Chapter 6

Effect of Metal Ions, Chemical Agents and Organic Compounds on Lignocellulolytic Enzymes Activities

Josiani de Cassia Pereira, Ellen Cristine Giese, Marcia Maria de Souza Moretti, Ana Carolina dos Santos Gomes, Olavo Micali Perrone, Maurício Boscolo, Roberto da Silva, Eleni Gomes and Daniela Alonso Bocchini Martins

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Abstract

Lignocellulolytic enzymes have been extensively studied due to their potential for industrial applications such as food, textile, pharmaceutical, paper, and, more recently, energy. The influence of metal ions, chemical agents, and organic compounds on these enzyme activities are addressed in this chapter, based on data available in the scientific literature.

Keywords: cellulases, hemicellulases, ligninases, enzymatic activities, metal ions, chemical agents, organic compounds

1. Introduction

Lignocellulolytic enzymes comprise cellulases, hemicellulases, and ligninases, which respectively degrade cellulose, hemicellulose, and lignin, the main constituents of plant cell wall, which collectively are named lignocellulose. Cellulases are employed in many industrial sectors, such as textile [1], detergents [2], animal feed, and vinification [2–5]. In the last years, the potential of these enzymes to saccharify cellulose from lignocellulosic residues has been extensively studied aiming the use of glucose for cellulosic ethanol production [6]. Hemicellulases are used in biobleaching of Kraft pulp for paper production [7, 8], bioclarification of fruit juices [9], and obtainment of C5 and C6 sugars from lignocellulosic residues, in the context of second-generation ethanol production [10]. Finally, ligninases are used in paper, textile, cosmetic, and pharmaceutical industries, in bioremediation and wastewater treatment [11, 12],

in organic synthesis, and in biological pretreatment of lignocellulosic residues [13] to be used for cellulosic ethanol production.

Many studies have elucidated how cellulases bind to their substrates, as well as their catalytic mechanisms [14–17]. The modes of action of hemicellulases and ligninases have also been explored [18, 19]. The knowledge about these enzymes activators and inhibitors is also relevant, mainly in the context of industrial applications. Metal ions, for example, influence

