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KRAFT LIGNIN POLYMERIZATION BY LACCASE

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SUMMARY

This work studied the enzymatic oxidation/polymerization of lignin extracted from the prehydrolysis kraft cooking of *Eucalyptus globulus* by exposure to laccase. The preliminary results strongly suggest that the balance between polymerization and depolymerization is dependent on the functional groups of lignin and the enzyme source. Fungal-derived laccase under a neutral pH medium led to inconclusive results; despite a decrease in the phenolic content of 16%, there were no significant changes in the lignin molecular weight. On the contrary, bacterial-derived alkaliphilic laccase, modified to work at high temperatures and alkaline pH, induced a more significant decrease in the phenolic groups (41%) and the polymerization was noticeable.

Both polymerization and depolymerization can have interesting applications. Depolymerization results in smaller fragments with important applications like biofuel, water coagulants, or even polymers, while higher size molecules can be applied as bioplastics, binders for the pulp and paper industry, or even as adhesives to increase the structural properties of composites.

Keywords: Biopolymers, biorefinery, enzymatic treatment, laccase, lignin valorization.

INTRODUCTION

Lignin is a tri-dimensional polymer found in plants, and due to the heterogeneity of its structure, is the most difficult wood component to understand and to find efficient processes for its valorization capable to be scaled to industrial units(1).

Wood lignin is produced by the oxidative polymerization of three monolignols: p-coumaric alcohol, coniferyl alcohol, and sinapyl alcohol(2). The three compounds work as building blocks for the lignin macromolecule, the polymerization process is catalyzed by enzymes, namely peroxidases and laccases. The monolignols are oxidated by the enzymes producing radicals that will link with other radicals, the sequence of radical coupling reactions will result in the high molecular weight compound(3). The lignin polymer formed is very resistant to degradation and gives mechanical strength to the plant cell walls.

For many years, lignin was thought to be a waste product of the paper and pulp industries. Only 1 to 2% of the 50 to 70 million tonnes of lignin produced each year is used to make value-added goods including concrete additives, dispersants, animal feed, resins, additives, and vanillin(4). The excess is being burned to produce energy. In recent years, a global effort has emerged to discover alternatives uses, namely as a source of phenols and other components that can rival the less eco-friendly chemicals(5, 6).

The increased research about this versatile compound is resulting in the development of lignin-derived polymeric materials with unique characteristics, such as high hydrophobicity and UV protection (7, 8).

In the chemical pulp production processes, lignin must be removed to liberate the cellulosic fibers and the extraction is normally done by chemical pathways. Kraft, soda, organosolv, and sulphite cooking

are the most used methods of lignin removal.

However, the harsh conditions required for lignin extraction provoke macromolecule degradation, affecting the physical and chemical structure of lignin which may affect future applications(9, 10).

Kraft cooking is the main process to produce cellulose fibers, and the use of Na_2S and NaOH conjugated with temperature allows the removal of almost 95% of the lignin in the raw material. Additionally, the lignin extracted in these conditions, despite having a heterogenous and high molecular weight, is yet reactive due to their phenolic content (higher than 30%)(11).

Kraft lignin contains both aliphatic and phenolic hydroxyl groups, where the phenolic content directly impacts the reactivity towards enzymatic and chemical modifications. The high molecular weight of this type of lignin has a negative effect on the reaction due to its dense structure and numerous linkages. However, the presence of phenolic content provides reactive sites that can be effectively utilized(12).

The modification of lignin can occur through enzymatic treatment, chemical modifications, and physical modifications. Each process with some advantages and disadvantages(13).

Lignin can be modified using enzymes (peroxidases and laccases); depending on the conditions employed the fragmentation or polymerization of lignin can be achieved. Figure 2 illustrates the polymerization process of a sulfonated lignin, by the oxidation of the hydroxyl groups associated with the phenolic groups in the lignin molecule (10).

