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Enzymatic Oxidation of Biorefinery Lignin

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Enzymatic Oxidation of Biorefinery Lignin

Abstract:

Lignocellulosic biomass is the main source of renewable chemicals in the future. Biorefinery methods can be used to separate the three major components, which are cellulose, hemicellulose and lignin. Treatment methods, where the polysaccharides are hydrolyzed, resulting in the production of hydrolysis lignin (HL). In order to valorize HL further enzymatic treatment is promising and can be a game changer in the biorefinery approach. The goal of the present work was to investigate possibilities to use enzymatic oxidation in valorization of HL. For the first time, the treatment of industrially fractionated HL with enzymes was used. Four different laccases and a peroxidase were tested. All enzymes showed activity in most conditions for different HL-s. A positive result was the observation of enzyme activity in conditions with several times higher HL content than previously described.

Keywords: Hydrolysis lignin, bacterial laccases, dye decolorizing peroxidase, lignin solubility

CERCS: P310 Proteins, enzymology, P390 Organic chemistry

Biorafineerimisligniini ensümaatiline oksüdatsioon

Lühikokkuvõte: Tulevikus saab lignotselluloosest biomassist põhiline taastuvate kemikaalide allikas. Biorafineerimise meetodeid kasutatakse kolme põhilise komponendi eraldamiseks, milleks on tselluloos, hemitselluloos ja ligniin. Töötlemise käigus hüdrolüüsitakse polüsahhariidid ning protsessi käigus toodetakse hüdrolüüsi ligniin (HL). HL väärindamiseks on paljulubavaks meetodiks ensümaatiline töötlemine, mis oleks läbimurdeks biorafineerimises. Käesoleva töö eesmärk oli uurida võimalusi ensümaatilise oksüdatsiooni kasutamiseks HL väärindamiseks. Selles töös kasutati esmakordselt tööstuslikult fraktsioneeritud HL töötlemist ensüümidega. Testiti nelja erinevat lakkaasi ja peroksüdaasi. Kõik ensüümid näitasid aktiivsust erinevate HL-de korral. Positiivne tulemus oli ensüümi aktiivsuse täheldamine tingimustes, kus HL sisaldus oli mitu korda suurem kui varem kirjeldatud.

Võtmesõnad: Hüdrolüüsi ligniin, bakteriaalsed lakaasid, värvainest värvust eemaldav peroksüdaas, ligniini lahustuvus

CERCS: P310 Proteiinid, ensümoloogia, P390 Orgaaniline keemia

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TERMS, ABBREVIATIONS AND NOTATIONS

ABTS - 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonate)

AmLac- *Amycolaptopsis* laccase

APL - Acid Precipitated Lignin

BL - Biorefinery lignin

B&R – Britton and Robinson buffer

Da - daltons

DyP Peroxidase - Decolorizing peroxidase

EPR - Electron Paramagnetic Resonance Spectroscopy

G - coniferyl

GS-type - Hardwood lignin–angiosperm

G-type - Softwood lignin–gymnosperm

HAA - 3-hydroxyanthranilic acid

H - p-coumaryl

HGS-type - Grass lignin

HL - Hydrolysis Lignin

KL - Kraft lignin

LS - Soda lignin or lignosulfonates

PU- Polyurethane

PDI- Polydispersity index

S - sinapyl

ScLac - *Streptomyces coelicolor* laccase

SEC – Size Exclusion Chromatography

SvLac - *Streptomyces viridosporus* laccase

UV-Vis - Ultraviolet-visible Spectroscopy

INTRODUCTION

Lignocellulosic wood biomass is a renewable resource with the potential to replace fossil-based feedstocks for sustainable energy, chemical compounds, and biomaterials production. Lignocellulosic biomass is mainly composed of three biopolymers: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose can be converted into C5 and C6 sugars and separated from the biomass to produce the polymeric hydrolysis lignin. Sugars can be converted into biofuels or biochemicals using known platforms. Valorization of lignin is crucial for the full utilization of lignocellulosic biomass. However, this has proven to be demanding task. Lignin depolymerization with selective bond cleavage is a major challenge for converting it into value-added precursors. Depolymerization of lignin can result in valuable products with high functionality and low molecular weights, which can be utilized in the preparation of polyurethane foams, coatings, epoxy resins etc.

Most of technical lignins from wood biomass pulping are often contaminated, have modified or highly condensed structures, thus the application of biological treatment methods is problematic. On the other hand, hydrolysis lignin, with its native nature could be a more promising target for enzymes. Recent works have shown a large array of bacterial and fungal proteins, which are able to target specific chemical bonds in lignin molecules. The major problems with these approaches are enzyme stability and possible inhibitory effect by lignin as well as solution compatibility between lignin dissolution, whilst retaining enzyme activity.

In the present work, the valorization possibilities of biorefinery lignin were investigated using enzymatic oxidation. First of its kind, the hydrolysis lignin produced from birch at an industrial scale is used in this thesis. Three different bacterial laccases as well as a peroxidase were tested to oxidize hydrolysis lignin. The experiments of this thesis were performed at the University of Tartu as well as at MetGen Oy, Finland.

1. LITERATURE OVERVIEW

1.1 Structural characteristics of lignin

Lignocellulosic biomass shows tremendous potential for generating biofuel and biochemicals. Lignocellulosic biomass is made up of three components cellulose, hemicellulose and lignin which make up the main structure of plant cell walls as shown in figure 1. Cellulose and hemicellulose can be depolymerized into monomeric sugars by standardized methods, but the degradation of lignin into monomers is more challenging (Chan, Paice and Zhang, 2020; Chukwuma *et al.*, 2020; Yu and Kim, 2020).

Lignin composition can depend on many factors such as methods used for its separation as well as species, age, and environmental conditions. For these reasons the structure is difficult to define. Lignin is categorized as the most interesting polymer to be used in biorefineries. However, at present lignin is mostly burned and used in energy production (Chio, Sain and Qin, 2019; Dessbesell *et al.*, 2020). There are several types of technical lignin generated as waste from different pre-treatment processes, which can be a source to originates aromatic monomers and oligomers. Lignin is a biopolymer, which is unique due to its heterogeneous structure, where it is composed of different aliphatic and aromatic groups and this type of structure is very difficult to define. Lignin is naturally crosslinked polymer with molecular weight of over 10 000 Daltons (Da) (Yinghuai, Tang and S., 2013). Therefore, lignin has complex cross-linked structure and within the molecule, it contains various chemical linkages such as aliphatic hydroxyl, phenolic hydroxyl, and methoxy groups (Chio, Sain and Qin, 2019; Wang *et al.*, 2020).

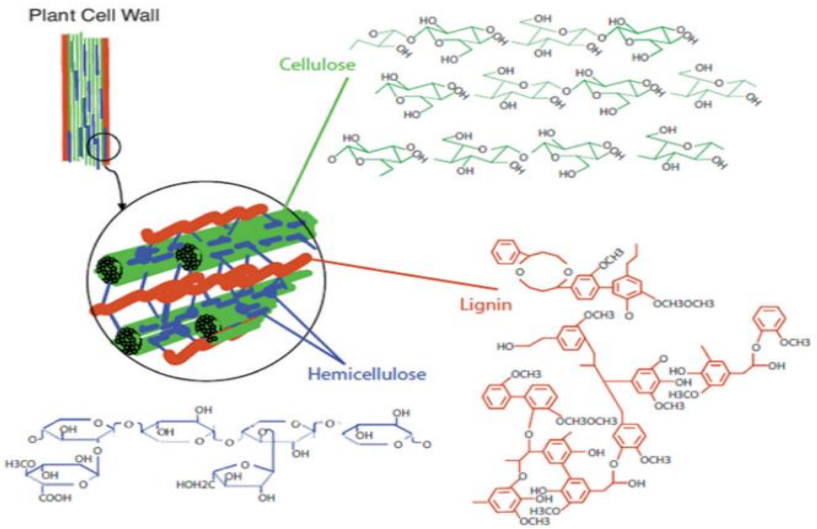

Figure 1. Structure of lignocellulosic biomass where a plant cell wall composed of **A** cellulose (green), **B** hemicellulose(blue) and **C** lignin(red) (Wang, Sun and Sun, 2019).

The lignin reactivity and chemical properties are majorly affected by the polymeric structure due to which it's hard to valorize lignin. The hydroxyl groups with aromatic structures are especially important since they are precursors to determine of the characteristics and properties of the polymers (Vanholme *et al.*, 2010). Synthesis of lignin in plant cells starts by the combination of monomeric heteropolymers, such as phenylpropanoid units (sinapyl (S), coniferyl (G), and p-coumaryl (H), alcohols), and this may contain methoxy groups at 3 and 5 aromatic carbons as shown in Figure 2. The lignin is classified as G-type (softwood lignin–gymnosperm), GS-type (hardwood lignin–angiosperm), HGS-type (grass lignin) based on the content of the three units—coniferyl alcohol units, sinapyl alcohol units, and p-coumaryl alcohol units (Gillet *et al.*, 2017). Because of its heteropolymer and recalcitrant nature lignin acts as protection against the microorganism's attack and transport water, nutrients, and metabolites through the plant.

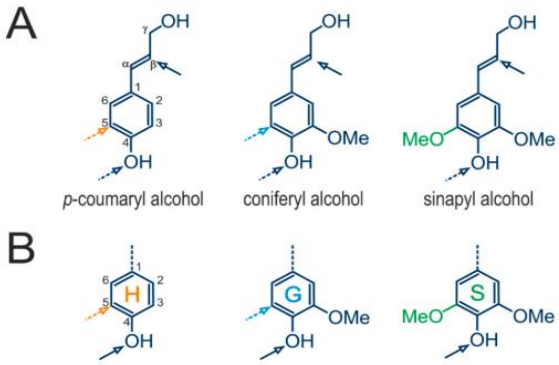

Figure 2. **A** represents heteropolymer alcohols while **B** corresponds to p-hydroxyphenyl, guaiacyl, and syringyl units in lignins. The arrows represent sites for radical coupling. Less prevalent coupling sites between monolignols are indicated by dashed arrows (Hasanov and Raud, 2020).

Softwood lignin is mainly derived from coniferyl alcohol units (95%), while hardwood may have between 25–50% of coniferyl alcohol units and 46–75% of sinapyl alcohol units. In the case of grasses, lignin is constituted between 5–33% (p-coumaryl), 33–80% (coniferyl), and 20–54% (coniferyl and sinapyl alcohols) respectively as shown in figure 2 (Hasanov and Raud, 2020). Therefore, lignin can be differentiated based on the number of methoxy groups arranged on the aryl rings of the polymer to define whether it's hardwood or softwood. As mentioned before softwood lignin is formed through the polymerization of coniferyl alcohol, which itself is formed from guaiacol and contains only one methoxy group per aromatic ring (Hasanov and Raud, 2020). Therefore, softwood biomasses have a more homogenous lignin composition while hardwood lignin is formed through polymerization of coniferyl and sinapyl alcohols, containing either one or two methoxy groups per aromatic ring (Luo, Fang

and Smith, 2014). Which concluded the fact that the common lignin interunit linkages include β-aryl ether (β-O-4'), phenyloumaran (β-5'), resinol (β-β'), dibenzodioxocin, 4-O-5', β-1, and α-O-4' as shown in figure 3. The connection among the structural units of lignin is primarily formed of ether bonds, which account for nearly 60–75% of total bonds where β-O-4 accounts for between 45–62% of all bond types. (Luo, Fang and Smith, 2014).