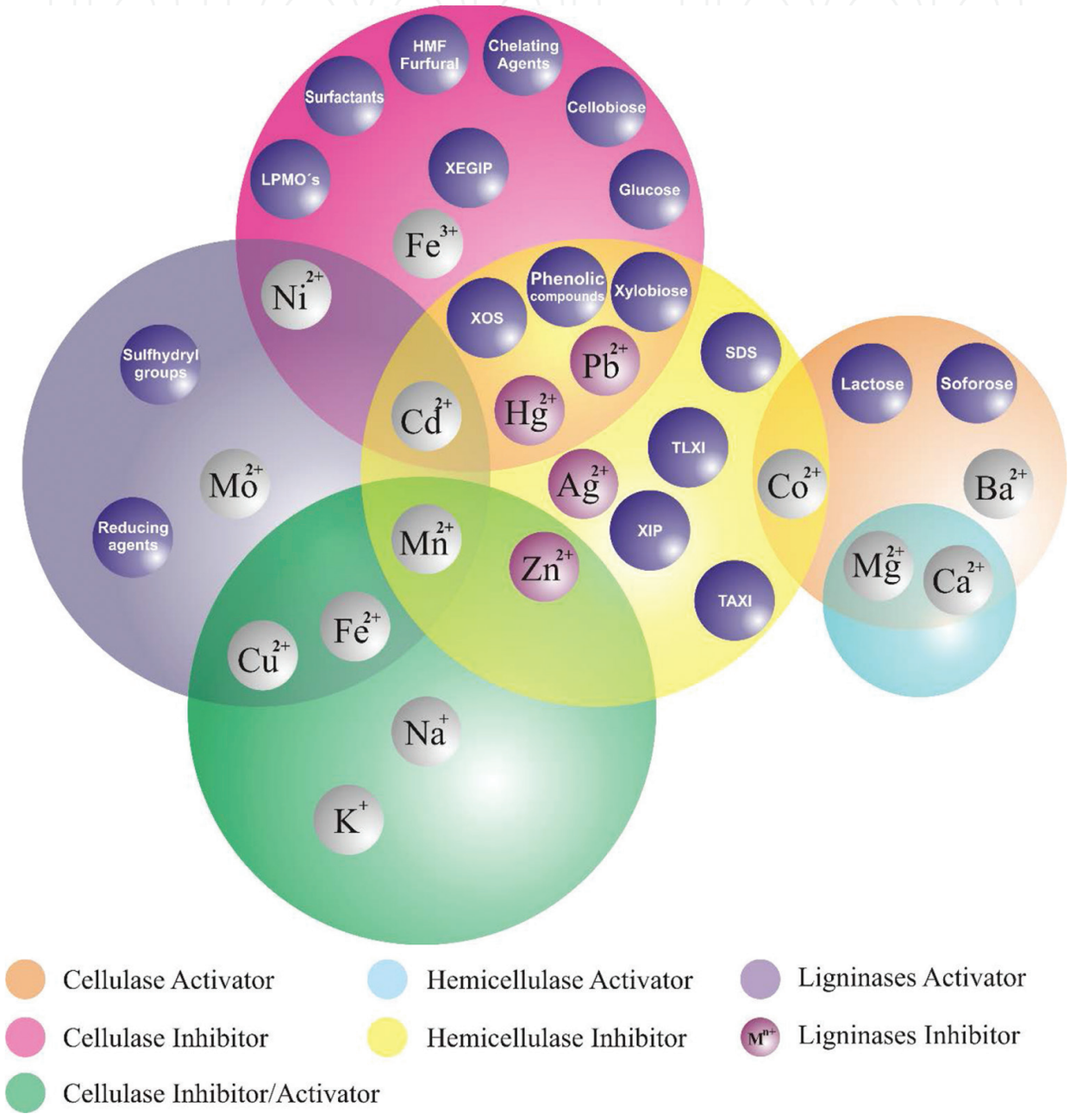

Figure 1. General distribution of activators and inhibitors of lignocellulases. HMF furfural: hydroxymethyl furfural; LPMOs: lytic polysaccharide monooxygenases; XEGIP: xyloglucan-endo-β-glucanase inhibitor proteins; XOS: xylooligosaccharides; SDS: sodium dodecyl sulfate; TAXI: *T*. xylanase inhibitor; XIP: xylanase inhibitor protein; TLXI: thaumatin-like xylanase inhibitor.

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these enzymes activities and may be present in water and/or other reagents employed in industrial processes or may result from equipment corrosion [20]. However, the interference mechanisms are not well understood. There is also a lack of data to corroborate if the inhibition or activation occurs via allosteric or nonallosteric mechanism. So, this chapter presents a brief review of the main activators and inhibitors of lignocellulolytic enzymes, which are summarized in **Figure 1**.

2. Cellulases

Cellulases are glycoside hydrolases produced mainly by microorganisms, especially filamentous fungi. Microbial cellulases include endoglucanases, exoglucanases, and β-glucosidases, which synergistically degrade cellulose.

The glycosidic bonds in cellulose molecule are not easily accessible to the active site of cellulases. So, many of these enzymes are modular, consisting of one or more noncatalytic carbohydrate binding modules (CBMs). CBMs associate the enzyme with the insoluble substrate and are connected to the catalytic module by linker peptides varying in length and structure [21, 22].

Endoglucanases (EG, endo-1,4-β-endoglucanases, E.C. 3.2.1.4) hydrolyze the amorphous fraction of cellulose, releasing cellodextrins and cello-oligosaccharides [22] decreasing the substrate polymerization degree. They are classified into 11 families of glycosil-hydrolases: GH 5, 6, 7, 8, 9, 12, 44, 45, 48, 51, and 74 [23]. Some endoglucanases have affinity with others substrates, besides cellulose, such as xyloglucan, xylan, and mannan [24].

Exoglucanases or cellobiohydrolases (CBH, exo-1,4-β-exoglucanases, E.C. 3.2.1.91) degrade the crystalline fraction of cellulose, releasing cellobiose, and are named Type I or II (action in nonreducing or reducing ends, respectively). Exoglucanases are clustered in two families of glycosil-hydrolases: GH 7 (CBH I) and GH 6 (CBH II) [22].

β-Glucosidases or cellobiases (beta-D-glucosideglucohydrolase, BG, E.C. 3.2.1.21) hydrolyze cellobiose to glucose and also remove the nonreducing terminal β-D-glucosyl residue from glycoconjugates [25].

2.1. Metal ions associate to cellulases activities

Metal ions can be associated to proteins and can also form complexes with other molecules linked to enzymes acting as electron donors or acceptors as Lewis´s acids, or as structural regulators [26]. These ions can either activate or inhibit the enzymatic activity by interacting with amine or carboxylic acid group of the amino acids [27].

Several studies have reported the activation or inactivation of microbial cellulases by metal ions (**Table 1**).

Mono-, di-, and trivalent metal ions such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} are commonly studied in the characterization assays of cellulases [46]. Besides

