, they are monomeric, dimeric, or tetrameric glycoproteins, which usually contain four copper atoms per monomer distributed in three redox sites named T1, T2, and T3. In the resting enzymes, all four copper ions are in the 2^+ oxidation state. Phenolic compounds, including lignin, polyphenols, methoxy-substituted phenols, or diamines, are oxidized by one-electron abstraction that leads to formation of radicals that can repolymerize or cause depolymerization [14]. In the presence of a mediator (2,20-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS)), that behaves as an electron shuttle, these enzymes can oxidize also nonphenolic compounds [15]. Because of these characteristics, laccases can be employed for delignification and removal of phenolic compounds in various fields such as biofuels and food production, pulp, and paper treatments, textile industry, nanobiotechnology, soil bioremediation, synthetic chemistry, and cosmetics [16]. The most laccases are produced by white-rot fungi that are secreted into the medium by the mycelium of filamentous fungi [17]. Some examples of fungi that produce laccase with high activity are *Trametes pubescens* (740 000 U/L) [18], *Coriolus hirsutus* (83 830 U/L) [19], *Trametes hirsuta* (19 400 U/L) [20], *Trametes versicolor* (16 000 U/L) [21], *Pycnoporus cinnabarinus* (10 000 U/L) [22], *Neurospora crassa* (10 000 U/L) [23], and *Pleurotus ostreatus* (80 000 U/L) [24].

Besides laccases, other important enzymes that can be used for delignification are peroxidases. Peroxidases by itself are too large to penetrate the dense lignocellulosic matrix. They are thought to generate small molecular radical species that catalyze the oxidation of the lignin [25]. Lignin peroxidases (LiPs; EC 1.11.1.14; CAZy AA2), known as peroxide oxidoreductases, oxidizing nonphenolic methoxyl-substituted lignin units (>90% of the lignin) in the presence of H_2O_2 [26]. Manganese-dependent peroxidases (MnPs; EC 1.11.1.13; CAZy AA2) oxidize a bound Mn^{2+} ion to Mn^{3+} in the presence of hydrogen peroxide generating an intermediate redox couple Mn^{2+}/Mn^{3+}. The product Mn^{3+} is released from the active site in the presence of a chelator (mostly oxalate and malate) that stabilizes it against disproportionation to Mn^{2+} and insoluble Mn^{4+}. The complex Mn^{3+} ion can diffuse into the lignified cell wall, where it oxidizes phenolic or nonphenolic lignin components [15].

Versatile peroxidases (VPs; EC 1.11.1.16; CAZy AA2) combine the substrate-specificity characteristics of the two other ligninolytic peroxidases (LiP and MnP). Unlike these two enzymes, it can oxidize phenolic and nonphenolic substrates including veratryl alcohol, methoxybenzenes, and lignin model compounds [26].

Peroxidases such as HRPs (EC 1.11.1.7, CAZy AA2), secreted by fungus *Phanerochaete chrysosporium*, are potential biocatalysts for bioremediation of environment polluted by harmful compounds (e.g. endocrine disrupting compounds: 17α-ethinylestradiol (EE2)) [27]. HRPs have also been implicated in the cell wall biosynthesis, indole-3-acetic acid (plant growth hormone) catabolism and oxidation of toxic compounds [28].

The lignin degradation can be further enhanced by the action of other enzymes such as: aryl alcohol oxidases (AAO; EC 1.1.3.7.; CAZy AA3) that oxidize many primary alcohols containing an aromatic ring and is described in *Pleurotus eryngii* [29], glyoxylate oxidase (GOx, EC 1.2.3.5) [30], pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4, CAZy AA3), and cellobiose dehydrogenase (CDH, EC 1.1.99.18; CAZy AA3). Fungal aryl-alcohol dehydrogenases (AAD; EC 1.1.1.90) and quinone reductases (QR, EC 1.6.99.2), tyrosinases (EC.1.14.18.1) and catechol oxidases (EC 1.10.3.1) are also involved in lignin degradation [31–33]. Table 1 shows an overview of fungi and enzymes involved in lignin disruption.