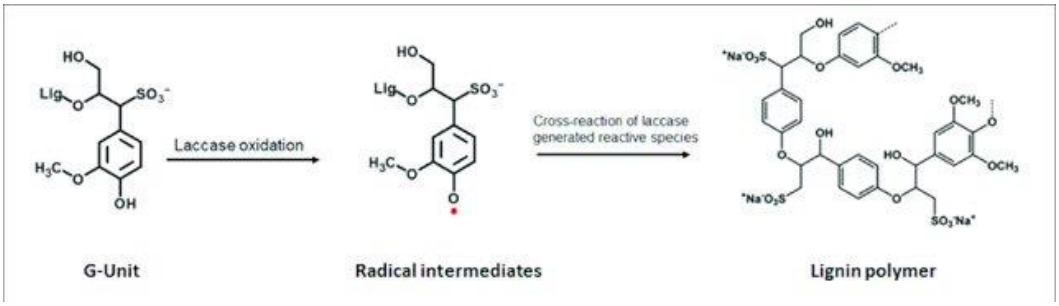

Figure 2 – Scheme of laccase catalyzed oxidation of lignin(14).

Regarding depolymerization, laccases and peroxidases are shown to have the capability to depolymerize lignin by the oxidation of the phenolic molecules into quinones(15), however, the process is still not well known (16). The use of laccase with lignin models compounds or lignosulfonates is shown to have preferentially polymerization reactions, yet, in the present of mediators such as 2,2,6,6-tetramethylpiperidine1-oxyl (TEMPO,) and, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) depolymerization occurs(15).

In addition to enzymatic modification, chemical changes can result in the synthesizing of new molecules with higher reactivity and more specific applications. Methylation and phenylation are the most used methods for this objective, both resulting in lignin that can more easily replace phenol in phenol-formaldehyde resins and polymers(17).

Other modification methods are being explored such as reduction, hydrolysis, mechanical treatments, and irradiation(13)... Despite most current methods are not economically attractive, the increased efforts are closing the gap for the utilization of lignin for value-added products, changing the view of lignin as a residue to a value-added by-product of the pulp industry.

Nowadays, there are already some companies focused on the production of lignin (mainly kraft lignin) such as Sodra® and Lignoboost®.

This research, including this article, aims to enhance the utilization of lignin for value-added compounds beyond the current range of 1 to 2%. In this study, the evaluation of two different laccases is tested considering the solubility of lignin in different pH solutions and the effect on their reactivity

towards oxidation by the laccase system and consequent polymerization. Additionality, different lignin's were tested to have a better understanding of the characteristics that may affect the polymerization.

EXPERIMENTAL

Raw Material and lignin isolation

Lignin was recovered from pre-hydrolysis soda cooking process, using *Eucalyptus globulus* wood chips supplied by Biotek S.A, Vila Velha de Rodão, Portugal.

A dissolving pulp production process was followed, in which, wood was pre-hydrolyzed for the removal of hemicelluloses followed by alkaline cooking, aiming for lignin extraction. Both phases were conducted in a batch reactor, with liquor circulation, using 1.0-kilogram (O.D) wood chips and a liquid:wood ratio of 7L:1kg.

The pre-hydrolysis was conducted with distilled water (auto-hydrolysis), for 1 hour at 148ºC and 6 bar. After hydrolysis, the sugar-rich solution was removed, and a NaOH solution was added into the reactor, after oxygen removal by nitrogen bubbling, aiming to reduce oxidation of the extracted compounds. The reaction conditions were as follows: maximum temperature: 148ºC; time at maximum temperature: 180 minutes; NaOH charge: 23% (based on the wood); L/W: 7/1; H factor: 400 (including the heating ramp time).

The lignin produced by this method is recovered by acidification (18). For this means, lignin was precipitated with 2M sulfuric acid at pH 6 and pH 2.

Total Phenolic Content, TPC

The determination of the Total Phenolic Content was performed by the Folin-Ciocalteu (FC) assay as described by A. Alzagameem(19).

For each assay, the lignin samples were dissolved in DMSO at 2 mg/mL. The blank sample used was a lignin-free DMSO solution.