Enzyme	Microorganism	Activator metals	Inactivating metals	Reference
Endoglucanase	*Aspergillus fumigatus*	Co^{2+} and Mg^{2+}	K^{+}, Mn^{2+}, Na^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Hg^{2+}	[28]
Endoglucanase	*Penicillium simplicissimum* H-11	Mg^{2+} and Sn^{2+}	Cu^{2+}, Co^{2+}, Li^{2+}, Fe^{2+}, Mn^{2+}	[29]
Endoglucanase	*Aspergillus niger*	Ca^{2+} and Mn^{2+}	Co^{2+}, Fe^{2+}, Cu^{2+}	[30]
Endoglucanase	*Aspergillusniger*ANL301	Mn^{2+}, Fe^{2+},	Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}	[31]
Endoglucanase exoglucanase	*Aspergillus niger NRRL 567*	Zn^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}	Mg^{2+}, Fe^{2+}, Hg^{2+}	[32]
Endoglucanase		–	Cu^{2+}	
Exoglucanase		Cu^{2+}		
Endoglucanase	*Daldiniaeschscholzii* (Ehrenb.:Fr.)	Ca^{2+}, Co^{2+}	Hg^{2+}, Cu^{2+}, Fe^{2+}	[33]
β-Glucosidase	*Melanocarpus* sp.	Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}	Cu^{2+}	[34]
Cellobiohydrolase	*Trichoderma reesei*	Mn^{2+}, Ba^{2+}, Ca^{2+}	Hg^{2+}	[35]
β-Glucosidase	*Aspergillus niger*322	–	Pb^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}	[36]
Endoglucanase	*Penicillium pinophilim* MS20	Co^{2+}, Zn^{2+}, Mg^{2+}	Na^{+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Cd^{2+}	[37]
Endoglucanase	*Mucor circinelloides*	Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}	Mn^{2+}	[38]
β-Glucosidase	*Penicillium citrinum* YS40-5	Na^{+}	Zn^{2+}, Cu^{2+}	[39]
β-Glucosidase	*Fusarium oxysporum*	Mn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}	Hg^{2+}	[40]
β-Glucosidase	*Monascus sanguineus*	–	Ca^{2+}, K^{+}	[41]
Exoglucanase	*Aspergillus fumigatus*	Ca^{2+}, Mg^{2+}, Zn^{2+}	–	[42]
Cellobiohydrolase	*Penicillium purpurogenum*KJS506	–	Fe^{2+}, Hg^{2+}	[43]
Cellobiohydrolase	*Agaricus arvencis*	Ca^{2+}, Cu^{2+}, Mg^{2+}	Zn^{2+}	[44]
Endoglucanase	*Aspergillus terreus*	Cu^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}	Fe^{2+}, Mn^{2+}, Zn^{2+}, K^{+}	[45]

Table 1. Effect of metal ions on microbial cellulases activities.

ionic charge, ion radius size has a great influence on the activity and stability of the enzyme. It was demonstrated that larger radius has less influence on catalytic amino acids, while the smaller radius can more intensely attract charged amino acids changing the enzyme's overall conformation with damage on the catalytic site [47, 48].

The studies reported inhibitory effects of Fe^{2+} and Cu^{2+} on endoglucanases, exoglucanases, and β-glucosidases activities. However, the effect of other divalent ions on cellulases activities seems to be variable among the enzymes secreted by different microorganisms (e.g., **Table 1**). The effect of divalent ions on cellulases is not well elucidated, and possibly occurs by redox effects on the amino acids, increasing or decreasing their activities [49].

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Inhibition of cellulases by Hg^{2+} is related to the interaction with catalytic amino acid residues containing sulfur, leading to oxidation and irregular formation of disulfide bonds [45, 46, 49]. Fe^{2+} can complex with D/L-lysine and L-methionine [50], Cu^{2+} with histidine [51], and Ba^{2+} with arginine, glutamine, proline, serine, and valine [52].

Sajadi [53] evaluated the interaction of amino acids, such as arginine and glutamine, with metal ions and established the following order of interaction degree: $Ca^{2+} < Mg^{2+} < Mn^{2+} < Co^{2+} < Cu^{2+} > Zn^{2+}$.

2.2. Chemical agents and organic compounds associate to cellulases activities

Cellulases activities may also be affected by drugs (2,3-dichloride-1,4-nafthoquinone, for example), fungicides (such as phenylmercury acetate and ethylen-bis-dithiocarbamate), antibiotics and disinfectants (Phenylmercury nitrate and 8-hydroxiquinoline, among others), sugars (final product inhibition), protein (such as those secreted by plant as defense mechanism), CBM-binding organic compounds, products from sugar and lignin degradation (such as phenolic compounds) [54], food additives (such as Octyl gallate), plant hormones (auxins, such as indoleacetic acid), and ionic solids (Sodium azide) [55–58].

Cellulose degradation products such as cello-oligosaccharides and cellobiose can inhibit endo- and exoglucanase activities, respectively. Endoglucanases that act on xyloglucan and xylan can be inhibited by the xylooligomers released [59]. The addition of xylanase to the reaction media is an alternative to remove these products [60]. The inhibition of β-glycosidases activities by glucose is frequently observed [6, 61]. Disaccharides such as cellobiose and xylobiose, and monosaccharides such as mannose and galactose can inhibit some exoglucanases activities [22, 59, 62].

Gluconolactone, resulting from cellulose oxidation by lytic polysaccharide monooxygenases (LPMOs) activities, can act as β-glycosidases inhibitor. Cellobiose and also other substrates of β-glycosidases compete with gluconolactone and other LPMO-degrading products [63–65]. On the other hand, β-glycosidases can be activated by soforose and lactose [66, 67].

It is relevant to consider that sugars released by enzymatic hydrolysis of lignocellulose can be degraded and converted into inhibitory compounds. Under acidic conditions, glucose, mannose, and galactose can be converted into furan aldehydes such as hydroxymethylfurfurals (HMF). HMF, in turn, can be converted into levulinic and formic acids [68].

Lignin degradation during the hydrolysis of some lignocellulosic materials such as alkali or acid pretreatment, or else during enzymatic hydrolysis (by laccases action) can release phenolic compounds [68] such as vanillin, syringaldehyde, trans-cinnamic acid, and hydroxybenzoic acid. These compounds are potential inhibitors of endo/exoglucanases and β-glycosidases activities due to the presence of hydroxyl, carbonyl, and methoxyl groups [69, 70].