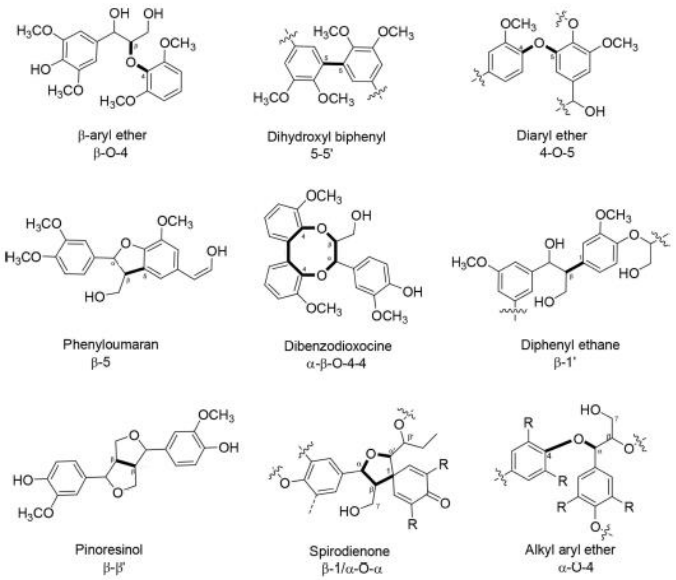

Figure 3. β-O-4, β-5, β-β', 5-5', 4-O-5 linkages present in lignin (Chan, Paice and Zhang, 2020).

1.2 Technical lignins

The expression biorefining lignin is used for various technical lignins as shown in table 1, where different types of technical lignins were broadly classified on the basis of scale, chemistry, sulfur content and purity. The term “Biorefinery” often refers to both biofuel processes and the new larger scale pulp and paper industries. This is due to the fact that in many European countries the pulp and paper industries use residue lignin as a source of “green energy”. However, the most common biorefinery concept (as used in North America) considers only the sugar-based biorefinery processes (biofuel process) and does not include pulp and paper. Mikhail Balakshin defined “Biorefinery” to biofuel biorefinery processes while classified lignins from pulping with inherent names such as (KL), soda lignin or lignosulfonates (LS) (Balakshin *et al.*, 2021). For the sake of clarity, it is better to use the term "hydrolysis lignin" (HL) to refer to technical lignin from sugar-based processes.

Presently, conventional pulp mills are the major suppliers of technical lignins, LS and KL. LS, highly sulfonated, generated by sulfite pulping processes, are the only lignin-based commercial products of significant volume. However, the structure and most of LS properties

are different from other technical lignins (Balakshin *et al.*, 2021). LS is soluble in water and has higher ash content compared with other technical lignins.

KL is produced in a sulphate pulping process, where active chemicals are sodium hydroxide (NaOH), and sodium sulfide (Na_2S), in the form of a by-product called black liquor. KL can be recovered by acid precipitation. KL structure is largely modified during the pulping process, KL is hydrophobic and not a reactive polymer unless it is modified. KL often contains the aliphatic thiol groups (R-SH) which cause a special odour. KL is soluble only in aqueous NaOH at high alkaline pH (Chio, Sain and Qin, 2019).

Soda lignin, generated in the soda pulping process, is also dissolving only in alkaline conditions. Organosolv lignin (OL) is produced from pulping processes where lignin in the biomass can be dissolved in the organic solvent under certain conditions. OL has modified structure, low molecular weight and it is soluble in organic solvents(Chio, Sain and Qin, 2019).

Table 1. Classification of technical lignins. (Berlin and Balakshin, 2014)

Lignin Type	Scale	Chemistry	Sulphur content	Purity
Kraft	Industrial	Alkaline	Moderate	Moderate
Soda	Industrial	Alkaline	Free	Moderate-low
Lignosulfonate	Industrial	Acid	High	Low
Organosolv	Pilot/demo	Acid	Free	High
Hydrolysis	Industrial/pilot	Acid	Free	Moderate-High

HL is mainly produced in cellulosic biofuel plants as a by-product of enzymatic hydrolysis processes. The structure of isolated HL depends on the pre-treatment process (Hasanov and Raud, 2020). For the separation of HL from the lignocellulosic biomass, the process includes cellulose decrystallization, total depolymerization of cellulose and hemicellulose, maximizing the enzymatic digestibility of the pre-treated material, minimizing the sugar-losses (Mahmood *et al.*, 2016).

However, HL can and should contribute dramatically to the biorefinery economy as a high-value product. In contrast to kraft pulp mills, there is no limitation to use all of the lignin produced in a sugar-based biorefinery mill for potential non-energetic purposes (Berlin and Balakshin, 2014). It is very important to look forward to the valorization of HL.

It should be noted that the current pulping processes are limited concerning variations in feedstock and process conditions, which are set according to the target properties of the chemical pulps (Kannangara *et al.*, 2012). Biological modification is challenging and can only be done through post-processing of technical lignins. In contrast, variations in biorefinery conditions are possible, as they target only the yield of different products which means sugars/ethanol and biofuels conversion but not their properties (Yu and Kim, 2020). Thus, sugar-based biorefineries offer much more opportunities and much greater flexibility for moulding and developing novel applications from lignin than the pulp and paper industry. Many current concepts argue that inconsistencies or variations in pulping and BRL as a chemical feedstock can be mitigated by lignin modifications with the new functionalities having a much stronger effect on lignin properties than the original functionalities (Balakshin *et al.*, 2021).

1.3 Hydrolysis lignin potential

There is a large diversity of lignins which are different in chemical composition, molecular structure, and physical properties. Not surprisingly, the behaviour of these lignins in various applications is different (Abdel-Hamid, Solbiati and Cann, 2013; Yu and Kim, 2020). Random selection of lignins for high-value applications is very unlikely to be successful. Therefore, directed development of the lignin-based product and the underlying lignin material with the best characteristics for a specific application is critical (Balakshin *et al.*, 2021).

Hydrolysis lignin is based on the Sunburst Pre-treatment technology and was capable of converting up to 94% of the carbohydrates in woody biomass to monomeric sugars and resulting in a clean lignin product (Blair, 2020) . The process depends on a modified twin screw extrusion technology which is globally used in many other industries reliably. Hydrolysis lignin was fractionated by converting biomass into slurry using acid and water (Hämäläinen *et al.*, 2018).

Many biorefinery schemes for lignocellulosic biomass conversion comprise a pre-treatment step followed by enzymatic hydrolysis of carbohydrates, resulting in simple sugars that will be the basis for producing biofuels (ethanol and higher alcohols) and chemicals (organic acids, alkenes, lipids and other chemicals) via fermentation (Gillet *et al.*, 2017; Barnhart-Dailey *et al.*, 2019; Moreno *et al.*, 2020). The effectiveness of pre-treatment technologies to improve enzymatic hydrolysis has been attributed where chemical pre-treatment methods assembles the role of biorefineries as a green source of chemicals and materials.

However, most of the methods to overcome the lignin barrier (e.g. physical technologies such as milling; chemical methods especially alkali- and acid-based pre-treatments and physicochemical pre-treatments as steam explosion) are very energy-intensive and involve harsh conditions (high temperatures and pressures) or even toxic and hazardous chemicals (Chukwuma *et al.*, 2020; Moreno *et al.*, 2020). As an alternative, biological delignification involves low energy demand, low environmental impact, and high product yield (Moreno *et al.*, 2015). Laccases are lignin specific which means we can oxidize and modify lignin to develop a bio-based process and to valorized the novel HL and show high reaction rates, significantly reducing the delignification process time without major consumption of energy and hazardous chemical process.

Over the past few decades, much research has been conducted to investigate the production of value-added bioproducts from KL. Recently, HL has generated a wide range of opportunities where the trend moves towards bio-based and sustainable process development. Also, a major point in valorizing HL is that its sulfur-free and its abundant availability from cellulosic ethanol plants and can also be utilized in anaerobic digestion (Mulat, Dibdiakova and Horn, 2018). Value-added utilization of lignin is critical for the accelerated development and deployment of the biorefinery. To improve the percentage of bio-replacement for example in PU the depolymerization of lignin is a feasible way to produce depolymerized lignin as bio-polyols with a lower Mw and better reactivity. Depolymerization of lignin not only reduces the molecular weights of the resulting products but also improves their functionalities, facilitating their utilization in PU preparation (Mahmood *et al.*, 2016).

The direct oxidation of lignin by laccases, restricted to phenolic units can lead to lignin elimination from lignocellulose (Hämäläinen *et al.*, 2018; Chan, Paice and Zhang, 2020). Different fungal laccases, including enzymes with low and high redox potential such as laccases from *Streptomyces ipomoea,* have shown this ability. In addition to lignin removal, laccases can depolymerize them into monomers (Moreno *et al.*, 2015). Also, the degradation effect of laccases on lignin was described by Zhang where combinations of enzymes were utilized in lignin degradation (Zhang *et al.*, 2020).

1.4 Bacterial laccases

Enzymes are proteins, which catalyze reactions. Enzymes are catalysts that speed up biochemical reactions in living organisms and are specific, which means it binds to specific substrates to catalyze the specific reaction (Bugg *et al.*, 2011). Laccases are bacterial en-

zymes that are thermally stable (active up to 60°C) and are primarily monomeric glycoproteins that contain four copper atoms in their active sites arranged into three Metallocentres (Majumdar *et al.*, 2014). Laccases can be found in various kinds of plants, fungi, bacteria, insects, and archaea, varying in both molecular structure and reduction potential (Zimbardi *et al.*, 2016). The laccases are well adapted to function under acidic conditions (Abdel-Hamid, Solbiati and Cann, 2013).

Laccases are important in lignin degradation where they catalyze the one-electron oxidation of phenolic hydroxyl or aromatic amino groups, forming free phenoxy radicals and amino radicals as well as decarboxylating phenolic and methoxy phenolic acids to attack the methoxy groups (Chukwuma *et al.*, 2020). Without the presence of a mediator, laccase can only oxidize phenolic lignin structures due to the low reduction potential (Chan, Paice and Zhang, 2020). Enzymatic processes have advantages over traditional chemical processes, including milder reaction conditions (temperature, pressure, and pH) and superior specificity and selectivity, which results in better use of energy and a lower generation of by-products and waste (Moreno *et al.*, 2015).