For the analysis, 0.1 mL sample solution was pipetted into a volumetric beaker, followed by 0.5 mL FC reagent, and shaken in a vortex for 30s. After the mixing, 7 mL of deionized water was added. One minute later, 1 mL of 20% (w/w) sodium carbonate solution was incorporated, and the beaker was filled with deionized water until reach 10 mL and re-mixed in the vortex. The solution was incubated for 30 minutes at 40º C before measuring the absorbance at 750nm.
For quantification, a calibration curve with syringic acid (0.1 – 1 mg/L) was prepared, intended for the results to be expressed as syringic acid equivalents (SAE) with the following equation:

$$SAE\ [mg/L\ ] = \frac{A_{lignin} - B_{cal}}{m_{cal}} \qquad (1)$$

where A_{lignin} is the absorbance of the lignin sample, B_{cal} is the intercept of the calibration curve, and m_{cal} is the slope of the calibration curve.
The following results are converted to total phenolic content based on the SAE and the lignin concentration (C_{lignin}) used, by the equation 2:

$$TPC\ [\%] = \frac{SAE}{C_{lignin}}\ 100 \qquad (2)$$

Lignin filtration (1.2 µm)

During the laccase treatment, various samples underwent filtration using a 1.2 µm glass microfiber filter (FiltraTech, France) to assess the accumulation of precipitated material in the reaction medium. Following filtration, the filters were dried, and their weights were recorded both before and after the process. Identical procedure was conducted on the enzymatic solution for each assay as well, and its value was subtracted from the laccase treated sample results. This methodology allows for the expression of results relative to the initial lignin, ensuring that the addition of the enzyme solution does not directly impact the measurement.

Lignin molecular weight distribution

The molecular weight of the lignin samples was determined by size exclusion chromatography (SEC). For this assay, two different Ultrahydrogel columns (Waters Co., Ltd., USA) were used with different pores ranges of 120Å and 250Å. Before injection in the SEC system, the samples were filtered in a 0.22 µm PTFE syringe filter. A NaOH solution, with a pH of 11, was used as eluent. The samples were analyzed at a flow rate of 0.3 mL/min by a UV (Accela PDA detector (80 Hz), from Thermo Scientific, EUA) and Refractive Index (RI, RefractoMax 520) detectors stabilized at 40ºC.

Laccase Treatment

Different conditions were employed based on the laccases used. Fungal-laccase was employed at pH 5 (optimum conditions for the enzyme) and 45ºC, while the Bacterial-laccase has the highest activity at alkaline pH, namely pH 10. Both systems were submitted to aeration as an oxygen source and as agitation of the reaction medium.

Laccase activity was measured by the oxidation of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The lignin assays were carried out at a laccase charge of 1 U/mg of lignin.

RESULTS AND DISCUSSION

Laccases are enzymes that play a crucial role in the breakdown and transformation of various organic compounds. Traditionally, fungal laccases have been extensively studied and utilized in various industrial applications. However, regarding lignin polymerization, our experimental data have shown inconclusive results regarding their efficiency and applicability. The limited lignin dissolution at pH 5 certainly also contributed to the observed performance of the fungal laccase. As a result, attention has shifted towards exploring the potential of bacterial laccases, working at alkaline pH.

Total phenolic content

The total phenolic content can be used as an indicator of lignin polymerization and depolymerization. Phenolic compounds are a key component of lignin, and changes in their concentration can reflect alterations in lignin structure and composition. In this assay, the comparison of fungal and bacteria laccase was assessed with soda lignin precipitated at pH 6. The phenolic content with fungal laccase decreased by 11% (27% to 24%) while the decrease with bacterial-derived alkaline laccase was 40 % (27% to 16%).

The main source for the significant difference is the pH where the enzyme is more active. Fungi-laccase used needs a slightly acidic medium, where the lignin is denser and can be clustered, making enzymes accessibility more difficult to the hydroxyl groups, decreasing the overall reactivity of lignin (20). On the other hand, the bacterial-derived laccase, being genetically modified to work at higher pH, can withstand the pH where lignin is completely soluble and more easily accessed by the enzyme. Based on these initial results, the bacterial-derived alkaline laccase was used in the following assays.

The reduction of phenolic content may be indicative of the increased molecular weight of lignin by the increased links between the different structures. However, additional techniques must be done to

confirm the results. Similar results were achieved by other studies with laccase-catalysed polymerization of lignin/phenolic compounds(11, 21).

Lignin polymerization

Laccase, as an oxidative enzyme, has the capability to modify lignin by catalyzing the oxidation of phenolic and non-phenolic lignin units. This enzymatic treatment alters the chemical structure of lignin, leading to increased molecular weight, improved hydrophobicity, and enhanced interactions with filtration media. These modifications can potentially enhance the retention of lignin particles during filtration processes(22) and can also be followed visually.

