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DEPOLYMERIZATION OF KRAFT LIGNIN WITH LACCASE AND PEROXIDASE: A REVIEW

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Abstract
Lignin is a complex aromatic polymer of phenyl propene units non-linear and randomly linked. The main building blocks are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is the third most abundant biopolymer on earth and usually accounts for 15-35% of the lignocellulose biomass. The degradation of lignin is extremely difficult due to the complexity of the chemical structure (variable upon the source) and the high molecular weight. Two major types of enzymes involved in the depolymerization of lignin are oxidoreductase: laccase and peroxidase, the main microbial producers being fungi and some bacteria. Due to its highly branched structure, lignin is considered to be the most recalcitrant component of lignocellulose, most of it not being recovered. Therefore, there's a demand for more effective methods for depolymerization of lignin in order to obtain value-added products. This review underlines the importance of valorization of lignin through enzymatic depolymerization with laccase and peroxidase.

Key words*: kraft, laccase, lignin, peroxidase.*

INTRODUCTION

Lignocellulose presents a special interest due to its structure and composition, consisting of complex biopolymers, such as: cellulose, hemicellulose and lignin, materials with potential applicability for energy production (Mitache et al., 2015).
Although cellulose and hemicellulose are relatively easy to hydrolyse to obtain their subunits, lignin depolymerization is difficult mainly because of its amorphic and complex three-dimensional structure and of its characteristics that make itself a binding polymer of the cells, fibres and vessels in wood or lignified parts of the plant.
Lignin plays an important role in plant's resistance, providing defence against pathogen attack, mechanical support, stress response and water transport (Li et al., 2016; Boerjan et al., 2003; Kilpeläinen et al., 2007).
Therefore, there's an imperative need to remove lignin from the biomass in order to have access to cellulose and hemicellulose. Initially, lignocellulose biomass was delignified through chemical pathways, but since lignin accounts for 15 – 30% of the biomass it wasn't an economical process, so scientists started to search for ways to valorise lignin with enzymatic degrading systems that will not affect cellulose and hemicellulose.
This review is focused on the importance of degradation of kraft lignin and highlights the main enzymes involved in the depolymerisation: laccase, lignin peroxidase, and manganese peroxidase.

LIGNIN

Native lignins have certain variations in their chemical composition based upon their source, thereby making it difficult to define the precise structure of lignin.
Generally, lignin is considered to be a network polymer containing building blocks of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (as observed in Figure 1), that are non-linear and randomly linked.

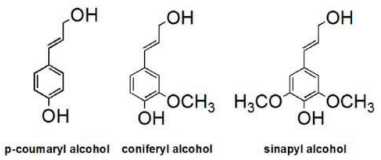

Figure 1. Lignin precursors

Kraft-lignin is a by-product obtained in the pulp and paper industries, through a Kraft

process. Kraft lignin differs considerably from natural lignin in its structure and chemical composition, but given the fact that native lignin's structure is so variable and complex, kraft lignin is often used as a substitute model for native lignin.

The content of lignin in the cell wall structure is the most significant contributor for the biomass recalcitrance to microbial and enzymatic deconstruction (Li et al., 2016). Over the years, several biological and chemical methods were conducted in order to convert lignin in value added products. One of the major challenge in using chemical methods for lignin degradation is related to catalyst selectivity (Xu et al., 2014), being difficult to develop a general catalyst that can specifically work on native lignins structures that are so randomly variable. On the other hand, biological methods cannot compete with chemical processes involved in lignin depolymerization because in the end they can't produce the desired products more economically. But, biological methods are still preferred due to their selectivity and mild reaction conditions. Therefore, enzymatic deconstruction of lignin is somewhat a less studied field that requires more research.

Being a large heterogeneous polymer and not containing hydrolysable linkages, lignin degradation requires the action of extracellular enzymes and more important oxidative enzymes (Hatakka, 2005).