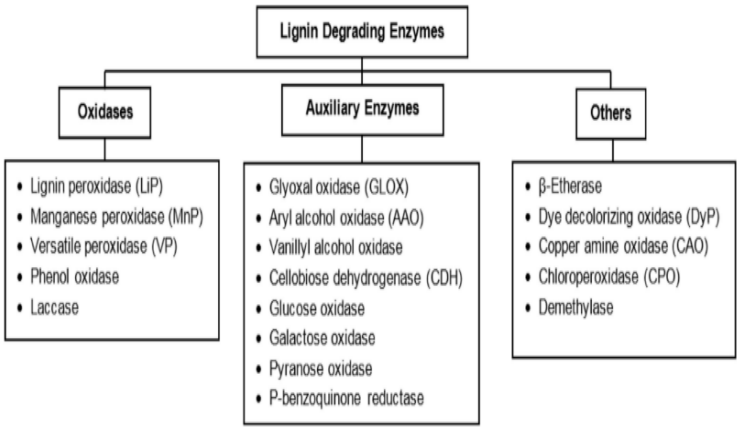

Figure 4. Lignin-degrading enzymes designate into three different semblances: Oxidases, Auxiliary enzymes and Others where laccase come under Oxidases. (Chan, Paice and Zhang, 2020)

However, as shown in figure 4 there are multiple lignin-degrading enzymes and particularly laccases that catalyze the oxidation of a wide range of substituted phenols, anilines and aromatic thiols and other aromatic compounds coupled to the reduction of molecular oxygen to water (Durão et al., 2006; Kallio et al., 2009; Brown et al., 2011; Bugg et al., 2011). Generally, laccases require a substrate and oxygen to catalyze the reaction (Majumdar et al., 2014). Laccases are known to bind four copper(II)ions where It binds with three different binding sites. each play an important role in substrate oxidation. (Christopher, Yao and Ji, 2014)

The four copper atoms of laccase are classified into three different groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy based on their coordination magnetic and spectroscopic properties (Chan, Paice and Zhang, 2020). In the native form, all the copper atoms are in a +2-oxidation state. This enables laccase to decarboxylate, demethylate, and demethoxylate phenolic and methoxy phenolic acids(Christopher, Yao and Ji, 2014). As shown in figure 5 the possible oxidative degradation pathway of β-O-4 lignin model compounds by laccases.

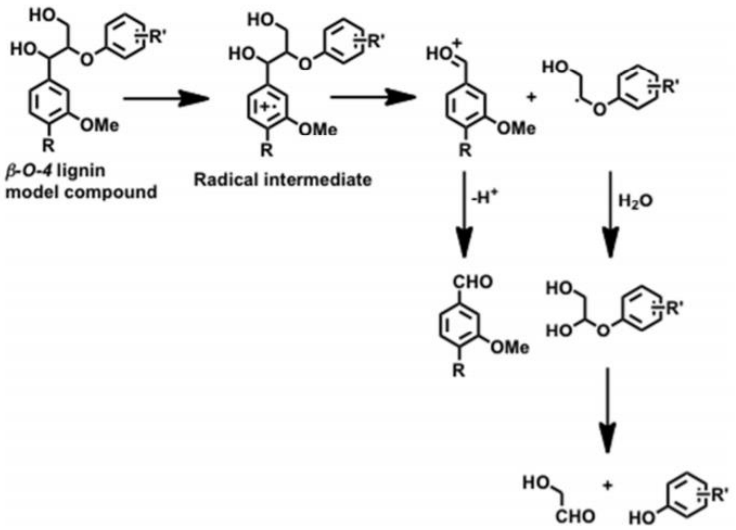

Figure 5. Oxidation mechanism of *(β-O-4)* lignin model compound by laccase through one-electron oxidation (Majumdar *et al.*, 2014).

On the other hand, the oxidation of non-phenolic compounds, which generally have a higher reduction potential, requires the presence of mediators due to the low oxidation potential of laccase (Dillies *et al.*, 2020) . Oxidation of a non-phenolic compound by laccase assumed through the removal of one electron produces cation radical intermediates followed by chemical decomposition (Christopher, Yao and Ji, 2014).

One explanation is that the mediator can facilitate electron transfer between the enzyme and substrate and thus enable the laccase to react with substrates of higher reduction potential than the enzyme (Hämäläinen *et al.*, 2018; Dillies *et al.*, 2020). It is also plausible that small-molecule mediators can easily diffuse into lignin macromolecule where the protein cannot access. A mediator can act as a carrier of electrons between the enzyme and the substrate by enabling the oxidation of the compounds that are not a substrate for laccase, thus enhance the catalysis process significantly(Bourbonnais and Paice, 1990; Dillies *et al.*, 2020).

It is generally agreed that mediators can help overcome the steric hindrance that exists between the substrate and the enzymes as well as the high reduction potential of the substrate

(Chan, Paice and Zhang, 2020). In addition, mediators increase the oxidation capabilities of laccase, thus initiating the oxidation of non-phenolic lignin compounds. Common redox mediators include 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (HBT), and 3-hydroxyanthranilic acid (HAA). ABTS was the first mediator described by Bourbonnais and Paice in 1990 where laccase from *T. versicolor* oxidized non-phenolic lignin compounds (veratryl alcohol) to veratraldehyde (Dillies *et al.*, 2020; Moreno *et al.*, 2020; Zhang *et al.*, 2020). Different depolymerization routes resulting in the formation of different intermediates can be stimulated by coupling different mediator systems with laccase (Christopher, Yao and Ji, 2014).

1.5 Lignin model substrates

A heavy work has been done on both phenolic and nonphenolic dimeric β-O-4 lignin model substrate which is widely used to study Cα−Cβ bond cleavage in lignin degradation (Kirk *et al.*, 1987). The ability of small laccases to oxidize the dimeric β-O-4 lignin model compounds indirectly reflects their lignin oxidative degradation ability, as more than 50% of lignin structure is composed of β-O-4 bonds (Villaverde *et al.*, 2009).

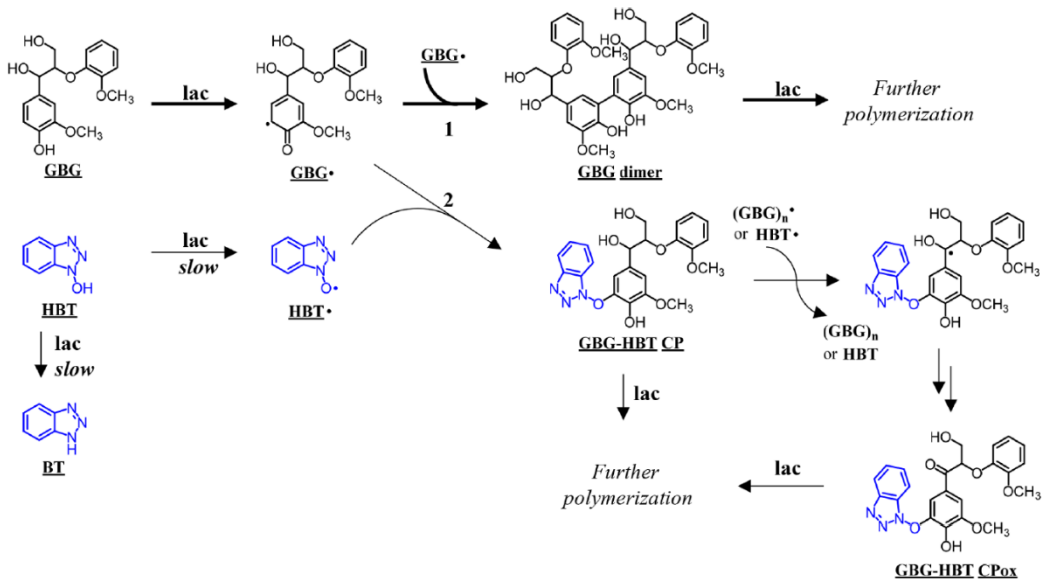

Figure 6. Overview of proposed reactions occurring after incubation of phenolic lignin dimer (GPG) with laccase alone and with a mediator (HBT) system. (Hilgers et al., 2018).

A mediator continuously oxidized by the laccase enzyme and subsequently reduced by the substrate. As the substrate due to its size cannot enter the laccase active site, the mediator acts as a carrier of electrons between the enzyme and the substrate thereby overcoming the steric hindrances that exist between them as shown in figure 6 route 2.

It was postulated that laccase reactivity decreases with the increase of the substrate size; therefore, the limited substrate accessibility is overcome through the use of appropriate laccase mediators. In the initial reaction step, the mediator is oxidized to stable intermediates with high redox potential by laccase. Thereafter, following diffusion-controlled reaction kinetics, the oxidized mediator diffuses away from the enzyme, and due to its small size is able to penetrate the pores of the plant cell walls to reach the target substrate.(Christopher, Yao and Ji, 2014)

Similarly, ABTS is readily oxidized by free radicals, various peroxidases, and laccase to the cation radical ABTS, and the concentration of the intensely colored, green–blue cation radical can be correlated to the enzyme activity(Christopher, Yao and Ji, 2014). The cation radical can be oxidized further to the dication ($ABTS^{2+}$) as shown in figure 7 where it shows the laccase-aided modification of ABTS during oxidization.

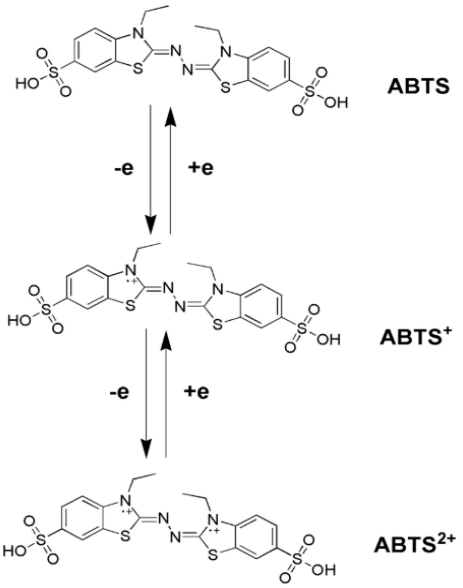

Figure 7. Oxidation of ABTS in presence of laccase where it can be oxidized further to the dication ($ABTS^{2+}$).(Christopher, Yao and Ji, 2014)

In the present work ABTS were used to oxidize the nonphenolic β-O-4 lignin model compound in the presence of small laccases. However, earlier work conducted with ABTS by Sudipta suggested that no C_α–C_β bond cleavage was observed while using ethanosolv lignin where result suggests that even in the presence of mediators neither bacterial nor fungal laccases were able to cleave the C_α–C_β bond, but they were able to modify the structure by forming the quinone product at different rates. (Majumdar *et al.*, 2014).