Significant amounts of peroxidases have been produced by fungi in the submerged or solid state fermentation. In the submerged culture, the fungus *Mucor racemosus* produced lignin peroxidases (75 376 U/L) and manganese peroxidases (4484 U/L) [34] while in a solid-state fermentation of steam exploded wheat straw by *Phanerochaete chrysosporium* manganese peroxidases (1375 U/L) were observed [35]. Submerged fermentation of versatile peroxidases (7300 U/L) was realized by genetically modified *Pleurotus ostreatus* [36].

3.2 Hemicellulose degradation by hemicellulolytic enzymes system

Hemicellulose hydrolysis demands cooperative action of several types of enzymes working at different levels of the hemicelluloytic matrix. This synergistic activity is necessary not only because of hemicellulose complexity but also because of its connection with the other plant cell wall components. According to their action on distinct substrates two types of enzymes are predominantly involved in hemicellulose degradation: endo-1,4-β-xylanase (EC 3.2.1.8) and exo-1,4-β-xylosidase (EC 3.2.1.37; alternative names: xylan β-1,4-xylosidase, 1,4-β-D-xylan xylohydrolase, β-xylosidase, or xylobiase) [4]. Endo-1,4-β-xylanases hydrolyse β-1,4-xylan chains, and generate xylo-oligosaccharides. Most of them belong to CAZy families GH10 and GH11 and some to GH5, GH7, GH8, and GH43 families. Xylan β-1,4-xylosidases cleave xylobiose and xylo-oligosaccharides releasing xylose [4].

Mannan, as the major component of hemicellulose in softwood, is comprised of mannose residues or a combination

Table 1. Overview of lignin-degrading fungi and enzymes involved in lignin modification and degradation [4, 27, 33–36]

Fungi	Enzyme	EC number	CAZy family
Trametes pubescens, Coriolus hirsutus, Trametes hirsute, Trametes versicolor, Pycnoporus cinnabarinus, Neurospora crassa, Pleurotus ostreatus, Botrytis cinerea	Laccase	EC 1.10.3.2	AA1
Phanerochaete chrysosporium, Mucor racemosus, Aspergillus sclerotiorum, Cladosporium cladosporioides, Stropharia coronilla, Bjerkandera adusta, Pleurotus eryngii	Manganese peroxidase	EC 1.11.1.13	AA2
Phanerochaete chrysosporium, Mucor racemosus, Aspergillus sclerotiorum, Cladosporium cladosporioides	Lignin peroxidase	EC 1.11.1.14	AA2
Pleurotus ostreatus, Pleurotus eryngii	Versatile peroxidase	EC 1.11.1.16	AA2
Phanerochaete chrysosporium, Pleurotus ostreatus, Pleurotus eryngii	Horseradish peroxidase	EC 1.11.1.7	AA2
Pleurotus sp.*(P. cornucopiae, P. eryngii, P. floridanus, P. pulmonarius, P. ostreatus)*	Aryl-alcohol dehydrogenase	EC 1.1.1.90	AAD
Phanerochaete chrysosporium	Glyoxylate oxidase	EC 1.2.3.5	AA5
Phanerochaete chrysosporium	Cellobiose dehydrogenase	EC 1.1.99.18	AA3
Gloeophyllum trabeum	Quinone reductase	EC 1.6.5.2	QR
Pleurotus eryngii	Alcohol oxidase	EC 1.1.3.7	AA3
Peniophora sp.	Pyranose 2-oxidase (glucose 1-oxidase)	EC 1.1.3.4	AA3
Myceliophthora thermophile, Agaricus bisporus, Pycnoporus sanguinensiss, Trichoderma reesei	Tyrosinases	EC.1.14.18.1	not defined
Aspergillus oryzae	Catechol oxidases	EC 1.10.3.1	not defined

of mannose and glucose residues also known as glucomannan. β-Mannanases (endo-β-1,4-mannanase; EC 3.2.1.78) are endohydrolases that hydrolyse mannan fibers by cleaving β-1,4 bonds and producing new reducing and nonreducing ends. Depending on the active site organization most β-mannanases are active on oligosaccharides consisting of three or four monomeric units. The hydrolytic action of β-mannanases on mannan is supported with β-mannosidase enzymes (exo-β-1,4-mannosidase; EC 3.2.1.25) that carry out hydrolysis of terminal, nonreducing β-D-mannose residues. In case of glucomannan degradation, β-glucosidases can cleave the bond between one mannose and one glucose residue. One should be aware that the action of these enzymes strongly depends on the number and pattern formed by the substituted galactoses and other substitutions and on the action of other enzymes [37].