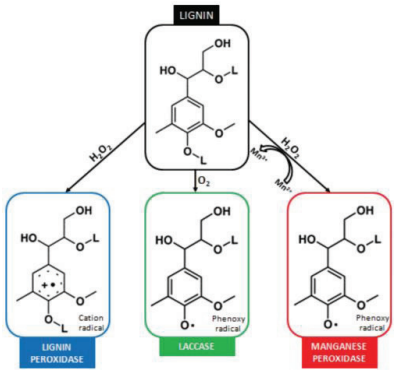

Figure 2. Main enzymes used for lignin depolymerization
Source: de Cassia Pereira et al., 2017

The main enzymes involved in lignin depolymerization are: lignin peroxidase, manganese peroxidase and laccase (Figure2). Other enzymes include: horseradish peroxidase and dioxygenases (protocatechuate 3,4-dioxygenase; 1,2,3-trihydroxybenzene 1,2-dioxygenase and catechol 1,2-dioxygenase) (Octavio et al., 2006).

These ligninolytic enzymes have an immense potential for several industrial and biotechnological processes, such as: food industry, textiles, pulp and paper industry, bioremediation, medical, pharmaceutical cosmetic applications etc. (Maciel and Ribeiro, 2010; dos Santos Barbosa et al., 2008; Kunamneni et al., 2008; Maijala et al., 2007).

LACCASE

Laccase (benzenediol: oxygen oxidoreductases, E.C. 1.10.3.2) is one of the most studied enzyme (Desai and Nityanand, 2011).

Laccases are multi-copper containing enzymes belonging to blue oxidases group and are able to catalyse one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water (Gochev and Krastanov, 2007).

Unlike most enzymes, laccases have the ability to display their activity on a wide range of substrates like monophenols, diphenols, polyphenols, methoxyphenols, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Desai and Nityanand, 2011; Ai et al., 2015).

Laccases from fungi are identified by their capacity to oxidize different substrates such as guaiacol, remazol brilliant Blue R, tannic acid, Poly R-478 etc. to specific coloured products (Desai and Nityanand, 2011).

Even though laccases have a broad substrate specificity on phenolic compounds, they cannot work on non-phenolic sub-units, unless in the presence of mediators - low molecular - weight organic compounds that act as „electron shuttles" (Desai and Nityanand, 2011).

When laccase, cannot oxidize alone a substrate, it will first oxidize a mediator that will form highly reactive and unstable cationic radicals, which will diffuse away from the enzymatic pocket and will oxidize more complex substrates that could not enter into the active site due to their size. After that, the co-mediator (oxidized mediator) will return to its original state and the electrons taken by laccases are

finally transferred back to oxygen to form water (as shown in Figure 3) (Desai and Nityanand, 2011; Gochev and Krastanov, 2007).

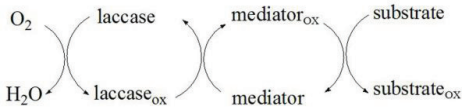

Figure 3. Mechanism of substrate oxidation by laccase with a mediator
Source: Christopher et al., 2014

The most often used mediators are: 2,2`-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (HBT), benzotriazole (BT), remazol brilliant blue (RBB), chlorpromazine (CPZ), promazine (PZ), 1-nitroso-2-naphtol-3,6-disulfonic acid (NNDS), N-hydroxyphtalimide (NHPI), 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS), 3-hydroxyanthranilic acid, N-hydroxyacetanilide (NHA), violuric acid (Octavio et al., 2006; Desai and Nityanand, 2011; Gochev and Krastanov, 2007; Lange et al., 2013; Brijwani et al., 2010).
Laccases can display their activity on a wide range of temperatures and pH. Optimum pH value is variable based on the reactions caused by the substrate employed, molecular oxygen or the enzyme itself (Desai and Nityanand, 2011). Laccases have a significant biotechnological potential due to their broad substrate specificity being used in: environmental bioremediation (removal of pollutants, such as alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene) (Gochev and Krastanov, 2007), food and beverages, biosensors, pulp and paper industry (pulp delignification), transformation of antibiotics and steroids, detergent manufacturing bioethanol (Octavio et al., 2006; Brijwani et al., 2010).