2 THE AIMS OF THE THESIS

1. To investigate possibilities to use enzymatic oxidation in to valorization of biorefinery hydrolysis lignin.
2. To find optimal conditions for enzyme treatment of lignin.
3. Optimization of the analysis for enzyme treatment of hydrolysis lignin.

3 EXPERIMENTAL PART

In the work, solubilization and acid precipitation of lignin was performed. Solubility of APL and HL was measured in two different buffers and NaOH solutions with various pH values. Enzyme treatment of HL and APL was analyzed with size exclusion chromatography (SEC). Enzymatic experiments were performed with different bacterial laccases on HL and APL. Analysis was performed on HL fractions provided by MetGen.

3.1 Materials

The HL is provided by Graanul Biotech, Estonia. AmLac, ScLac, SvLac and DyP enzymes are provided by Tiit Lukk group, TalTech and L371 enzyme is provided by MetGen Oy, Finland. 99.8% acetic acid (CH_3COOH) from Lachema, phosphoric acid (H_3PO_4) from Sigma Aldrich, 99.8% boric acid (H_3BO_3) was from Keemia Kaubandus AS, 97% sodium hydroxide (NaOH) from Lachner, Tris-HCl 99% from Sigma Aldrich, 30% peroxide (H_2O_2) was bought from Sigma Aldrich as well, ABTS was bought from Alfa Aesar. Filter paper used was Whatman Grade GF/B Glass microfiber, 460x 570 mm. MetGen lignin fractions (>70 KDa, 10-70 KDa, >3 KDa) was provided MetGen Oy, Finland.

3.2 Methods

3.2.1 Preparation of Acid Precipitated Lignin (APL)

Acid precipitated lignin (APL) was obtained from solubilizing crude lignin in 1% NaOH (wt.%) solution and 10% (wt.%) crude lignin for 30 min. After solubilization, the sample was centrifuged for 5 mins at 9000 rpm and the solution was filtered through a glass fiber filter paper. Following the filtration 50 ml of soluble lignin was treated with (3 M) sulfuric acid to precipitate lignin at pH 2. The precipitated lignin was centrifuged 5 min at 9000 rpm, the supernatant was discarded. The obtained APL pellet was washed with MQ H_2O. Washing entailed the addition of 5 ml MQ H_2O, vortexing and centrifugation at 9000 rpm for 5min, the supernatant was discarded. This step was repeated three times.

After centrifugation, acid precipitated lignin and insoluble lignin was dried under vacuum overnight at 50 °C. The weight of APL and insoluble crude lignin was measured by weight. The yield of APL was calculated from solubilized lignin by weight. As shown in figure 8 APL attained after drying overnight**.**

Figure 8. APL after drying overnight

3.2.2 Solubility measurements of HL and APL in different buffers.

Vacuum filtration is used to determine the solubility of HL and APL in different buffers. 20 mM Tris-HCl 7.75 pH and B&R buffer was prepared as described previously by Carlos and Victor (Islands, 2016). 0.1g HL and APL was weighted into the vials and 1 ml B&R buffer was added. The samples were solubilized with magnetic stirring for 1 h. After solubilization, the samples were centrifuged for 5 mins at 9000 rpm. The Whatman grade GF/B glass microfiber filter papers were cut and placed on the filtration system and after centrifugation the samples were vacuum filtered at 450 torr. The insoluble residue was left on the filter paper as shown in figure 9. After filtration, all the filters were oven-dried (105°C) overnight and the insoluble lignin was measured on a dry weight basis.

Figure 9. Insoluble lignin left over the filter paper

3.2.3 Lignin solubility measurements with UV-Vis spectrophotometer

Thermo Evolution 160 UV-Vis pectrophotometer was used to measure the UV absorption of lignin solutions. In NaOH solutions dissolved lignin samples were diluted with different dilution ratio using 15 ml vials and sufficient amount transferred to quartz cuvette (3 ml). Where different dilution ratios were administered following the calibration curve which is used to calculate the concentration of lignin in NaOH solution at 280 nm, while considering

the absorption coefficient ε (17.62 $l \cdot g^{-1} \cdot cm^{-1}$) and path length (1 cm). The reference sample was made using mQ water (2 ml).

3.2.4 Experiments with enzymes

Enzymatic hydrolysis experiments were performed described previously (Zhang *et al.*, 2020). However, we investigated higher concentration of lignin in hydrolysis process compare to the research done previously. 0.1g HL was weighted and 1ml of buffer was added. The samples were vortexed and solubilized for 1h with magnetic stirring. Following the magnetic stirring centrifugation was performed to separate insoluble lignin. The enzyme reactions were performed in 100 µl: 90 µl soluble lignin. 10 µl of the enzyme (10 µl MQ H_2O control) at room temperature with a reaction time of 22 h.

In presence of H_2O_2 and ABTS, an 80 µl sample was taken to treat it with 10 µl of enzyme and 10 µl of ABTS orH_2O_2 (in the case of DyP). The change in molecular weight was analyzed by SEC. While at MetGen Oy the reaction was performed in a 40 ml Flask with 3 g/l lignin concentration in presence of 1 ml L371.

3.2.5 Size Exclusion Chromatography (SEC)

The weight-average (Mw) and number-average (Mn) molecular weights of the lignin, as well as their polydispersity index (PDI), were determined by SEC. Polystyrene sulfonate salt standards (PSS) were used at the University of Tartu for the calibration at the different molecular weight. Before analysis in SEC, the enzyme-treated lignin samples were centrifuged for 10 minutes at 10 000 rpm and 90 µl supernatant was taken to mix with 90 µl of 0.1 M NaOH to make a final volume of 180 µl for analysis. SEC configuration is as follows: Communication bus module Shimadzu CBM-20A, Column oven Shimadzu CTO-10AS, pump Shimadzu LC-20AD, Refractive index detector Shimadzu RID-20A, Autosampler Shimadzu SIL-20A, Diode array detector Shimadzu SPD-M20A, Column PSS MCX 1000Å 5 um 8mm x 300mm, 0.1M NaOH mobile phase, the flowrate of 0.5 ml/min with a run time of 35 min and an injection volume of 20 µL.

While at MetGen Oy, the SEC configuration used at MetGen Oy, Finland is Agilent 1260 Infinity II, MCX 10µm 1000Å+100 000Å, UV detector (280+358nm), 0.1M NaOH was used as the mobile phase, the flowrate of 0.6 ml/min with a run time of 35 min and an injection volume of 20 µL.

4 RESULTS AND DISCUSSION

4.1 Preparation of lignin samples

In this study, two different types of birch lignin were subjected to solubility experiments and enzyme treatment. The first is so called crude HL (later on just HL). HL is obtained directly from the production process of birch biomass hydrolysis, which can be produced with different "impurities". These impurities are usually free sugars, cellulose residues, enzyme proteins and minerals from hydrolysis process. In the first part of this work, HL with approximately 80% purity was used as determined by the research group (free sugars 6 %, cellulose 7.5 %, proteins 5 % and minerals 1.5 %). Crude HL was dried under vacuum overnight at 50°C and grinded with disintegrator (DESI-11, 200 Hz).

Second type is acid precipitated lignin (APL). Acid precipitation is a well-known and simple method that uses strong acid to separate lignin from kraft black liquor. Precipitation is based on the dissociation equilibrium of weak acid groups, which affects the solubility behavior of lignin-like species (Jardim *et al.*, 2020). All biorefinery HL is slightly soluble in pure water as most other technical lignins. In industrial applications this lignin is mostly solubilized by using alkaline conditions (NaOH). Dissolution of HL in NaOH solution and acid precipitation of APL are described in the Experimental section (see 3.2.2). Solubility of HL in 1 % NaOH, was determined by UV-Vis spectrophotometric protocol used by research group (see 3.2.3). Average solubility of HL was determined 55 ± 1.5 g/l, which is 55 % of the starting material. The goal was not to dissolve all of the HL, but to separate the cellulose and proteins. The choice of soft conditions was also to avoid large changes in the structure of the lignin occurring at high content of NaOH. The prepared HL solutions were immediately treated with 3 M H_2SO_4 to precipitate APL.

The dry mass of APL was used to compare the APL yield to the originally solubilized HL. As an average result of multiple experiments, it was found that approximately 91% of dissolved HL can precipitate to produce APL. With this procedure free sugars, some proteins and ach compounds were separated from lignin. APL is different from the starting birch HL in some aspects, such as solubility and purity. This creates more possibilities for analysis of the enzyme activity on lignin. The crude HL has higher heterogeneity and consist impurities, whereas the APL is free of additives but could consist more ash and be also modified due to the solubilization-precipitation process. With the exception of the molecular weight distribution analysis that follows, no further structural analyzes were performed for lignin samples

in this work. These HL and APL samples were used in the solubility trials and enzymatic treatment at varied conditions.

4.2 Solubilization and solubility of HL and APL in enzyme suitable buffers

Enzymatic reactions typically require specific conditions to perform their function. However, laccases are reported to be extracellular, which means they are generally more stable and have the potential to be active in harsher conditions (Majumdar *et al.*, 2014). To verify these hypotheses, different buffers and solutions were chosen for testing as a preliminary screen for laccase activity on lignin.

To perform this test of laccases, stable the lignin solutions have to be formed. Recent reports have shown that in some cases lignin precipitates when enzyme-catalyzed reactions occur. In experiments of this study, we also wanted to pay attention to this type of detail, which means that no heterogenous (colloidal) solutions can be used. In ideal conditions, lignin concentration would be high, and we achieve complete dissolution. Both of these are a problem in many cases and the same is true here (Wang *et al.*, 2020).

The types of lignin used in this study are more soluble in alkaline conditions (pH >8). For the reasons listed here and above, different buffers were used for both birch HL and APL. These include B&R buffer, which allows for different pH values to be used, as well as 20 mM Tris-HCl at pH 7.5.

The average experimental solubility of APL in Tris-HCl was found to be 18 ±1.2 g/l whereas for HL was 17 ±1.5 g/l. Solubilization experiments were conducted with solid lignin to Tris-HCl buffer solution ratio 1/10 (10%). As expected, lignin solubility in these conditions is moderate. However, it is important to note the difference between the two lignin samples. In the case of APL, only the soluble lignin fractions enter the Tris-HCl buffer solution, HL also contains free sugars and minerals in the solution, the actual lignin content is lower.

Solubilization of HL and APL in B&R buffer were carried out with solid lignin to buffer solution ratios 1/10 (10%) and 0.5/10 (5%). The idea for using different solid to liquid ratios was to achieve the highest possible lignin content in the buffer (Evstigneev, 2011). Solubilities of HL and APL in B&R buffer were presented in table 2. Solubility of both types of lignin as a function of the pH in B&R buffer are shown in figure 10.