To efficiently hydrolyse wood xylans and mannans hemicellulose degradation is supported with the help of the other enzymes whose acting synergistically. These accessorial enzymes are acetylxylan esterase (EC 3.1.1.72), feruloyl esterase (EC 3.1.1.73) and p-coumaroyl esterase (EC 3.1.1.B10), α-L-arabinofuranosidase (EC 3.2.1.55), xylan α-1,2-glucuronosidase (EC 3.2.1.131), and α-glucuronidase (EC 3.2.1.139). For the complete degradation of oligomer arabinoxylan, which is one of the components of wheat straw, it is necessary to employ enzyme α-L-arabinofuranosidase (EC 3.2.1.55). This enzyme hydrolyses covalent bonds between L-arabinose and D-xylose and removes residues substituted at C2 and C3 position of xylose residues [38]. Table 2 summarizes the fungi and enzymes involved in hemicellulose disruption.

3.3 Cellulose degradation by cellulolytic enzymes system

In order to enable utilization of insoluble cellulose as such, multiple enzymatic activities are required. Fungi able to degrade cellulose produce an array of enzymes with different specificities. Different characteristics of the cellulose, like the degree of polymerization (DP), crystallinity, particle size and surface area, influence the efficiency of enzymatic hydrolysis. Fungal cellulose degradation is accomplished by a set of glycoside hydrolase (GH) enzymes with complementary catalytic activities. These enzymes are listed in the carbohydrate-active enzymes (CAZy) database that provides compilation of carbohydrate-degrading or -modifying enzymes and describes families of structurally related enzymes.

Cellulases have different specificities to hydrolyse the β-1,4-glycosidic linkages bonds that connect glucose units in the cellulose fiber. Most of them have an independently folded carbohydrate binding module (CBM) connected to the catalytic domain by a flexible linker. The CBM is responsible for binding the enzyme to the crystalline cellulose. They are divided into three major classes: endoglucanases (endo-1-4-β-glucanase; EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21).

Endoglucanases (EC 3.2.1.4) internally cleave β-1,4-glycosidic bonds in the amorphous regions of cellulose thereby releasing reducing and nonreducing chain ends. Because of the artificial substrate, used for their detection, they are often called carboxymethylcellulases (CMCase) [39]. They belong to CAZy families GH5, GH6, GH7, GH9, GH12, GH45, and GH74. Exoglucanases (EC 3.2.1.91) also known as cellobiohydrolases

Table 2. Overview of fungi and enzymes involved in hemicellulose degradation [4]

Fungi	Enzyme	EC number	CAZy family
Trichoderma longibrachiatum	Endo-1,4-β-xylanase	EC 3.2.1.8	GH5, GH7, GH8, GH10, GH11, GH43
Aspergillus nidulans	Exo-1,4-β-xylosidase	EC 3.2.1.37	GH3, GH39, GH43, GH52, GH54
Sclerotium rolfsii	Endo-β-1,4-mannanase	EC 3.2.1.78	GH5, GH26, GH113
Aspergillus niger	Exo-β-1,4-mannosidase	EC 3.2.1.25	GH1, GH2, GH5
Aspergillus niger	Feruloyl esterase	EC 3.1.1.73	CE1
Neocallimastix sp.	p-Coumaroyl esterase	EC 3.1.1.B10	CE1
Aspergillus niger, Phanerochaete chrysosporium, Rhizomucor miehei	Endo-α-1,5-arabinanase	EC 3.2.1.99	GH43
Aspergillus niger	α-L-arabinofuranosidase	EC 3.2.1.55	GH43, GH51, GH62
Phanerochaete chrysosporium	α-Glucuronidase	EC 3.2.1.139	GH67
Aspergillus fumigatus, Phlebia radiate, Pleurotus ostreatus, Trichoderma sp. (T.hamatum, T. harzianum, T. viride, T. longibrachiatum)	Xylan α-1,2-glucuronosidase	EC 3.2.1.131	GH67
Mortierella vinacea	α-Galactosidase	EC 3.2.1.22	GH4, GH27, GH36, GH57, GH97, GH110
Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae	Endo-galactanase	EC 3.2.1.89	GH53
Humicola insolvens	β-Glucosidase	EC 3.2.1.21	GH1, GH3
Trichoderma reesei	Acetyl esterase	EC 3.1.1.6	CE16
Aspergilus sp., *Schizophyllum commune, Trichoderma reesei*	Acetylxylan esterase	EC 3.1.1.72	CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12, CE16
Acremonium alcalophilum, Phanerochaete chrysosporium, Trichoderma reesei	Glucuronyl methyl esterase	EC 3.1.1	CE15