As mentioned above, another class of cellulolytic inhibitors has a proteic origin. Specific xyloglucan endo-β-glucanase inhibitor proteins (XEGIPs) are presented in the cell walls of some vegetables such as tomatoes, tobacco, and wheat and inhibit endoglucanases that act on xyloglucan [71–73]. These proteins are part of the plant protecting mechanism against pathogens and act by forming high-affinity complexes with the enzyme [73].

Another factor that affects the catalysis by cellulases is the enzymes interaction with lignin, the phenomenon called "nonproductive adsorption" or "nonspecific binding." Cellulases can adsorb lignin through their CBMs [21, 74–77], more specifically by its alanine residues [76]. Some cellulases show higher catalytic activity when CBMs are removed by decreasing nonproductive adsorption on lignin [74].

Nonproductive adsorption of cellulases on lignin can also be decreased by adding surfactants to the reaction media, which increases the efficiency of enzymatic catalysis [78–81]. Tween 20, 40, 60, 80, and 100, Triton X-100, polyethylene glycol (PEG), among others surfactants, tend to decrease the surface tension of aqueous systems, which may alter the properties of liquids such as detergency, emulsification, greasing, and solubilization. Surfactant properties can decrease the nonproductive adsorption of cellulases on lignin, acting as "activators agents" of these enzymes [78].

Chelating agents such as EDTA (ethylene diamine tetra acetic acid), ethylene glycol (or β-mercaptoethanol), and DPPE (1,2-bis diphenylphosphino-ethylene) may activate some enzymes activities, especially cellulases, by sequestering inhibitors' metal ions from the aqueous system [82]. When chelating agents complex with metals in the reaction media, the active site of enzyme is available to react with the substrate, which represents the positive effect of these compounds on cellulases activities. In contrast, the negative effect of chelating agents on enzymatic activity suggests that enzyme activities depend on the inorganic ion that was sequestered [20, 33, 45].

3. Hemicellulases

Since hemicellulose is very heterogeneous, its complete degradation requires the synergic action of several enzymes, mainly endoxylanases and β-xylosidases as well as a variety of accessory enzymes that act in substituted xylans and include α-D-glucuronidases, acetyl xylan esterases, ferulic acid esterases, α-galactosidases, acetyl mannan esterases, and α-L-arabinofuranosidases [83].

α-L-Arabinofuranosidases (EC 3.2.1.55.; AFases) are exopolysaccharide hydrolases which remove side chains containing arabinose residues linked by α-1,2, α-1,3, and α-1,5 glycosidic bonds to the main chain of arabinananas or arabinoxylans [84]. AFases are grouped into six families of glycoside hydrolases: GH 3, 10, 43, 51, 54, and 62 [85]. A variety of AFases have been purified from fungi, bacteria, and plants [86–88]. These enzymes' activities can be affected by metal ions, ionic and nonionic detergents, and by chelating and reducing agents [85].

Xylans with acetyl and methyl glucuronic acid (MeGlcA) as substituents groups are named *O*-acetyl-4-*O*-methylglucuronoxylans. On the other hand, when α-4-0-methylglucuronic acid and α-arabinofuranose are the substituent groups, xylans are named as arabino 4-*O*-methylglucuronoxylan [89]. α-glucuronidases (EC 3.2.1.139.) hydrolyze α-1,2-glycosidic bond of MeGlcA in the side chain [90]. Among xylan-degrading enzymes, α-glucuronidases are the less studied and characterized ones. They are grouped into three families of glycosyl-hydrolases: GH 4, 67, and 115 [91].

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Endoxylanases (E.C. 3.2.1.8; endo-β-1,4-xylanases) hydrolyze β-1,4 glycosidic linkages in the backbone of xylans that are composed of xylose residues [92]. According to the similarities of amino acid sequences, the majority of xylanases are grouped into glycoside hydrolases (GH) families 10 and 11 and are also classified into families GH 5, 7, 8, and 43 [93].

β-Xylosidases (E.C. 3.2.1.37; β-1,4-xylosidases) release β-D-xylopyranosyl residues from the nonreducing end of xylobiose and some small 4-β-D-xylooligosaccharides [92]. These enzymes have been classified into 10 families: GH 1, 3, 30, 39, 43, 51, 52, 54, 116, and 120, based on the predicted structural motifs of the enzyme's catalytic domain. β-Xylosidases play a crucial role in endoxylanases activities, since their substrates, such as xylobiose, can inhibit endoxylanases action [94, 95].

3.1. Metal ions associate to hemicellulases activities

The inhibitory effect of Hg^{2+} on AFases activities has been reported [96–99]. Besides Hg^{2+}, Ag^{2+}, and Pb^{2+} are mixed inhibitors, which do not bind to the active site, but to another region of the enzyme, and thus do not interfere with substrate binding to the catalytic site. In addition, Hg^{2+} is known to react with histidine and tryptophan residues, reducing the enzyme availability to metabolic function [100]. Zn^{2+}, Cd^{2+}, and Co^{2+} have also been described as potential inhibitors of AFases [88, 99, 101].