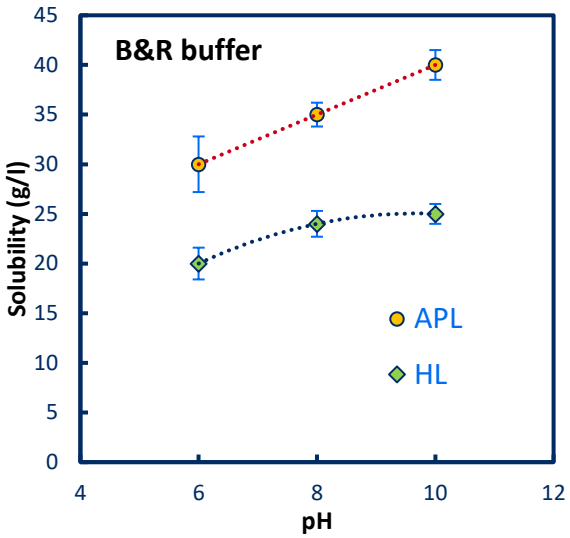

Figure 10. Solubility of APL and HL in B&R buffer at pH 6, 8, 10.

Table 2. Solubility of APL and HL in B&R buffer at pH 6, 8, 10.

pH	Solubility (g/l) Solid to liquid ratio 0.05		Solubility (g/l) Solid to liquid ratio 0.1	
	HL	APL	HL	APL
6	12	20	20±1.6	30±2.8
8	12.5	25	24±1.3	35±1.2
10	13.5	35	25±1.0	40±1.5

If there is a higher amount of solid lignin, there are potentially more lignin molecules with smaller molecular weight that could dissolve. This result can also be seen in table 2 and figure 10. As the pH of the buffer solution increases, the solubility of lignin also increases in all cases. The behavior of HL is interesting in figure 10, which shows the decrease in the effect of pH on the solubility in the R&B buffer. However, since the lignin content was the highest when 10% solid lignin was dissolved, these solutions were used in further experiments with enzymes.

4.3 Enzymatic treatment of HL and APL in buffers and NaOH solutions

In order to obtain solutions of HL and APL in different buffers, the lignin was first solubilized as described previously. The solution was separated from the insoluble residue, this solution is the material for enzyme treatment and also serves as a control. HL and APL were treated with 10% solution of different enzymes provided by Tiit Lukk group (TalTech). At first, experiment was performed where only enzymes were analyzed using SEC without containing lignin in the buffer. The reason behind this experiment was to see whether enzymes disturb SEC analysis and was found it has no effect. At second, mediator (ABTS) were analyzed similarly and was found no influence on molecular weight distribution measurements.

For enzyme experiments, time experiments were first performed to find the optimal experimental time by sampling at hourly intervals. APL and ScLac in R&B buffer were used in these assays. It was found that after 22 hours, the SEC no longer records changes in the molecular weight distribution of lignin.

Also, while treating HL and APL with H_2O_2 and mediator (ABTS) the different substrate and enzymes solution concentration was 10%, meaning if the reaction has made in presence of H_2O_2 and ABTS. Then 10µl (APL) + 10µl H_2O_2 (2 mM) +10µl ABTS (1.5 mM) were in incubated to reach 100 µl solution.

4.3.1 Experiments in Tris-HCl buffer

Different enzymes were investigated to treat HL and APL in Tris-HCl buffer where the reaction time was postulated 22 h as mentioned before. The result showed it is possible to treat HL in 20 mM Tris-HCl buffer at 7.75 pH. Where we discover increase in molecular weight of HL after incubating ScLac for 22h at room temperature. As shown in figure 11(a) their is an increase in molecular weight which resembles the fact that, it is possible to oxidize HL at pH 7.75. The increase in molecular weight define the area in the figure 11 where compare to the control the area treated lignin increased. It is interesting to note that we introduce higher concentration of lignin i.e based on our solubility results, we treat 17 g/l lignin mass in 20mM Tris-HCl buffer compare to the literature, where concentration was about 2 g/l (Zhang *et al.*, 2020).

Also, as shown in figure 11(b), APL was treated with ScLac at pH 7.75 in Tris-HCl buffer. Under Tris-HCl buffer condition the molecular weight distribution did not show much effect, which drive the fact that this condition is not favorable to treat APL compare with HL where enzyme is showing effect by increasing the molecular weight.

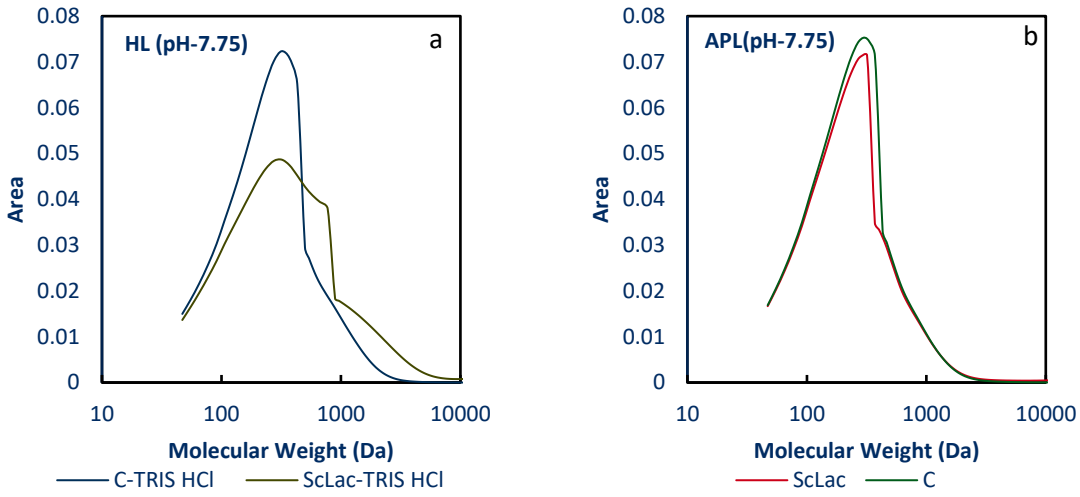

Figure 11. HL(a) and APL(b) in 20mM Tris-HCL buffer at pH 7.75.

The Average molecular weights i.e number average (Mn), weight average (Mw) and PDI are all showing molecular weight increased as shown in table 2. PDI is calculated by Mw/Mn and it shows the dispersity of the distribution. For example- A monodisperse polymer where all the chain lengths are equal has an Mw/Mn = 1.

Table 2. Mn, Mw and PDI values of HL and APL in presence of ScLac at pH 7.75.

Time	Mn (Da)	Mw (Da)	PDI
Start (HL)	211	448	2.11
ScLac (HL)	253	1594	6.30
Start (APL)	194	392	2.02
ScLac (APL)	200	409	3.04

This means the enzyme was cleaving specific bonds which leads to radicalization and due to radicalization it leads to repolymerization. One explanation for the increase in molecular weight could be, where enzymes are only cleaving a specific bond and immediately due to a higher concentration of lignin inside, it leads to make bonds with other cleaved bonds. This means repolymerization occurs simultaneously with depolymerization with increases in reaction time which leads to increase in the molecular weight (Chan, Paice and Zhang, 2020; Kumar and Chandra, 2020; Moreno *et al.*, 2020).

We also tested HL with DyP in 20mM Tris-HCl buffer at pH 7.75 where we found no significant change in molecular weight. However, there is a minor decrease in molecular weight as shown in figure 12, but it does not lead to any proper conclusion. There is a possibility of depolymerization but because it is not solid we cannot say DyP is showing some effect on HL under Tris-HCl buffer. Also, because DyP required H_2O_2 to react with substrate, it might be the effect of H_2O_2 which influence depolymerization (Brown, Barros and Chang, 2012).

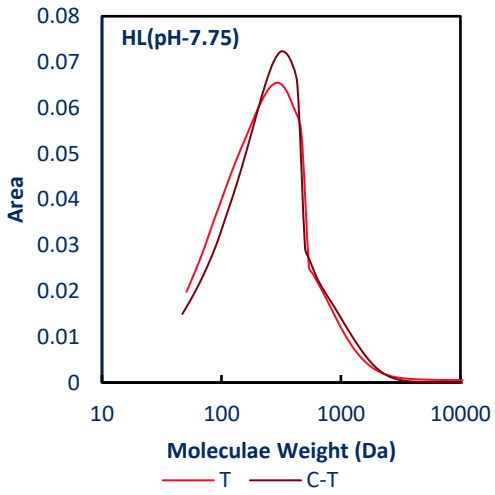

Figure 12. HL treated with DyP in 20mM Tris-HCL buffer.

Average molecular weights (Mn, Mw and PDI) as shown in table 3 did not reveal any significant change in molecular weight.

Table 3. Mn, Mw and PDI values of DyP in 20mM Tris-HCL buffer with H_2O_2.

Time	Mn (Da)	Mw (Da)	PDI
Start	216	463	2.14
DyP	203	542	3.15

APL and HL also treated with AmLac and SvLac in Tris HCl buffer at pH 7.75. The results can be found in Appendix Figures 2 and 7. Also, Mn, Mw and PDI values of APL and HL treated with AmLac and SvLac in Tris HCL buffer can be found in Appendix Table 1 and 2. where the results are showing the enzymatic effects on HL in Tris-HCl buffer.

4.3.2 Experiments in B&R buffer

The results showed it is possible to treat HL and APL at higher pH with higher lignin concentration in B&R buffer. As shown in figure 13 (a) and (b) the molecular weight distribution

increased. The increase in molecular weight resembles the fact that it is possible to oxidize HL and APL at alkaline pH.

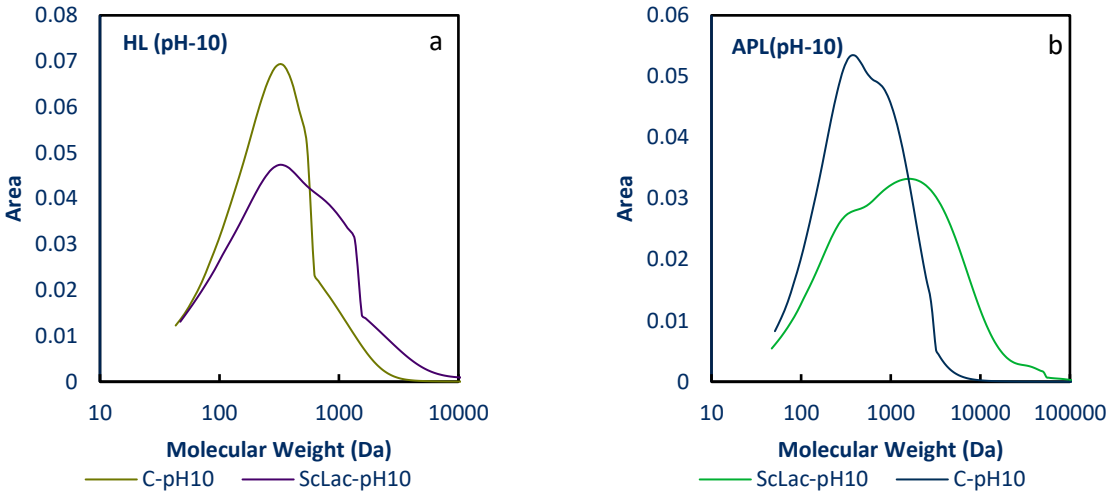

Figure 13. HL(a) and APL(b) treated with ScLac at pH 10 in B&R buffer.