Table 3. Overview of fungi and enzymes involved in cellulose degradation [4, 39]

Fungi	Enzyme	EC number	CAZy family
Trichoderma reesei, Trichoderma harzianum, Aspergillus niger, Pesralotiopsis sp., *Phanerochaete chrysosporium, Fomitopsis palustris, Neocallimastix frontalis*	Endoglucanase	EC 3.2.1.4	GH5,GH6, GH7, GH9, GH12, GH45, GH74
Trichoderma reesei, Trichoderma harzianum, Pesralotiopsis sp., *Phanerochaete chrysosporium, Fomitopsis palustris*	Exoglucanase	EC 3.2.1.91	GH5, GH6, GH9
Trichoderma reesei, Phanerochaete chrysosporium, Fomitopsis palustris	β-Glucosidase	EC 3.2.1.21	GH1, GH2, GH3, GH5, GH9, GH30, GH39, GH116
Penicillium brefeldianum	1,6-β-d-Glucosidase	EC 3.2.1.75	GH5, GH30
Rhizopus chinensis	1,3-β-d-Glucosidase	EC 3.2.1.39	GH5, GH16, GH17, GH55, GH64,GH81, GH128
Achlya bisexuals	exo-1,3-β-Glucanase	EC 3.2.1.58	GH3, GH5, GH16, GH17, GH55
Orpinomyces sp.	1,3-1,4-β-d-Glucosidase	EC 3.2.1.73	GH16, GH50, GH86, GH118

(CBH), remove dimers (cellobiose) from the end of the cellulose chain. Some CBHs are able to work only on reducing ends while others cleave cellobiose units only at the nonreducing ends [10]. They belong to families GH5 and GH6. The main product of cellobiohydrolases is the disaccharide cellobiose, which is cleaved into glucose units by the enzyme β-glucosidase [7]. β-Glucosidases (EC 3.2.1.21) hydrolyse glucose dimers and in some cases gluco-oligosaccharides to glucose. They belong to families GH1 and GH3. Except for the above, the role of other enzymes engaged in cellulose degradation is also important (Table 3).

The synergistic action of these enzymes is essential in the hydrolysis of lignocellulose. It is revealed that except cellulases the other enzymes may have an important role in the degradation (Fig. 1). The recent discovery of oxidative enzymatic processes that augment cellulose degradation prompted the introduction of a new CAZy family, termed as "auxiliary activity (AA)" (before GH61 family) [40]. They are known to remarkably improve the hydrolysis of lignocellulose by acting in synergy with other cellulolytic enzymes. Especially the cellulose-active lytic polysaccharide monooxygenases (LPMOs; CAZy: AA9) attracted attention due to their ability to directly oxidize crystalline substrate surfaces, which extremely enhances the overall degradability of cellulose [41–43]. Several studies have demonstrated that LPMOs are oxidative enzymes acting in synergy with cellobiose dehydrogenases (CDH) that gives a new view on a cellulose degradation [7, 44–46].

4 Fungal delignification strategies

Examples of lignin degrading fungi and their enzymes used in various processes are listed in the following section.

A study of Kerem *et al.* [47] compared lignocellulose degradation ability of two fungi: *Pleurotus ostreatus* and *Phanerochaete*