Most scientific works about α-glucuronidases purification and characterization report that these enzymes do not require metal ions for their activities [102–106]. On the other hand, various metal ions exert inhibitory effects on α-glucuronidases activities, such as Ag^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, and Fe^{3+} (e.g., **Table 2**).

Some GH 10 family enzymes require metal ions for their stability and activities. For example, *Pseudomonas fluorescens* sub sp. produces a xylanase that is one of the first GH 10 enzymes found to contain a calcium-binding site [93]. On the other hand, there are many GH 43 enzymes with crystal structures that showed tightly bound metal ions such as Ca^{2+}, with structural roles [107]. Besides, many studies have reported the apparent activation of fungal β-xylosidases by Mn^{2+} and Ca^{2+}, suggesting that these ions activate and protect the active site [95].

The negative effect of heavy metals, such as Hg^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Ag^{2+}, Cu^{2+}, and Pb^{2+} on xylanases activities have been reported [108]. Inhibition by heavy metal ions (such as Zn^{2+}, Pb^{2+}, and Hg^{2+}) may occur due to the formation of a complex with the reactive groups of the enzyme. Metals from group Ilb exhibit high affinity for SH, $CONH_2$, NH_2, COOH, and PO_4 [109]. Furthermore, inhibition of xylanase by Hg^{2+} has been reported as related to the presence of tryptophan residues, which oxidize indole ring, thereby inhibiting the enzyme activity [110]. Xylanase from *Bacillus halodurans* TSEV1 was strongly inhibited by Hg^{2+}, Cu^{2+}, and Pb^{2+}, probably due to the catalysis of the cysteine thiol group autooxidation, which leads to the formation of intra- and intermolecular disulfide bonds or to the formation of sulfenic acid [111].

3.2. Chemical agents and organic compounds associate to hemicellulases activities

Some authors have reported that the addition of chelating agents such as EDTA and reducing agents such as β-mercaptoethanol and DTT (dithiothreitol) does not affect AFases activity [85,

Metal ions	Microorganism	Referees
Ag^{2+}	*Bacillus stearothermophilus*	[105]
	Saccharophagus degradans 2-40	[106]
Zn^{2+}	*Bacillus stearothermophilus*	[105]
Cd^{2+}	*Thermotoga maritime*	[104]
Hg^{2+}	*Thermotoga maritime*	[104]
	Bacillus stearothermophilus	[105]
	Aspergillus niger	[102]
	Helix pomatia	[103]
	Saccharophagus degradans 2-40	[106]
Mn^{2+}	*Thermotoga maritime*	[104]
	Bacillus stearothermophilus	[105]
	Aspergillus niger	[102]
Fe^{2+} and Fe^{3+}	*Aspergillus niger*	[102]
Ni^{2+}	*Bacillus stearothermophilus*	[105]
	Saccharophagus degradans 2-40	[106]
Cu^{2+}	*Thermotoga maritima*	[104]
	Bacillus stearothermophilus	[105]
K^{+}	*Geobacillus stearothermophilus*	[105]

Table 2. Metal ions that exert inhibitory effects on α-glucuronidases activities.

99, 112]. Such agents are well known as inhibitors of thiol groups, and these data suggest that sulfhydryl groups are not related to the active site of AFases.

There are few studies reporting the action of ionic detergents in AFases activities. At low concentrations (1–2 mM), ionic detergents such as SDS can stimulate the enzyme activity, whereas in higher concentrations (20 mM) they can cause an inhibitory effect [113]. Since SDS interferes in hydrophobic regions of the enzyme, it alters its three-dimensional structure [114], indicating that these concentrations may be critical and cause enzyme denaturation.

Among the compounds that significantly activate the enzyme activity there are 2-mercaptoethanol, DTT (dithiothreitol), L-cysteine, and NAD^{+} indicating that these reducing agents are required for maximal activities of α-glucuronidases [115]. Some of the family 4 enzymes are known to be NAD^{+} dependent. The role of NAD^{+} for the activity of the hydrolytic GHF4 is not well known. The pyridine nucleotide cofactor could have structural and/or catalytic function and, in addition, could also be important for the regulation of enzyme activity [116].

Xylanases have received great attention in recent years, mainly due to their potential for the application in the processes of xylooligosaccharides (XOs) production, pulp bleaching,

removal of antinutritional factors of animal feeds, bread making (improving the separation of wheat or other cereal gluten from starch), juice extraction from fruits or vegetables, clarification of fruit juices and wines, and extraction of more fermentable sugar from barley to produce beer [111, 117].

Xylanase proteic inhibitors might hamper their efficacy when used in industrial application. Two distinct types of xylanase inhibitors have been identified in barley, wheat, and rye: XIP (xylanase inhibitor protein), a monomeric and glycosylated protein (XIP-I most widely studied in the XIP class), that can inhibit all GH 10 and GH 11 fungal xylanases, except that from *Aspergillus aculeatus*. The other type of xylanase inhibitor, TAXI (*Triticumaestivum* xylanase inhibitor) is a mixture of two proteins, TAXI I and TAXI II, which differ according to xylanase specificities and p*I*. TAXI inhibitors seem to be specific for GH 11 bacterial and fungal xylanases. More recently, a third class of inhibitor called TLXI (thaumatin-like xylanase inhibitor) also purified from wheat, showed variable activities against most of GH 11 xylanases and does not inhibit GH 10 microbial xylanases [117, 118].