It is believed that laccases catalyze the oxidation of lignocellulosic substrate to produce aryl cation radicals where these radicals spontaneously rearrange, leading to further polymerization of oxidized materials by the fission of carbon-carbon or carbon-oxygen bonds of the alkyl side chains or to the cleavage of aromatic rings as discussed by (Marzullo *et al.*, 1995).

Also, if we acknowledge the Mn, Mw and PDI values for HL and APL in B&R buffer at pH 10 as shown in table 4, we can see the increases in Mn, Mw and PDI values after 22 hours of enzymatic oxidation of HL and APL.

Table 4. Mn, Mw and PDI values of HL under ScLac enzyme at pH 10 in B&R buffer.

Time	Mn (Da)	Mw (Da)	PDI
Start (HL)	215	492	2.28
ScLac (HL)	265	1322	4.98
Start (APL)	288	708	2.458
ScLac (APL)	468	3462	7.417

HL and APL was also treated with AmLac, SvLac at three different pH (6, 8 and 10) in B&R . The oxidation results of APL with AmLac and SvLac in B&R buffer at different pH conditions can be found in Appendix Figure, 3, 4. Also, HL treated with SvLac in B&R buffer the reults can be found in Appendix Figure 5.

The increase in molecular weight of HL and APL as shown in figure 13 (a) and (b) was due to the repolymerization of depolymerized lignin.

Nevertheless, it can be concluded that during lignin oxidation there is a competition between de-polymerization and re-polymerization, which would shift towards repolymerization when higher concentration of lignin was treated in presence of bacterial laccases.

The treatment results of APL in presence of ScLac in B&R buffer at pH 6 and 8 can be found in Appendix Figure 1. Also, if we see the Mn, Mw and PDI values of HL in Appendix Table 2, we see increases in Mn, Mw and PDI after 22 hours of enzymatic oxidation. Also, if we see Mn, Mw and PDI values of APL in B&R buffer found in Appendix Table 1, we also see increase in molecular weight.

DyP needs H_2O_2 to catalyse the reaction as discussed previously. Enzymatic hydrolysis of HL in presence of DyP with H_2O_2 is possible and disclosed repolymerization in B&R buffer. The result of DyP-treatment at pH 6 and pH 8 in B&R buffer can be found in Appendix Figure 8.

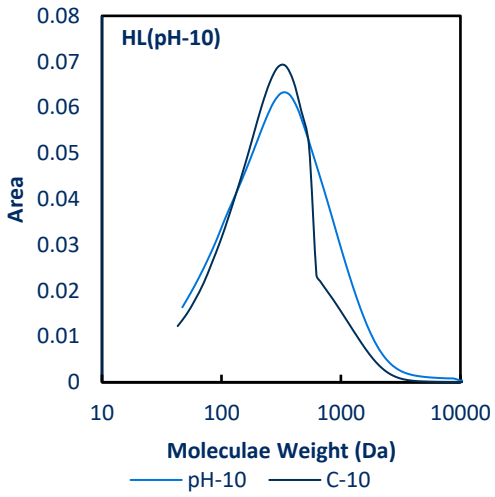

Figure 14. Treatment of HL with DYP enzyme in B&R buffer.

Mn, Mw and PDI results can be found in Appendix Table 3.

4.3.3 Experiments in 1 wt% and 0.4 wt% NaOH solutions

It is very fascinating to see laccases performing oxidation in NaOH solution. As it was found out laccases can oxidize lignin up to an optimum pH and concentration, where NaOH concentration plays a very critical role in estimating the theory behind oxidation (Hämäläinen *et al.*, 2018) . It is possible to say that low concentration of NaOH can lead to enzymatic hydrolysis up to pH 10 with both HL and APL in NaOH solution.

4.3.3.1 Experiments in 1 wt% NaOH solution

The optimum pH and concentration of HL and APL at which the laccases can catalyze the reaction was important to investigate. It is found out while decreasing the pH with 3M H_2SO_4 does not help to increase the rate of oxidation in 1% NaOH when compare to 0.4% NaOH. As shown in figure 15 and 16(a) there was no change in molecular weight with APL and HL under pH 12, 11, 10 where 1% of NaOH was utilized.

However, there is an increase in molecular weight with HL at pH 9 shown in figure 16(b). It might be because of lower concentration of NaOH or addition of acid (3 M H_2SO_4) to drop the pH.

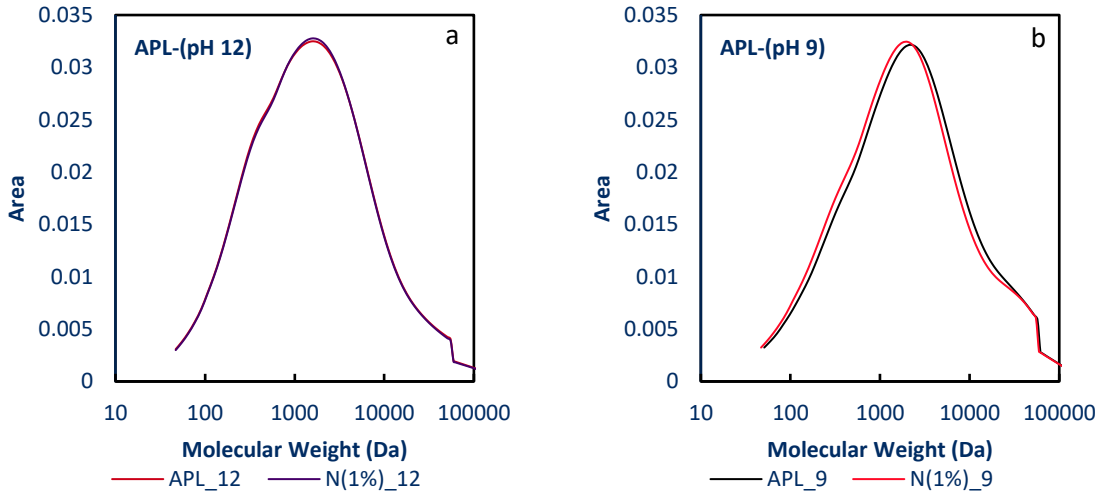

Figure 15. APL treated with ScLac in 1% NaOH solution at pH 12(a) and 9(b).

The results of 1% NaOH solution at pH 11, 10 of APL and HL which shows no sign of change in molecular weight can be found in Appendix Figures 9, 10.

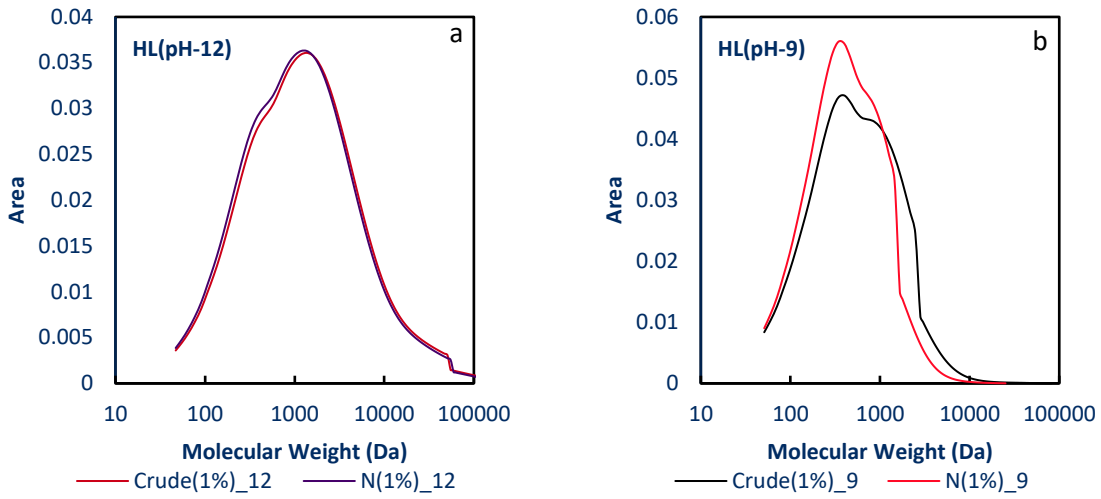

Figure 16. HL treated with ScLac in 1% NaOH solution at pH 12(a) and 9(b).

4.3.3.2 Experiments in 0.4 wt% NaOH solution

0.4% concentration of NaOH was used with ScLac enzyme at four different pH 12, 11, 10, 9 where the concentration of HL and APL was constant but the pH was dropped using 3M sulfuric acid as discussed before. The result showed till pH 10 the enzymes perform oxidation with both APL and HL. Therefore an increase in molecular weight can see figure 17 and 18. However, while increasing the pH or NaOH concentration the enzymes did not show any effect.

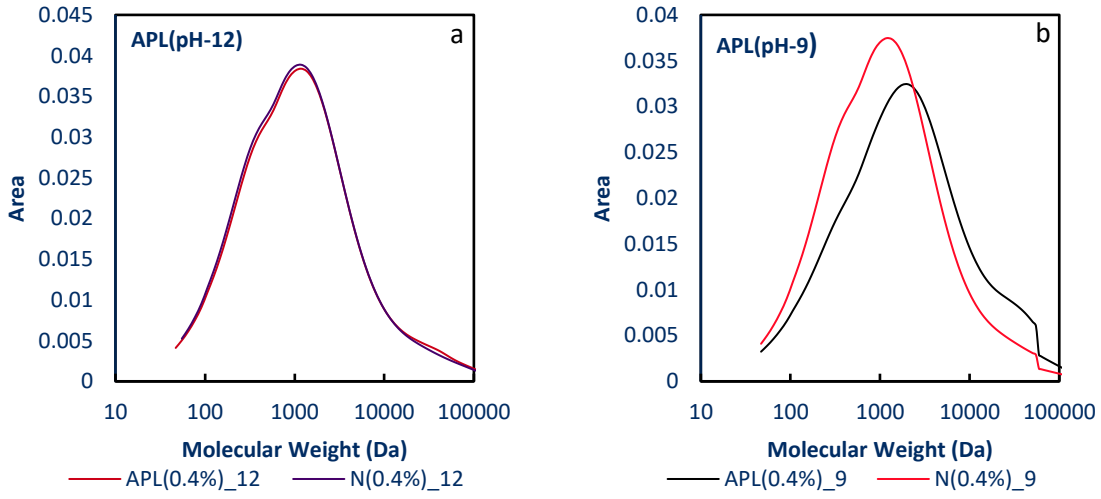

Figure 17. APL treated with ScLac in 0.4% NaOH solution at pH 12(a) and 9(b).