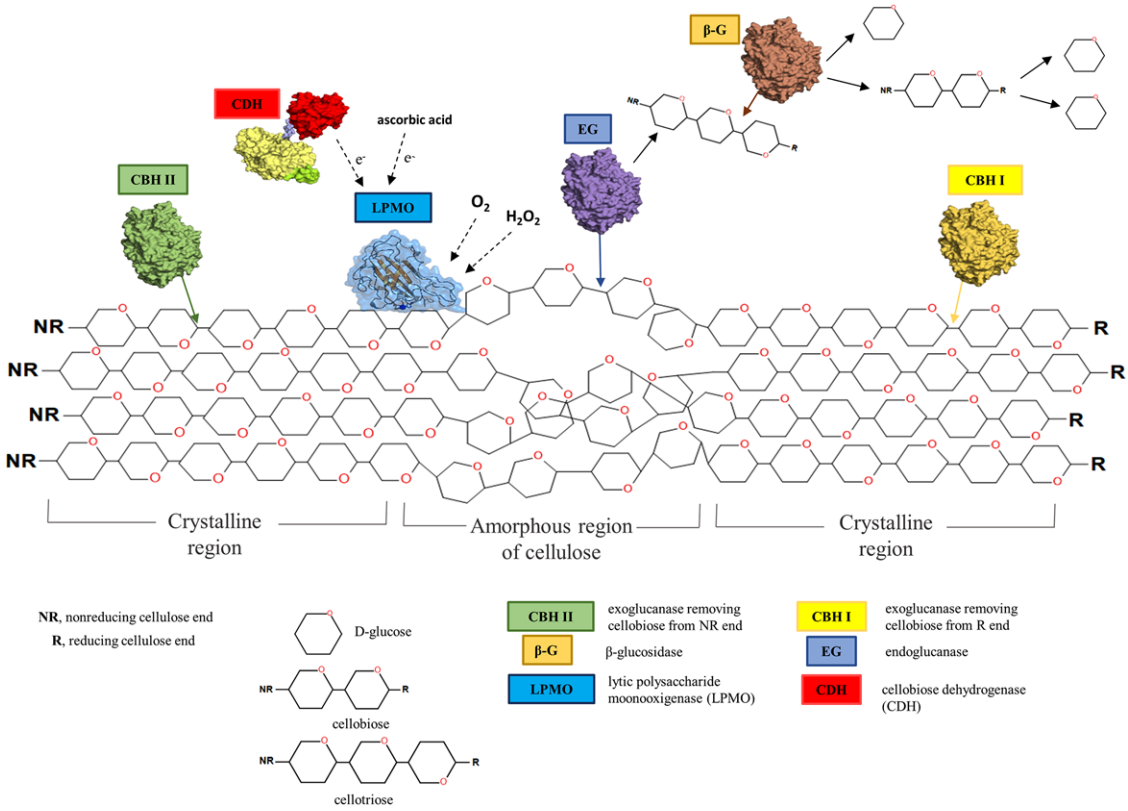

Figure 1. Scheme of the enzymatic degradation of cellulose chain via synergistic interaction of cellulases (endoglucanase, exoglucanase, and β-glucosidase) and LPMO (AA9 or 10) enzymes.

chrysosporium, during solid state fermentation on cotton stalks as a substrate. The growth of *P. chrysosporium* resulted in the loss of 55% of the initial dry organic matter within 15 days of fermentation, while the lignin loss equalled to 35% of the initial lignin content in the substrate. The growth of *P. ostreatus* resulted in the loss of only 20% of original dry organic matter, while the lignin loss was 45% of the initial lignin content in the substrate.

Li et al. [48] analyzed compositional changes of cottonseed hull substrate during *P. ostreatus* growth. After 45 days of incubation, lignin content decreased from an initial 17% to a final of 11% of dry matter. Moreover, they performed delignification of wheat straw by *Fusarium concolor* that is able to produce laccase, LiP and MnP enzymes when grow on a lignocellulosic medium. After 5 days of incubation they observed removal of 13.07% of the lignin and loss of 7.62% of the total polysaccharide fraction [49].

Herpöel *et al.* [50] investigated wheat straw pulp degradation combining commercial xylanases and laccases from *Pycnoporus cinnabarinus,* separately, followed by alkaline treatment. This two stage treatment was effective to remove 60% of lignin in wheat straw pulp. Also, xylanases and laccases, previously used for enzyme-aided bleaching, contribute improvements in the following chemical delignification step [51]. The same fungus was used in delignification of lignocellulose-containing raw materials (*Prosopis juliflora/Lantana camara*) reported by Gupta et al. [52]. The results showed that lignin removal improves the saccharification of cellulose. The fungal delignification was the highest during the first 15 days. An increase of 21.1–25.1 % sugar release was obtained when fungal treated substrates were enzymatically hydrolysed as compared to the hydrolysis of untreated substrates.