Many other substances, such as EDTA (a chelating reagent), β-mercaptoethanol, and DTT (both disulfide bonds reducing agents) have been extensively investigated regarding their influence on xylanases activities. Xylanase from *Talaromyces thermophile* is inhibited by EDTA and DTT, suggesting that disulfide bonds are essential to maintain the enzyme conformation [119]. On the other hand, the activation of xylanases in the presence of β-mercaptoethanol and DTT was reported and indicates the presence of a reduced thiol group of cysteine in these enzymes [120].

The effect of different modulators on the activity of xylanase from *B. halodurans* TSEV1 has been investigated. These modulators include *N*-bromosuccinimide (N-BS), ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC), iodoacetate (IAA), and Woodward's reagent K (WRK). The inhibition of xylanase activity in the presence of NBS suggests the presence of tryptophan residues in their active site. EDAC and WRK inhibited the enzyme activity, which indicates the importance of carboxylic groups in enzyme catalysis [111].

Treatments for deconstruction of the lignocellulosic structure are frequently employed in the use of biomass as sugar's source for ethanol production and can generate besides soluble sugars, other sources such as furan derivatives, organic acids, and phenolic compounds that can act as xylanases inhibitors, as described for cellulase [121].

Significant inhibition of xylanase activity by vanillic acid, syringic acid, acetosyringone, and syringaldehyde has been observed [121]. Boukari et al. [122] reported that endoxylanase from *Thermobacillus xylanilyticus* was inhibited by phenolic compounds, including cinnamic acid, p-coumaricacid, caffeic acid, ferulic acid, and 3, 4, 5-trimethoxy-cinnamic acid by the noncompetitive multisite inhibition mechanism.

Studies on the inhibitory effect of sugars on xylanases (mainly β-xylosidases) are essential for a better understanding about the decrease in the enzyme activity during biomass conversion. This kind of inhibition was subject of research for a long time, bringing up many different opinions about its mechanism. Jordan et al. [123] studied the active site of the GH 43 β-xylosidase from *Selenomonas ruminantium* and reported that it comprises of two subsites and

a single access route for ligands. The authors classified the inhibitors into two groups: I, single binding inhibitors including cellobiose (4-O-β-D-glucopyranosyl D-glucose), D-glucose, maltose (4-O-a-D-glucopyranosyl-D-glucose), D-xylose, and L-xylose; II, double binding inhibitors including D-arabinose, L-arabinose, D-erythrose, and D-ribose. Both groups have presented competitive or noncompetitive inhibition.

4. Ligninolytic enzymes

Microorganisms that colonize on living and decaying wood are capable of producing oxidative extracellular enzymes which together play a fundamental role in lignin biodegradation. The ligninases, or lignin-degrading enzymes, can oxidize lignin and several related compounds, e.g., environmental pollutants containing polycyclic aromatic hydrocarbons, dyes, and chlorophenols [124].

Lignin-peroxidase (LiP, E.C. 1.11.1.14), manganese-peroxidase (MnP, E.C. 1.11.1.13), and laccase (E.C. 1.10.3.2) are the major lignin-modifying enzyme systems of white-rot fungi and have also been described in actinomycetes and bacteria. These enzymes oxidize phenolic compounds and reduce molecular oxygen to water, generating intermediary radicals as illustrated in **Figure 2** [125, 126].

Accessory enzymes involved in the main reactions of degradation of lignin have also been described and comprise the following: cellobiose-quinoneoxireductase (E.C. 1.1.5.1), aryl alcohol oxidase (E.C. 1.1.3.7), glyoxal oxidase (GO, E.C. 1.2.3.5), manganese-independent peroxidase (E.C. 1.11.1.7), versatile peroxidase (VP, E.C. 1.11.1.16), and cellobiose dehydrogenase (E.C. 1.1.99.18) [127, 128].

Besides ligninolytic enzymes have been used to reduce the lignin content in several feedstock and to degrade recalcitrant aromatic compounds, due to the high chemical similarity of these compounds with lignin [13, 129, 130], the lignin-degrading enzymes have been applied in various industries such as textile dye bleaching, pulp and paper delignification, food, brewery, animal feed, laundry detergents, and xenobiotic compound degradation. Phenol oxidases such as laccases, particularly, have been applied in immunoassay, biosensors, biocatalysts, and oxygen cathode manufacturing [127, 131].

The performance of these enzymes is easily affected by environmental factors including metal ions and other chemical compounds usually found in the aforementioned industries. Ligninases with stronger tolerance to metal ions and organic solvents exhibit high potential for the application in the recalcitrant xenobiotics biodegradation and also improve the effectiveness of biotechnological and industrial enzymatic process [132, 133].