Mechanism of action

Laccases contain 4 copper atoms, that are classified into three types, referred to as type 1 (T1), type 2 (T2) and type 3 (T3). The copper atoms differ from each other in their electron paramagnetic resonance (EPR) signals (Gochev and Krastanov, 2007).
The type 1 Cu is responsible for the blue colour of the protein, type 2 Cu does not confer colour and the type 3 Cu atoms consists of a pair of Cu atoms in a binuclear conformation (Desai and Nityanand, 2011).
Type 2 and Type 3 copper sites forms a trinuclear centre responsible for the catalytic mechanism of the enzyme (Desai and Nityanand, 2011).
Laccase catalysis pathway implies three major steps: the type 1 Cu is reduced by a reducing substrate, the electron is transferred from the type 1 Cu to the trinuclear cluster of type 2 Cu and type 3 Cu and at the trinuclear centre will take place the reduction of oxygen to water (Brijwani et al., 2010).
Laccase is considered to act as a battery, that stores electrons from individual oxidation reactions in order to reduce molecular oxygen. Therefore, there are required four molecules of reducing substrate for the complete reduction of molecular oxygen to water (Desai and Nityanand, 2011).

Sources

Amongst all of the large blue copper containing proteins, laccases are the most widely distributed in sources such as: bacteria, fungi, higher plants and insects (Desai and Nityanand, 2011; Gochev and Krastanov, 2007).
Laccase was first characterized when it was extracted from the Japanese lacquer tree *Rhus vernicifera* in 1883. Later, in 1896, it was demonstrated that laccases were also present in fungi (Desai and Nityanand, 2011).
In higher plants, laccases can be found in *Rhus vernicifera*, *Rhus succedanea*, *Lactarius piperatus*, *Prunus persica* (Octavio et al., 2006), *Acer pseudoplatanus, Chaetomiaceae* sp. (Christopher et al., 2014).
Laccase activity has been reported only in a few bacteria such as: *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus*, *Bacillus subtilis* (Octavio et al., 2006), *Streptomyces lavendulae*, *Streptomyces maltophilia, Streptomyces coelicolor*, *Bacillus licheniformis* (Desai and Nityanand, 2011; Christopher et al., 2014).
The most studied laccases are the ones from fungal sources, including genera of *Ascomycetes*, *Deuteromycetes*, *Basidiomycetes* and cellulolytic fungi (Christopher et al., 2014). Amongst these, the most frequently described were the laccases from the white-rot

basidiomycetes such as: *Trametes versicolor, T. hirsuta, T. ochracea, T. villosa, T. gallica, Phlebia radiata, Coriolopsis polyzona, Lentinus edodes, Pleurotus ostreatus* (Desai and Nityanand, 2011; Brijwani et al., 2010), *Pycnoporus cinnabarinus, Coprinus cinereus* (Christopher et al., 2014). Other fungal strains include: *Agaricus blazei, Melanocarpus albomycea* (Christopher et al., 2014), *Stereum ostrea, Lentinus tigrinus, Ganoderma* spp., *Polyporus versicolor, Pholiata* spp., *Podospora anserine, Neurospora crassa, Aspergillus nidulans, Pyricularia oryzae* (Octavio et al., 2006), *Trichoderma harzianum, Trichoderma atroviride, Trichoderma longibrachiatum, Aspergillus niger, Phanerochaete chrysosporium, Theliophora terristrus, Stereum ostrea* (Gochev and Krastanov, 2007). Marine derived fungi that display laccase activity were: *Coriolopsis byrsina, Cerrena unicolor, Diaporthe phaseolorum, Pestalotiopsis uvicola* (Desai and Nityanand, 2011).

Bacterial laccases are more stable to high pH and temperature compared with the fungal ones (acidic optimum pH). The optimal temperature for laccases is usually between 50 - 70°C (Christopher et al., 2014).

Depolymerization of Kraft lignin

At first, some genera of basidiomycetes involved in lignin depolymerisation were found to lack lignin peroxidases, indicating that different enzymes were responsible for the degradation. After some research, it was suggested that laccases could play a key role in lignin depolymerisation (Gochev and Krastanov, 2007).

Laccases importance in this degradation is due to their capacity to work on both phenolic and non-phenolic compounds (Desai and Nityanand, 2011).

Regarding the depolymerization of lignin, laccase will first attack the phenolic lignin moiety (<20% of total lignin), releasing phenolic residues (as shown in Figure 4) with oxidized side chains (phenolic aldehydes, ketones and acids). After that, through a mediator facilitated process, laccase will oxidize the non-phenolic benzylic structures (Christopher et al., 2014). The phenolic fragments resulted in the first oxidation are able to infiltrate in the bulk lignin polymer and act as a natural mediator, thus helping the enzyme to oxidize more recalcitrant non-phenolic lignin (Christopher et al., 2014; Reddy et al., 2003).