The Mn, Mw and PDI values of APL and HL treated with ScLac, can be found in Appendix Table 4.

It is observed that enzymes can oxidize the novel industrial HL in NaOH solution. Concentration of NaOH plays a vital important role in oxidizing the lignin at alkaline pH.

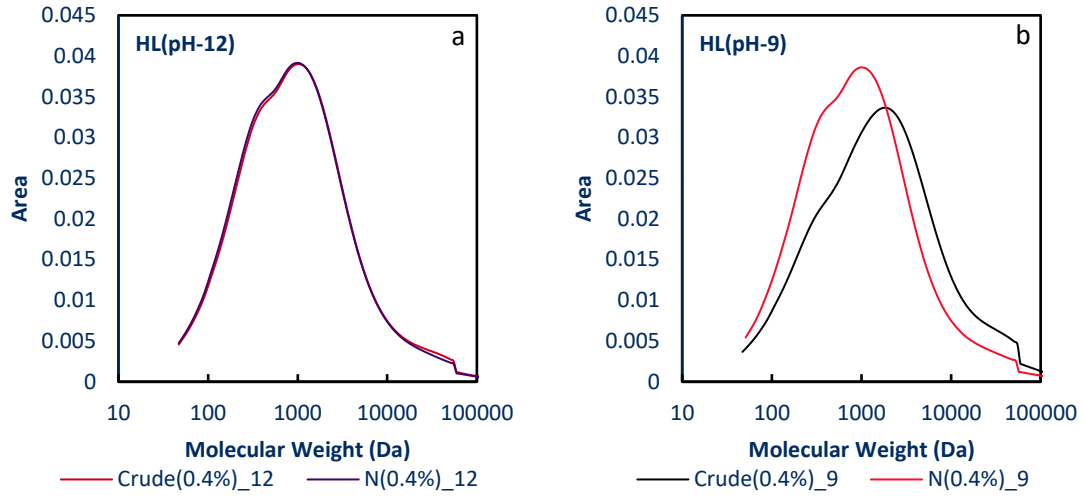

Figure 18. HL treated with ScLac in 0.4% NaOH solution at pH 12(a) and 9(b).

APL and HL in 1% NaOH at pH 11 and 10 can be found in Appendix Figure 11 and 12.

4.3.4 Mediator effects

According to the literature, ABTS act as a mediator to influence the non-phenolic oxidation of lignin in different buffers (Li, Xu and Eriksson, 1999; Hilgers *et al.*, 2018; Dillies *et al.*, 2020). Here ABTS was present with all three laccases in form of a cocktail which demonstrated the increase in molecular weight that mimics the repolymerization phenomenon as discussed in previous chapters. It was found out ABTS influence oxidation whilst using B&R buffer and NaOH solution at different pH (figure 19). It could be assumed that ABTS augment the reaction which leads towards more condensed polymeric lignin.

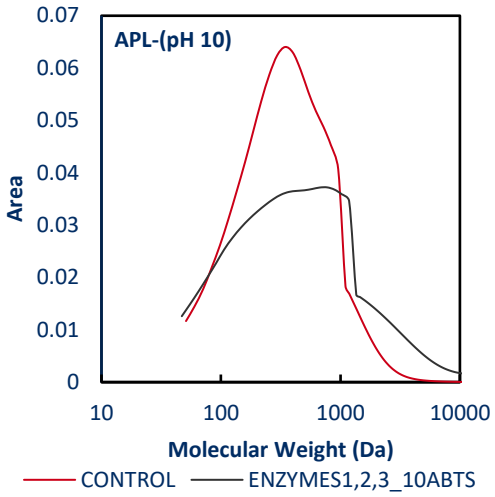

Figure 19. APL in presence of AmLac, SvLac and ScLac and ABTS increases molecular weight at pH 10 NaOH Solution.

It is found out ABTS influence oxidation of APL in presence of DyP enzyme in B&R buffer that leads to an increase in molecular weight as shown in figure 20. However, it is interesting to note that HL in presence of DyP shows a sign of depolymerization in presence of ABTS as shown in figure 20.

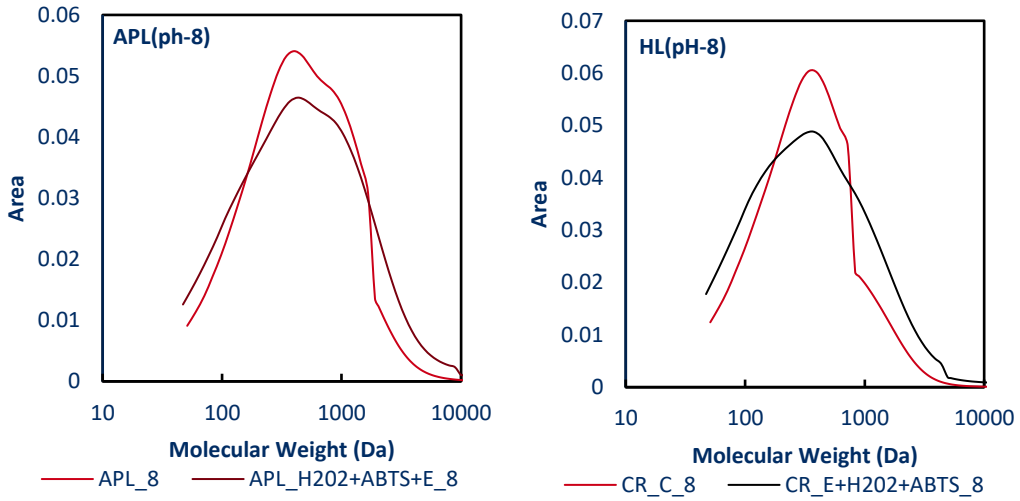

Figure 20. APL and HL in presence of DyP which defines repolymerization and some de-polymerization with ABTS in B&R buffer at pH 8.

The Mn, Mw and PDI values can be found in Appendix Table 5.

4.4 METNIN™ fractions in NaOH solution

MetGen is a Small Medium Enterprise (SME) in Finland, METNIN™ Lignin Refining Technology allows removing the complexity of lignin molecule where biotechnology potentiality was administered e.g. METNIN™ breaks down any type of lignin gently and affordably into specific fractions US Patent for method for lignin depolymerization patent (Patent # 10,626,553). METNIN™ fractions have the chemical characteristics required for the final applications. METNIN™ Products include intermediate lignin fractions for endless uses for aromatics as well as ready-to-use formulations for industrial materials and chemicals (Hämäläinen *et al.*, 2018). In the work fractions from METNIN™ Lignin Refining Technology were studied in order to get more information about working with enzymes and to develop the analysis. Also, solutions were investigated in higher dosages of the enzyme.

4.4.1 Enzymatic treatment of HL in presence of L371

First, the reaction was performed with HL where 3g/l concentration of lignin was incubated with 1ml of enzyme L371 for 22 h at room temperature with a pH of 10.5. Samples were taken at different time intervals i.e. Start, when enzyme was added (S+E), after 1 h, 3 h, 5 h, and 22 h. It was found out that there is an increase in molecular weight as shown in figure 21 and in table 5.

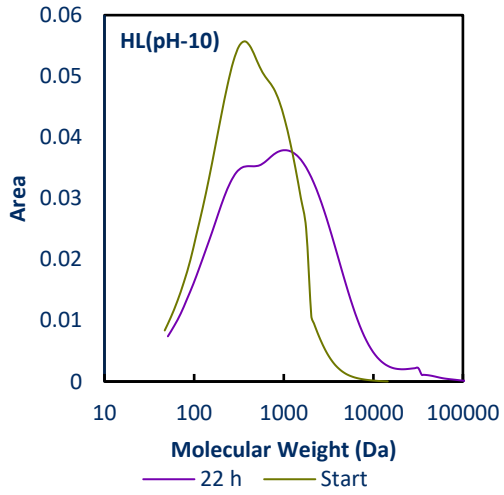

Figure 21. Repolymerization of HL in presence of L371 after 22 hours. The analysis is done in SEC at 280 nm.

Table 5. Mn, Mw and PDI of repolymerized HL. The analysis is done in SEC at 280 nm. Where S+E defines (Substrate + Enzyme).

Time	Mn (Da)	Mw (Da)	PDI
Start	403	661	6.91
S+E	410	691	8.23
1H	560	800	7.41
3H	580	1090	8.9
5H	640	1578	13.05
22H	700	4133	17.24

Also, the SEC analysis was done at two wavelengths 280 and 358 nm at MetGen Oy, where Mn, Mw and PDI values at 358 nm were analyzed in the same way as 280 nm of HL. Results for 358 nm wavelengths can be found in Appendix Figure 13 and table 6.

The results depicts the repolymerization as discussed earlier in the work. However, it was found the similar results with different bacterial laccases in same solutions.

4.4.2 Enzymatic treatment of >70 kDa fraction of HL

We found similar results with fractions of birch hydrolysis lignin fractionated by MET-NIN™ Lignin Refining Technology. The enzymatic treatment of >70 kDa fractions was performed with the same solution conditions as described before where >70 kDa fraction was solubilized at pH 10 in NaOH solution.

As shown in figure 22 the same result was discovered where increase in molecular weight mimics repolymerization and polycondensation of fractions.

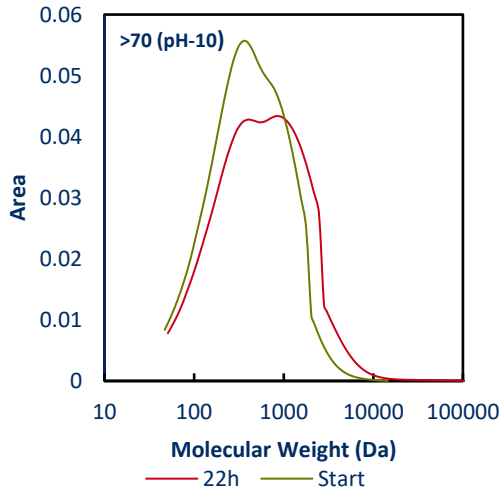

Figure 22. Repolymerization of >70 kDa treated with L371 and analyzed in SEC at 280 nm.

Samples were taken at a specific time and analyzed in SEC at 280 nm. Mn, Mw and PDI values is shown in table 6. As shown in table 6, all of these parameters increase over time.

Table 6. Mn, Mw and PDI of repolymerized >70 kDa fractions of HL treated with L371 and analyzed in SEC at 280 nm.