4.1. Laccases (E.C. 1.10.3.2)

Laccases are multicopper blue oxidases that catalyze the one-electron oxidation of a wide range of substrates with a concomitant four-electron reduction of molecular oxygen to water [126]. The active site of laccase comprises four copper atoms in three groups: T1 (mononuclear

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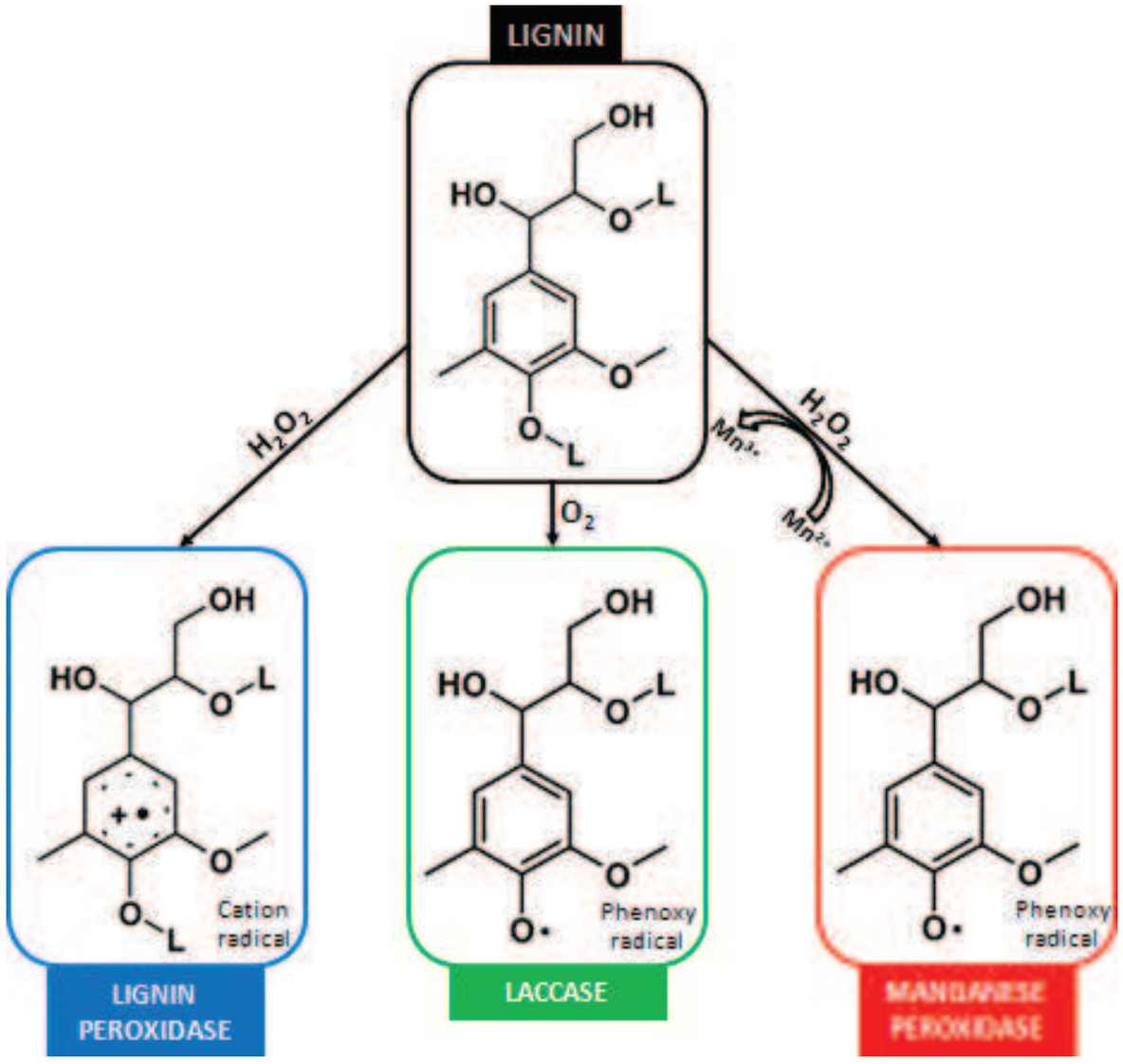

Figure 2. Simplified reactions of lignin peroxidase, manganese peroxidase, and laccase.

copper), T2 (normal copper), and T3 (coupled binuclear copper). The T1 and T2 Cu^{2+}-sites contribute as the primary electron acceptors while T3 is reduced by an intramolecular two-electron transfer from T1 and T2 Cu^{2+} sites [126, 134].

4.1.1. Metal ions associate to laccase activity

Although laccases are efficient on a wide range of substrates without cofactors, in most cases, the addition of Cu^{2+}, Cd^{2+}, Ni^{2+}, Mo^{2+}, and Mn^{2+} ions increases the activity of laccases, whereas Ag^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, NaN_3, NaCl, and H_2O_2 inhibit their activity [126].

Apart from the inhibition problem, the influence of metal ions on the performance of enzyme-catalyzed reaction is also important, in addition to the study of effects of single metal ions on the enzyme activity. Lu et al. [135] observed that monovalent and trivalent metal ions inhibited the 4-nitrophenol degradation by laccase-Cu^{2+}, as well as the addition of low concentrations

of divalent ions. The suppressive effects of cations on laccase activity comprised $Mg^{2+} > Na^{+} > Al^{3+} > K^{+} > Mn^{2+} > Hg^{2+} > Co^{2+}$.