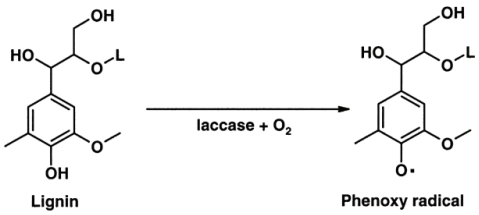

Figure 4. Simplified reaction of lignin depolymerisation with laccase
Source: Hatakka, 2005

An important factor in lignin degradation with laccase is the molecular weight of lignin and its phenolic content (Niku-Paavola et al., 2002). It was implied that during laccase action, both polymerization and depolymerization reactions can take place, as the phenoxy radicals produced can either lead to oxidation or polymerization (Tamminen et al., 2003).

The most common products obtained after lignin depolymerization with laccase and mediators are: 2,6-dimethoxy-4-methylbenzaldehyde, 4-ethyl-2,6-dimethoxybenzaldehyde and 2,6-dimethoxy-4-((E)-prop-1-enyl) benzaldehyde) (Du et al., 2013).

White rot fungi are the main microbial strains involved in the lignin degradation due to their extracellular high laccase activity (Christopher et al., 2014).

In comparison to peroxidases, laccases have a broad substrate specificity and are able to display their activity using only atmospheric oxygen as electron donor, instead of hydrogen peroxide used by peroxidases (Christopher et al., 2014). By not using hydrogen peroxide, laccases show a greater stability which allows them to be used more efficiently in an immobilised way (Octavio et al., 2006).

The ability of laccase to work with mediators in order to oxidize both phenolic and non-phenolic compound of lignin is considered to be a significant participant in lignin valorisetion (Christopher et al., 2014).

PEROXIDASE

Peroxidases (E.C. 1.11.1.7) involved in lignin degradation are heme-containing enzymes that

can oxidize a variety of organic and inorganic substrates in the presence of hydrogen peroxide as electron acceptor (O'Brien, 2000; Falade et al., 2017).

These enzymes are a group of oxidoreductases that catalyses the reduction of peroxides such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds. Heme-peroxidases are extracellular enzymes associated with lignin depolymerization. They include three types of enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP).

Lignin-peroxidase (diaryl propane oxygenase; LiP, E.C. 1.11.1.14) is a monomeric hemo-protein. This enzyme has the capacity to catalyse hydrogen peroxide dependent oxidative depolymerization of lignin (Falade et al., 2017).

Manganese peroxidases (Manganese-dependent peroxidases; MnP; E.C. 1.11.1.13) are extracellular glycoproteins that are considered to be the most common ligninolytic enzymes produced by white-rot fungi (Falade et al., 2017). The production of these enzymes is regulated by nutrients availability and environmental factors (Silva, 2014).

Versatile peroxidases (VP; E.C. 1.11.1.16) are a group of enzymes belonging to the class II subfamily of peroxidases. Its name derives from its versatile nature, being able to oxidize directly diverse substrates from hydroquinones, substituted phenols to bulky lignin, without redox mediators (Ravichandran and Sridhar, 2016).

VP was first mistaken with MnP in 1996 by Martínez et al. and in 2000 by Giardina et al. (Ruiz-Dueñas et al., 2009).

VP is also known as a hybrid peroxidase or manganese-lignin peroxidase, because of its ability to combine the catalytic properties of both MnP and LiP, being able to oxidize both phenolic and non-phenolic compounds.

These three types of peroxidases can work together on lignin degradation if they are produced by the same organism. While LiP oxidize the non-phenolic components of lignin and MnP targets the phenolic ones, VP has the ability to oxidize both phenolic and non-phenolic structures (Falade et al., 2017).

Ligninolytic peroxidases are used in a variety of applications such as: removal of contaminants, organic and polymer synthesis, pulp and paper industry, biosensors, analysis and diagnostic kits, enzyme immunoassays, biofuel production (Hamid, 2009).