Figure 3 illustrates the increased retention of lignin on a 1.2 µm filter, for increasing reaction times.

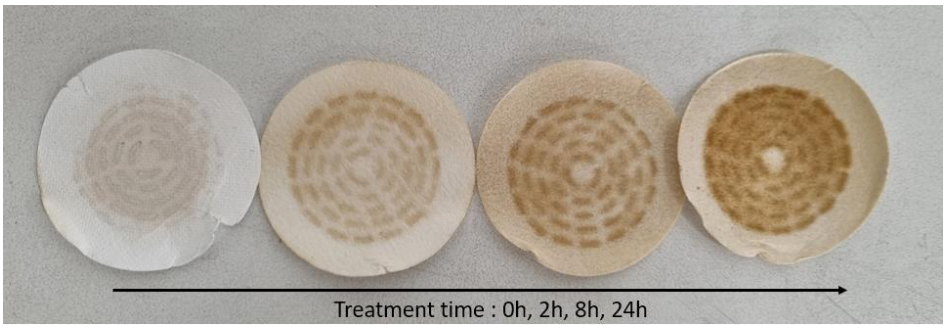

Figure 3 – The increase in retention of lignin on a 1.2 µm filter

This empiric procedure was also used to evaluate the effect of the laccase treatment for different lignin samples. The study included lignin from soda black liquor (BL) at two concentrations (1 g/L and 17.5 g/L), ethanol soluble lignin (1 g/L) and acid soluble lignin from the pre-hydrolysis stage in the dissolving pulp production method (7 g lignin/L).

The black liquor lignin can also be submitted to a fractionation process, aiming to produce lignin fraction with different characteristic and/or as characterization procedure. In this study, a sequential extraction was conducted using isopropanol, ethanol, and methanol. The fraction soluble in ethanol constituted roughly 50% of the total lignin and was considered the most representative component of the entire sample. The other lignin assays were conducted by directly treating black liquor and pre-hydrolysate, with already soluble lignin.

Table 1 shows the percentage of the initial lignin retained in the 1.2 µm filter along the laccase treatment, for the different lignin.

Table 1. Lignin retention in 1.2 µm filter by the time

Time, hours	BL 1 g/L (%)	BL 17.5 g/L (%)	Ln_ethanol 1g/L (%)	Pre-hydrolysis 7 g/L (%)
0	5.0	6.0	5.0	30.0
1	14.1	11.1	11.3	27.9
2	24.1	14.3	15.6	30.7
5	31.4	24.9	21.0	47.1

8	25.7	15.1	18.8	26.4
24	31.2	12.0	21.0	20.0
72	63.3	9.4	46.0	17.1

Initially, we examine two samples, BL_1 g/L, and Ln_Ethanol, both having a concentration of 1 g/L. It is evident that these samples exhibit increased retention compared to the other groups throughout the treatment duration. Over the course of 72 hours, there was an observed increase of 58% and 41% in the total lignin withheld in the filter, respectively.

The black liquor lignin sample is derived from the dilution of lignin extracted through the alkaline process. This particular sample encompasses a wider range of molecular weights of lignin, resulting in diverse interactions with the laccase enzyme. On the other hand, the ethanol-soluble lignin has already undergone fractionation with isopropyl alcohol, which removes smaller and more reactive portions of lignin. Consequently, this fractionation process can potentially reduce the reactivity of the lignin toward the laccase oxidation reaction and explain the difference between the withheld material between the same concentration samples.

BL_17.5 g/L assay showed a significant increase in the retained compounds in the first 5 hours, but afterward there is a decrease until the end of the treatment resulting in a small increase of 3.4% throughout the treatment. During the initial 5 hours, the lignin undergoes polymerization, leading to the formation of larger molecular structures. However, after the initial 5 hours, a phenomenon of depolymerization occurs. This can be tentatively attributed to various factors, such as enzymatic or chemical reactions, temperature changes, or the action of microorganisms. When compared with the more diluted sample (BL_1 g/L) the significant difference between the results shows that the black liquor raw sample or the enzymatic solution may have some inhibitors that impact the behaviour of the enzyme. Similar behaviour is visible in the pre-hydrolysis sample. This sugar-rich sample has a lower lignin concentration than the black liquor raw sample, but the lignin polymerization also reaches a pike at 5 hours, and it proceeds to decline until it reaches the minimum withheld at 72h for 17.4%. In both higher concentration samples, enzymes that were initially promoting lignin polymerization now exhibit a tendency towards depolymerization. The concentration of lignin its proven to affect the enzyme activity and the results go in concordance with other studies(11, 23), where the inhibitory effects of lignin impact different enzymes.