4.1.2. Chemical agents and organic compounds associate to laccase activity

The Michaelis-Menten equation has been suitably used to describe the laccase kinetics and apparent binding constant (K_m) and maximal reaction rate (V_{max}) values. In water-miscible solvents, these kinetic parameters can be affected by the changes in water thermodynamic activity. In the case of laccase from the white-rot fungus *Phlebiaradiata*, e. g., pK_l values show the linear dependence on solvent hydrophobicity (log*P*) in a system of 2,6 dimethoxyphenol as substrate in the presence of methanol, ethanol, *n*-propanol, acetonitrile, acetone, and DMSO [136].

Previously, the changes in V_{max} by the addition of solvents have been compared to free and immobilized laccases. The activity of laccase from *P. radiata* was rather similar to both forms of the enzyme in the presence of 10% of ethanol, methanol, acetone, DMSO, and dioxane. The immobilized laccase was less vulnerable to Cu-chelatorthioglycolic acid, 2,6-dimethoxy-1,4-benzoquinone [128, 137].

In the conditions of low water content, which is the case of water/organic mixtures, the values of the apparent K_m tend to grow exponentially with water concentration. The apparent V_{max} of immobilized laccase from *Coriolusversicolor* increased two orders of magnitude values with a linear increase in water content [138].

4.2. LiP (E.C. 1.11.1.14)

Lignin-peroxidases are heme-containing glycoproteins that contain Fe^{3+} in their active site. LiP catalyzes the H_2O_2-dependent oxidative depolymerization of nonphenolic lignin and lignin-model compounds as well as a variety of phenolic compounds [139].

4.2.1. Metal ions, chemical agents, and organic compounds associate to LiP activity

The decrease in LiP activity is described as inhibition or denaturation according to the concentration of inhibitor compounds in an aqueous reaction system. The hydrogen bonding and anion stabilization are important characteristics to describe the effect of compounds on the active sites of enzymes, as well as water activity (a_w), log*P*, and solvation [140].

The addition of Cu^{2+}, Mn^{2+}, and Fe^{2+} ions increases the activity of LiP, whereas Ag^{2+} inhibit their activity [141]. On the other hand, different solvents and organic compounds have been described as LiP potential inhibitors: alcohols, aldehydes, ketones, esters, ethers, amines, acids, amides, acetonitrile, cysteine, DMSO, EDTA, DMF, TEMED, CTAB, sodium azide, and H_2O_2 [140–144].

Vazquez-Duhalt et al. [145] chemically modified a LiP from the white-rot fungus *Phanerochaete chrysosporium* by reductive alkylation with benzyl, naphthyl, and anthracyl moieties, thereby increasing its superficial hydrophobicity. These modifications altered the kinetics and increased the yield of oxidation of pyrroles, pyridines, and aromatic amines in 10% acetonitrile.

4.3. MnP (E.C. 1.11.1.13)

Manganese-peroxidases catalyze the H_2O_2-dependent oxidation of Mn^{2+} into Mn^{3+}, which is stabilized by fungal chelators such as oxalic acid or different organic acids. Then, the oxidation of various phenolic substrates (e.g., amines, dyes, lignin related compounds) occurs under the action of chelated Mn^{3+} ions that comprise a diffusible charge-transfer mediator in these reactions [141, 146].

4.3.1. Metal ions associate to MnP activity

MnP activity is completely inhibited by Hg^{2+}, Pb^{2+}, Ag^{+}, lactate, NaN_3, $CaCl_2$, TEMED, ascorbic acid, β-mercaptoethanol, and dithreitol [147, 148]. Partial inhibition of MnP activity was observed with EDTA, a metal chelating compound that complexes with inorganic cofactors and prosthetic groups of enzymes. High concentrations of Cu^{2+} and Fe^{2+} (~4 mM) could enhance MnP activities [148]. Youngs et al. [149] related that Cd^{2+} is a reversible competitive inhibitor of Mn^{2+} to MnP activity. The inhibition was not observed in reaction systems containing 2,6-dimethoxyphenol or guaiacol in the absence of Mn^{2+}.

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Author details

Josiani de Cassia Pereira[1]*, Ellen Cristine Giese[2], Marcia Maria de Souza Moretti[1], Ana Carolina dos Santos Gomes[1], Olavo Micali Perrone[1], Maurício Boscolo[1], Roberto da Silva[1], Eleni Gomes[1] and Daniela Alonso Bocchini Martins[3]

*Address all correspondence to: josianipereira@gmail.com

1 São Paulo State University, UNESP, Cristovão Colombo, CEP, São José do Rio Preto, SP, Brazil

2 Coordination for Metallurgical and Environmental Process, Centre for Mineral Technology, CETEM, Pedro Calmon, CEP, Rio de Janeiro, RJ, Brazil

3 São Paulo State University, UNESP, Prof. Francisco Degni, CEP, Araraquara, SP, Brazil

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