Mechanism of action

Peroxidases catalyse the oxidation of a wide variety of substrates, using H_2O_2 or other peroxides. In general, the peroxidase catalytic cycle involves distinct intermediate enzyme forms and the activation of molecular oxygen is achieved in a two-step process. First, the native ferric enzyme is oxidised by hydrogen peroxide to form an unstable intermediate called compound I (Co I), which has a heme structure of Fe IV=O-porphyrin p-cation radical (Hamid, 2009), with consequent reduction of peroxide to water. Then Co I oxidise electron donor substrate to give compound II (Co II), releasing a free radical. Co II is further reduced by a second substrate molecule, regenerating the iron (III) state and producing another free radical.

Lignin peroxidase oxidize various non-phenolic structures of lignin including β-O-4 linkage-type arylglycerol-aryl ethers. The oxidation mechanism involves the formation of radical cation through one electron oxidation and this action leads to side-chain cleavage, demethyla-tion, intramolecular addition and nonetheless rearrangements (Falade et al., 2017).

Although, LiP mainly oxidize non-phenolic structures, it can also act on a variety of phenolic compounds such as: guaiacol, acetosyringone, catechol, vanillyl alcohol, syringic acid etc. (Falade et al., 2017).

An important redox mediator for LiP activity in lignin depolymerization is veratryl alcohol, a non-phenolic metabolite and a high redox potential substrate (Falade et al., 2017).

The catalytic capacity of LiP has been attributed to its exposed tryptophan residues, that forms a tryptophanyl radical on the surface of the enzyme through long-range electron transfer to the heme (Falade et al., 2017).

The variation in the tryptophan environment can influence the enzyme activity, stability and substrate specificity (Falade et al., 2017).

The mechanism of action of LiP involves three steps: oxidation of the resting ferric enzyme by hydrogen peroxide resulting in formation of compound I (oxo-ferryl intermediate), transfer

of one electron from the substrate to the compound I, to form compound II and subsequent donation of a second electron from the reduced substrate to compound II (Abdel-Hamid et al., 2013).
Similar to laccase, manganese peroxidase can have the capacity to oxidize non-phenolic compounds in the presence of mediators such as thiol or lipid radicals.

Sources
Peroxidases are widely distributed in nature in plants, animals and microorganisms.
LiP was first discovered in the extracellular medium of white-rot fungus *Phanerochaete chrysosporium* in 1983 (Falade et al., 2017). After that, several sources have been reported such as: *Trametes versicolor, Phanerochaete sordida, Phlebia radiata* (Falade et al., 2017), *Trametes villosa, Trametes trogii, Phlebia tremellosa, Phlebia ochraceofulva, Junghuhnia separabilima* (Hatakka, 2005).
Several white-rot basidiomycetes have displayed exclusively MnP activity as extracellular peroxidase, such as: *Ceriporiopsis subvermispora, Dichomitus squalens, Lentinula (Lentinus) edodes, Phanerochaete sordida, Pleurotus ostreatus* (Gasser et al., 2012), *Pleurotus eryngii* (Camarero et al., 1999), *Abortiporus biennis, Agaricus bisporus, Bjerkandera* sp., *Cyathis stercoreus, Heterobasidion annosum, Nematoloma frowardii, Panus tigrinus, Rigidoporus lignosus* (Hatakka, 2005).
Pleurotus eryngii is considered to be a model organism for studies regarding biodegradation of lignin, due to its selectivity in removing lignin when cultivated on natural substrates (Camarero et al., 1999).
Some reports indicate that some white-rot fungi can produce both LiP and MnP: *Phanerochaete chryosporium, Phanerochaete flavido-alba, Phlebia radiata, Bjerkandera adusta* and *Trametes versicolor* (Gasser et al., 2012; Hatakka, 2005).
VPs have been detected mainly in *Pleurotus* and *Bjerkandera* species (Gasser et al., 2012). Some reports suggest that VP's can also be found in *Panus, Calocybe, Trametes, Lepista, Dichomitous* and *Spongipellis* species (Ravichandran and Sridhar, 2016).

Depolymerization of Kraft lignin
In nature, lignin is efficiently mineralized by multiple enzymes produced mainly by white-rot fungi, which are found in forest litter and fallen trees (Gasser et al., 2012).
Initially, LiP was considered as the main enzyme connected to the oxidative breakdown of lignin due to the highly amount of non-phenolic units in lignin structure (Camarero et al., 1999).
The discovery of lignin peroxidase was a major step in understanding the mechanism of lignin depolymerization (O'Brien, 2000).
Lignin peroxidase has a high redox potential for the oxidation of non-phenolic structures which represent up to 90% of lignin (Figure 5) (Martinez, 2005).