During a solid state cultivation on wood chips, the main oxidative enzyme produced by *Cereporiopsis subvermispora* is manganese peroxidase (MnP). This fungus also secretes a number of low molecular mass compounds including oxalic acid and several fatty acids [53]. MnP-initiated lipid peroxidation reactions can explain degradation of nonphenolic lignin substructures by *C. subvermispora.* Cellulose is more resistant to the attack by this fungus and their enzymes because this fungus tends to remove lignin in advance of cellulose and hemicellulose. The lack of cellobiohydrolases (CBHs) and the presence of systems able to suppress Fenton's reaction in the cultures can explain nonefficient cellulose degradation by this fungus. Although the low permeability of the wood cell walls to endo-cellulases, the cellulose depolymerization, induced by low-molecular weight agents as reported for brown-rot fungi, should not be excluded. The abilities of *C. subvermispora*, used for biochemical pulping of agricultural residues (rice, wheat, and barley straws), was compared with chemical process [54]. Although the delignification of rice, wheat, and barley straws was not as efficient as chemical process, the quality of papers produced by biochemical pulping of straws was high and satisfactory. The reduction of the amount

of chemicals and the mechanical energy used in the process was lower what makes this process a better choice. The same fungus was used in a large-scale biopulping process of wood chips [55].

A two-stage fungal biopulping method, studied by Giles *et al.* [56], was investigated to improve enzymatic hydrolysis of wood for ethanol production. In a two-stage wood treatment a liquid culture suspension, consisting of white-rot fungus *C. subvermispora* and brown-rot fungus *Postia placenta*, was used. The treatments resulted in $6 \pm 0.5\%$ mass loss and increased the yield of enzymatic hydrolysis by 67–119% [56].

The delignification of wood (*Eucalyptus globulus*) and nonwood (*Pennisetum purpureum*) feedstocks employing laccase from *Trametes villosa,* mediator 1-hydroxybenzotriazole (HBT) and alkaline extraction was examined by Gutiérrez *et al.* [57]. When using 50 U/g laccase and 2.5% HBT they aimed to remove 48 and 32% of the lignin from *E. globulus* and *P. purpureum*, respectively. The enzymatic pretreatment (25 U/g) increased the glucose yields by 61 and 12% in 72 hours and ethanol yields by 4 and 2 g/L in 17 hours, respectively.

Several studies have demonstrated the effectiveness of ligninolytic fungal enzymes to delignify lignocellulose-containing raw materials. Very promising results have been obtained using ligninolytic enzymes in processes such as delignification and bleaching systems. Unfortunately, there is still no real concept capable to fully implement such enzymes into industry because of its high costs of application, limitations in performance, and technical feasibility, which depends on the enzymatic system. Possible alternatives to these existing enzymatic concepts could be:

(1) oxidation system mediated with lipases, special ketone compounds, fatty acid or fat compounds, and H_2O_2
(2) new enzymatic approaches with methods that generate reactive oxygen species (ROS) or reactive nitrogen species (RNS) [58].

Call and Call [59] reported a new generation of enzymatic systems for delignification and bleaching, as already mentioned. They developed two systems: one mediated oxidoreductase system is capable to delignify with the aid of the active components: peroxynitrous acid (PNA) or dicyclopentadienyl transition metal complexes (ferrocene) for obtained peroxide activation and another with special generated organosulphonic peracids or enzymatically activated sulphite that can generate in combination with ketones dioxirane [8].

5 Carbohydrate active enzymes (CAZymes)

The large diversity of monosaccharides, the multiple types of intersugar linkages and the fact that almost all organic macromolecules can be glycosylated results in an enormous amount of carbohydrate structures and conjugates [60]. Furthermore, since all such carbohydrates must both be synthesized and broken down, the amount and especially the complexity of enzymes performing such activities is enormous. Enzymes that are involved in the synthesis, modification, or breakdown of glycoconjugates or complex polysaccharides are summarized as called Carbohydrate-Active enZymes (CAZymes; the CAZy database www.cazy.org/) [61]. The CAZy classification groups the proteins in families according to amino acid sequence similarity and was introduced to obtain a classification system that was more meaningful than the EC system, which is solely based on the reaction mechanism. Due to the modular structure of many CAZymes it is possible to find one protein in several families [62].