Size Exclusion Chromatography

SEC (Size Exclusion Chromatography) HPLC is a technique commonly used to separate molecules based on their size. The stationary phase of the SEC column contains pores of a specific size, allowing smaller molecules to enter the pores and therefore elute later, while larger molecules are excluded and elute earlier. Figure 4 shows the results from black liquor at 0h of the treatment and at 24h (BL_1 g/L).

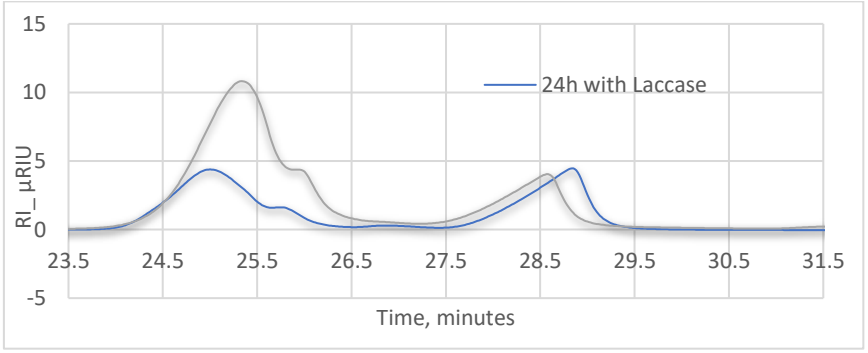

Figure 4 – Chromatogram of lignin-laccase treated and non-treated.

When lignin is subjected to laccase treatment, it undergoes enzymatic modification, resulting in the production of larger lignin fragments (24). These modified lignin fragments have an increased molecular size compared to the untreated lignin. In fact, the maximum pick corresponding to the fraction with high molecular weigh was moved to lower retention time, i.e. higher molecular weight. The chromatogram also reveals the presence of material with very low molecular weight.

In this particular case, it is important to consider not only the retention time but also the overall area of the metabolite signal. After 24 hours of laccase treatment, the yield of soluble lignin in the sample is reduced. This reduction is primarily attributed to the increase in molecular weight, which causes the compound to be retained in the filter used for sample preparation. Table 1 demonstrates that approximately 60% of the total lignin is withheld in a 1.2 µm filter during the laccase treatment. Furthermore, when preparing HPLC samples, an additional filtration step through a 0.24 µm syringe filter is required. This further filtration step results in a decreased quantification of lignin throughout the treatment process.

CONCLUSION

The results indicate that the enzymatic treatment with laccase leads to structural modifications in lignin, resulting in the generation of modified lignin fragments. These fragments have altered physical and chemical properties compared to the original lignin, making them potentially more accessible for further processing or utilization.

The choice of enzymes used in the study influences the complex of enzyme-substrate. Different enzymes may exhibit varying degrees of substrate specificity and catalytic activity towards specific phenolic moieties within lignin. The preliminary results also highlight the importance of working with an alkaline solution during the laccase treatment. Lignin has limited solubility in many solvents (including water), but it’s soluble and more easily accessed in alkaline conditions.

The retention of lignin on a 1.2 µm filter was used to simply follow the laccase treatment of different lignin solutions and SEC analysis brought additional information. Black Liquor lignin_1g/L eluted earlier in SEC analysis after laccase treatment, suggesting that the enzyme has effectively modified the lignin molecule, leading to the formation of bigger fragments. This finding provides evidence of the enzymatic polymerization of lignin and highlights the potential of laccase treatment.

The same enzyme when applied in black liquor lignin at much higher concentration exhibited initial lignin polymerization followed by depolymerisation.

Overall, these results underscore the importance of selecting appropriate enzymes for lignin treatment and shed light on the intricate dynamics of lignin modification. The findings contribute to our understanding of lignin's behaviour under enzymatic conditions, which can ultimately aid in the development of more efficient and sustainable strategies for lignin utilization in various industries. Further research in this area is warranted to explore and optimize the enzymatic treatment of lignin for its potential applications.

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