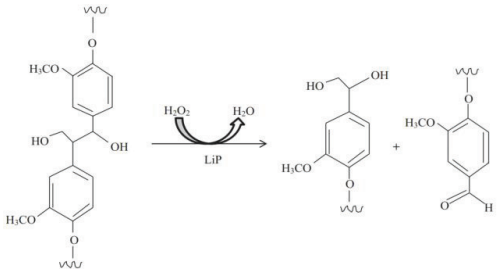

Figure 5. Simplified lignin depolymerization with lignin peroxidase
Source: Falade et al., 2017

Besides the involvement in degradation of non-phenolic structures of lignin, LiP can also oxidize the aromatic rings of lignin via long-range electron transfer (Gasser et al., 2012), which results in formation of unstable cation radicals, that will undertake different non-enzymatic reactions (Falade et al., 2017).
In comparison with laccase, LiP doesn't require mediators to degrade high redox potential compounds, but it does need hydrogen peroxide to initiate the catalysis (Maciel and Ribeiro, 2010).
There are many reports on depolymerisation of both native and synthetic lignins with manganese peroxidase (Falade et al., 2017). The action of manganese peroxidase is started by the activation of hydrogen peroxide with the iron protoporphyrin IX, that in return will oxidize the manganese co-factor from Mn^{2+} to the highly reactive Mn^{3+}. The Mn^{3+} centre is chelated by carboxylic acid anions that will

produce small, freely diffusible species, which will act as redox mediators, oxidizing phenolic lignin structures, as seen in Figure 6 (Falade et al., 2017; Gasser et al., 2012; Lange et al., 2013).

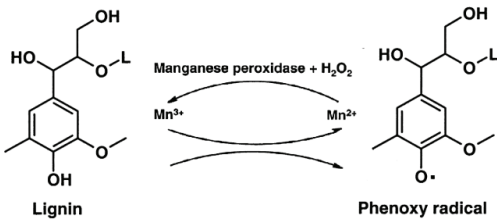

Figure 6. Simplified lignin depolymerisation with MnP
Source: Hatakka, 2005

Additionally, it was reported that lipid peroxidation by MnP might be an important factor in degradation of non-phenolic structures of lignin, due to the fact that the formed peroxyl radicals (Figure 6) can act as agents that will promote the oxidation of non-phenolic β-O-4-linked lignin compounds (Gasser et al., 2012).

In comparison with LiP, MnP has displayed a preference to oxidize *in vitro* phenolic substrates, due to its lower redox potential. On the other hand, some reports suggest that unlike LiP, MnP may be able to oxidize Mn^{2+} without hydrogen peroxide with decomposition of acids and concomitant production of peroxyl radicals (Maciel and Ribeiro, 2010; Hofrichter et al., 1999).

The interesting part about versatile peroxidase is that due to its unique molecular structure given by the presence of different oxidation-active sites (Falade et al., 2017; Ruiz-Dueñas et al., 2008), it's able to oxidize the substrate without redox mediators, unlike MnP and LiP (Ravichandran and Sridhar, 2016). Therefore, VP can have a great potential for future biotechnological applications (Busse et al., 2013).

The non-enzymatic reactions include aromatic ring cleavage, hydroxylation, demethoxylation, ether bond cleavage, side chain cleavage and phenol formation (Busse et al., 2013).

CONCLUSIONS

Lignin is the second most abundant biopolymer that represents 15-30% of lignocellulosic biomass.

There's an imperative need to find new and improved methods to valorise lignin, without affecting the other major components of lingo-cellulosic biomass.

The difficulty in degrading lignin is reflected by lignin complex and variable structure.

The most important enzymes involved in lignin depolymerisation are: laccase, lignin peroxidase and manganese peroxidase.

While lignin peroxidase attacks the non-phenolic structures of lignin, laccase and manganese peroxidase catalyse, without mediators, the oxidation of phenolic fragments of lignin.

These enzymes can work together if they are produced by the same microorganism. Therefore, an improvement on this subject could be the exploration and optimization of novel microbial sources that can produce these enzymes capable of depolymerizing lignin.

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