In 2008, the CAZy database covered approximately 300 protein families divided into five classes: glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, and noncatalytic carbohydrate-binding modules. This database grows progressively and is constantly updated with new sequence information, 3D structures and biochemical characterizations [62]. In 2013, a novel enzyme class was introduced, covering redox-enzymes that work in concert with CAZymes, which have been named Auxiliary Activites [40, 62]. Currently, the CAZy database holds about 400 protein families divided into six classes and provides a consistent nomenclature for CAZymes.

Glycosyltranferases (GTs) are responsible for the enzymatic formation of glycosidic bonds using an activated donor sugar substrate with a phosphate leaving group. Other sugars or lipids, proteins, nucleic acids, and small molecules can act as the acceptor substrate [63]. GTs show great diversity in donor, acceptor, and product specificity and can potentially generate an infinite number of glucoconjugates, oligo-, and polysaccharides [64].

Carbohydrate esterases (CEs) are a class of CAZymes that remove ester-based modifications by de-*O* or de-*N* acylation of a substituted saccharide in a hydrolytic manner.

Polysaccharide lyases (PLs) use β-elimination instead of a hydrolytic mechanism to cleave uronic acid containing polysaccharides. PLs form a complimentary strategy to the degradation of C-6 carbohylated polysaccharides by glycoside hydrolases [65].

Glycoside hydrolases (GHs) form the enzyme class with most families, comprising 153 at present. These enzymes are responsible for the hydrolysis of glycosidic bonds between two carbohydrate moieties or one carbohydrate and one noncarbohydrate moiety. The variation of activities in the GH family is large, including enzymes that predominantly target insoluble substrates, soluble oligosaccharides of variable or strictly defined chain length and branch points of branched polysaccharides. GH activity on polysaccharides can be endo- or exo-, reffering to their ability to cleave the polysaccharide chain randomly or from the chain end.

A noncatalytic class of proteins found in the CAZy database are the carbohydrate-binding modules (CBMs). CBMs are connected with other CAZymes in multimodular structures and promote association with the substrate. By recognizing and binding the target structure, the catalytic domain is brought in close proximity to the substrate that may potentiate catalysis [61]. CBMs recognize their target structure within their natural context, e.g. the plant cell wall [66]. Interestingly, binding to nonsubstrate polysaccharides in an intact plant cell wall, potentiates degradation of the substrates as well by means of the proximity effect [67].

As already mentioned, AAs are the latest addition to the CAZy database. AAs involve proteins that are potentially able to aid other CAZymes in degrading a complex substrate. Hence, they comprise a wide array of enzymes that are active on polysaccharides and nonpolysaccharides like lignin, which, without exception is found in combination with polysaccharides in the

plant cell wall [40]. This class of enzymes includes laccases, cellobiose dehydrogenases (CDHs), copper radical oxidases, and other enzymes that utilize a redox mechanism. LPMOs are enzymes that were previously classified as family 61 of the GHs and family 33 of the carbohydrates-binding modules. The finding that these proteins were oxidative enzymes acting on chitin [68] or cellulose [44, 69, 70] was one major reason for extending the CAZy database in order to reclassify these proteins. These enzymes work in synergy with many GHs and stimulate their activity by increasing the accessibility to the substrate [42].

6 Lytic polysaccharide monooxygenase enzymes

LPMOs are copper-dependent enzymes that catalyze the oxidative cleavage of a glycosidic bond in the presence of hydrogen peroxide or dioxygen and an external electron donor [71]. They are able to degrade insoluble polysaccharides such as crystalline cellulose, chitin and starch [71–74]. Also, they depolymerise noncrystalline or soluble hemicellulosic substrates such as xyloglucan, xylan, and beta-glucans [75]. LPMOs are important in biomass conversion because they act in synergy with glycoside hydrolase (GH), thereby enhancing overall polysaccharide conversion efficiency. Although these enzymes have been intensely investigated since their discovery in 2010, several aspects of their catalytic mechanism and their mode of action remain unclear. LPMOs are abundant and show high sequence diversity, which suggests functional roles beyond biomass degradation.

They are currently categorized as families 9, 10, 11, 13 14, and 15 of the auxiliary activities. Family AA9 contains only fungal enzymes that were previously referred to as GH61. Family AA10 proteins (previously CBM33) are found in all domains of life, namely archaea, bacteria, and eukaryote. Family AA11 has mainly fungal members while AA13 exclusively comprises fungal LPMOs. The latest addition to the auxiliary activities was when Couturier *et al.* identified the existence of a previously unknown family of LPMOs. This new family, named AA14, is distantly related to other LPMO families with the main activity to cleave xylan with oxidation of C-1, which has been demonstrated for two AA14 LPMOs from fungus *Pycnoporus coccineus* [75]. Likewise, another new family was recently created in CAZy database after Sabbadin *et al.* showed the copper-dependent LPMO activity of two AA15 enzymes from *Thermobia domestica* [76].

The broad occurrence of LPMOs indicates biologically important roles that may include tasks beyond breaking down cellulosic and chitinous materials. Genomes of biomass degrading fungi usually encode several LPMO genes with numbers up to over 40. The transcription and expression of fungal LPMOs are influenced by the growth conditions of the organism and seem to be upregulated in the presence of biomass [76–78].

6.1 Substrate preferences

The first LPMO activity discovered was the oxidative cleavage of crystalline β-chitin by CBP21 (chitin-binding protein) [68]. Another study on this enzyme revealed activity on crystalline α-chitin, yet to a lower extent, and that synergy with *Serratia marcescens* chitinases decreased with a lowering degree of crystallinity of the substrate [79]. Further studies uncovered activity of an AA10 from *Streptomyces coelicolor* and an array of AA9s on cellulose [44, 46, 69]. In subsequent studies, LPMO activity on additional substrates has been revealed. For example, NcLPMO9C from *Neurospora crassa* exhibits not only activity on crystalline cellulose but also on soluble cello-oligomers [80]. The same LPMO was found to cleave β-1,4-glucan bonds in hemicellulose, in particular xyloglucan, showing its ability to accept substitutions in various positions in the β-glucan backbone [81]. Later, it was shown that an AA9 from *Myceliophora thermophila* (MtLPMO9A) is active on xylan-coated cellulose and cleaves the β-1,4-xylosyl bonds in xylan as well as the β-1,4-glucosyl bonds in cellulose [82]. All these substrates show a common feature, namely the β-1,4 linkages connecting the single sugar moieties in the backbone. Vu et al. [83] discovered that LPMOs were not only restricted to cleave β-1,4 linkages by showing activity of an *N. crassa* family AA13 LPMO toward starch (i.e. α-1,4 bonds). Later, a starch active LPMO was also identified from the fungus *Aspergillus nidulans* [84]. Thus, LPMO substrates are indeed more diverse that initially assumed. A fast and sensitive spectrophotometric (using 2,6-dimethoxyphenol as chromogenic substrate and H_2O_2 as cosubstrate) assay for LPMO activity determination was developed to follow the production and purification as well as to study enzyme-binding constants or thermal stability [85].

7 Concluding remarks

Fungi involved in complex lignocellulose-containing raw materials degradation express a broad spectrum of enzymes. According to the enzyme composition and degradation mechanisms, three groups of fungi are described: soft-rot, brown-rot, and white-rot fungi. A huge effort has been done and is still ongoing to classify and group these fungi enzymes in a central database called the Carbohydrate-Active enzyme database (or short CAZy). The CAZy classification groups the proteins in families according to amino acid sequence similarity.

In the last few years, investigation attention was focused on the synergistic action of the fungal enzymes involved in the lignocellulose degradation. One of the key players in those actions are LPMOs. These enzymes work in synergy with glycoside hydrolases (GHs) and stimulate their activity by increasing the accessibility to the substrate and enhancing overall polysaccharide conversion efficiency. In addition, LPMOs are able to degrade insoluble polysaccharides such as crystalline cellulose and soluble cello-oligomers. Importance of these enzymes is also confirmed by the phylogenetic investigations. Genomes of lignocelluloses degrading fungi usually encode several LPMOs gens.

Many cultivation strategies were studied to enhance efficiency of lignocellulose-containing raw materials degradation whereas delignification was indicated as the main barrier for the successful lignocellulose utilization. To remove lignin and enhance hemicellulose and cellulose degradation, one of the strategies was the solid state cultivation of brown-rot and white-rot fungi. This strategy approved the hypotheses of the synergistic action of the fungal enzymes and enhancement of the delignification efficiency. However, for the industrial utilization of the

fungi and their enzymes more collaborative effort and synergy has to be attained between molecular and genetic biotechnologies, enzymologist, and bioprocess engineers.

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