Time	Mn (Da)	Mw (Da)	PDI
S	352	400	6.06
S+E	389	430	6.1
1h	450	600	6.8
3h	480	869	7
5h	530	908	7.2
22h	560	1350	7.3

4.4.3 Enzymatic treatment of 10-70 kDa fraction of HL

The same result was obtained with 10-70 kDa fractions as shown in figure 23. where 10-70 kDa fractions were tested with 1ml L371 laccase and the samples were taken at Start, S+E, 1 h,3 h, 5 h and 22 h which is used to compare the Mn, Mw and PDI.

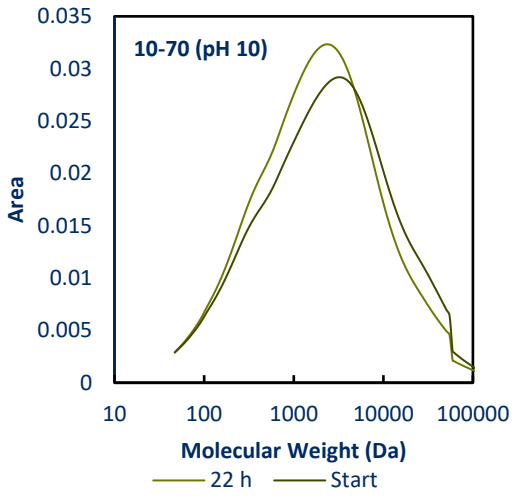

Figure 23. Repolymerization of 10-70 kDa treated with L371 and analyzed in SEC at 280nm.

As shown in table 7 the measurement of Mn, Mw and PDI shows increase in molecular weight during the reaction referring to repolymerization of 10-70 kDa fractions.

Table 7. Mn, Mw and PDI of 10-70 kDa treated with L371 and analyzed in SEC at 280 nm.

Time	Mn (Da)	Mw (Da)	PDI
S	241	443	1.84
S+E	230	480	1.9
1H	235	500	1.9
3H	240	690	2
5H	365	800	2.2
22H	440	850	2.34

Similarly, L371 was tested with >3 kDa fractions. Results can be found in Appendix Figure 14 and the Mn, Mw and PDI can be found in Appendix Table 7.

5 SUMMARY

Lignocellulosic biomass is the main source of renewable chemicals. Biorefineries allow the fractionation of polysaccharides and hydrolysis lignin (HL), which can already be utilized in high value products. Biorefineries are presently focused on the valorization of sugars from polysaccharides, while neglecting the potential of HL. It is suggested that in the near future the value-added utilization of HL will be a game changer for the biorefinery idea. One possible way for the valorization can be the treatment of HL by enzymes.

However, there are many barriers to using enzymes in lignin solutions. Many of them require acidic or near neutral pH, however lignin solubility in these solutions is very low. While there are some enzymes working in alkaline conditions, these are only used with low lignin concentration. For these reasons the application of enzymes on an industrial scale has remained elusive.

The goal of the present work was to investigate possibilities to use enzymatic oxidation to valorize biorefinery HL. For the first time, the treatment of high-purity industrially fractionated birch HL with enzymes was used in this work. Beside crude HL, three solubilized HL fractions and acid precipitated lignin was used. Multiple reaction conditions were tested including different buffers and NaOH solutions at specific pH values. Four different laccases as well as a peroxidase were tested in these conditions. All enzymes showed activity in most conditions for different HL fractions. The optimal enzyme treatment conditions were found, which encompass a range of pH values, high lignin solubility and enzyme activity. Although, when the enzymes showed activity the lignin repolymerized. This leads to new challenges, which require our attention in order to resolve the repolymerization issue. These first steps are still promising as the obtained knowledge in terms of the analysis and treatment methods, which allows us to further progress in this field. A positive result was the observation of enzyme activity in conditions with several times higher lignin content than previously described.

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Appendix

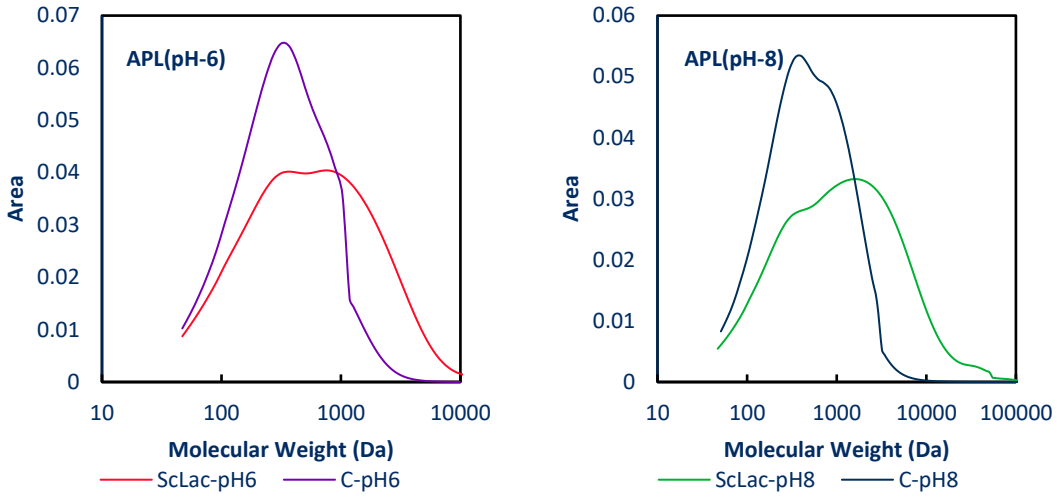

Figure 1. APL treated with ScLac at pH 6 and 8 in B&R buffer.

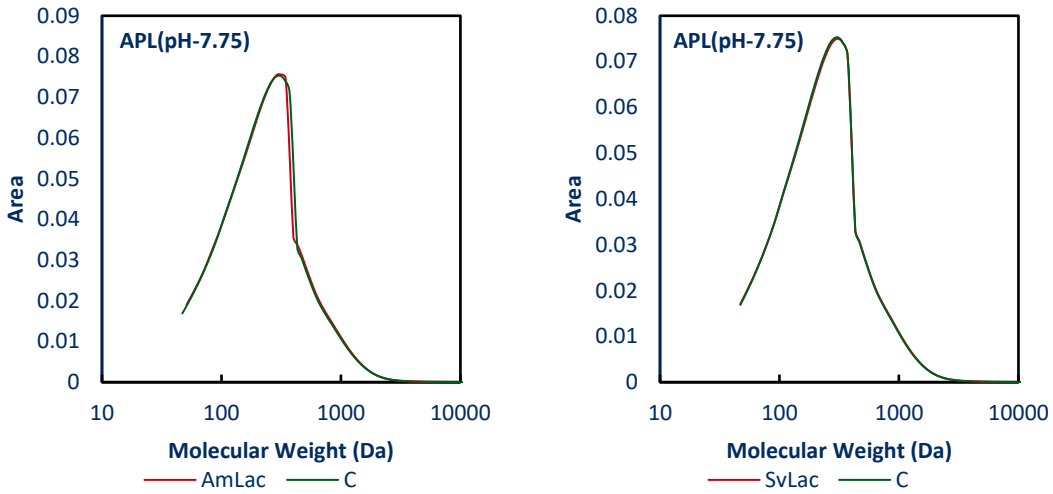

Figure 2. APL treated with AmLac and SvLac in Trsi-HCl buffer.

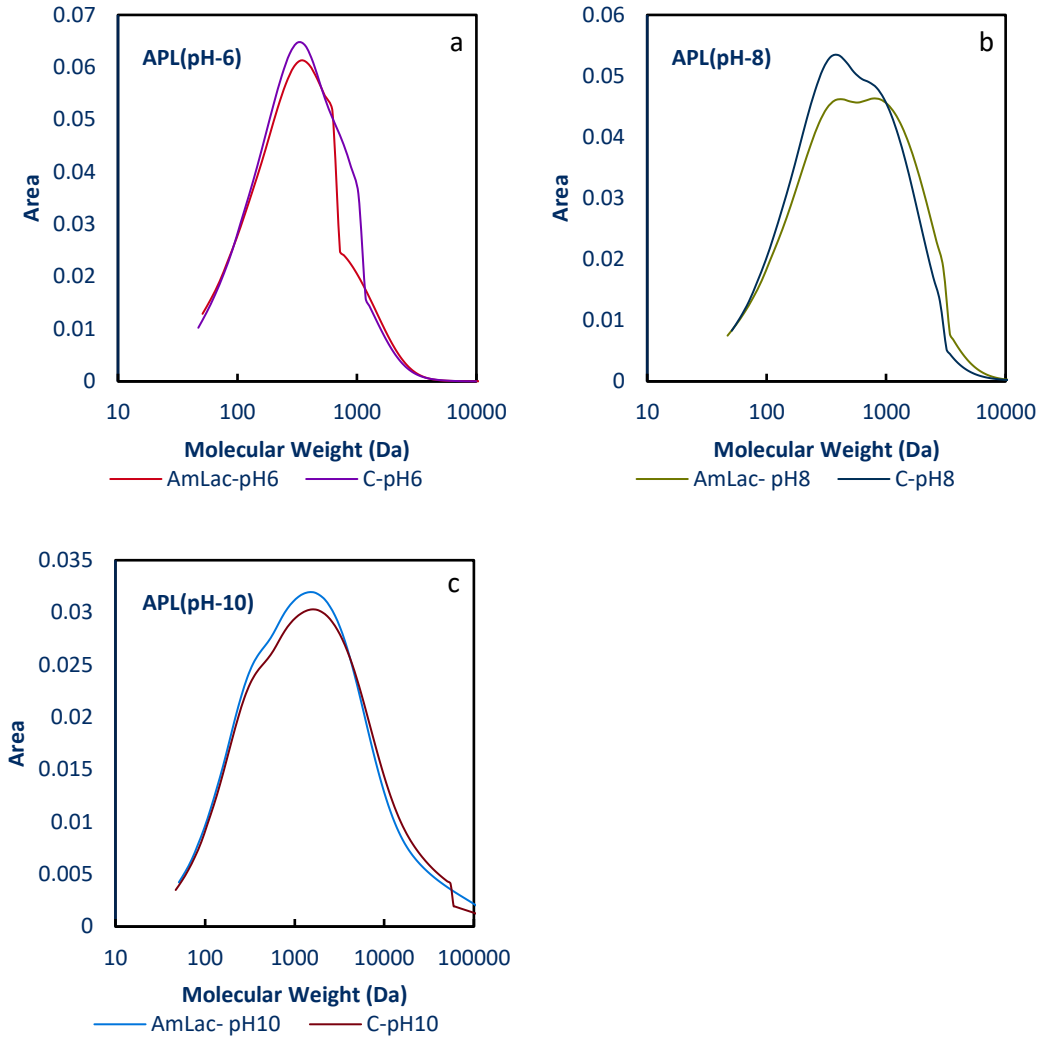

Figure 3. APL treated with AmLac at three different pH. a- pH 6 , b- pH 8, c- pH 10 in B&R buffer.

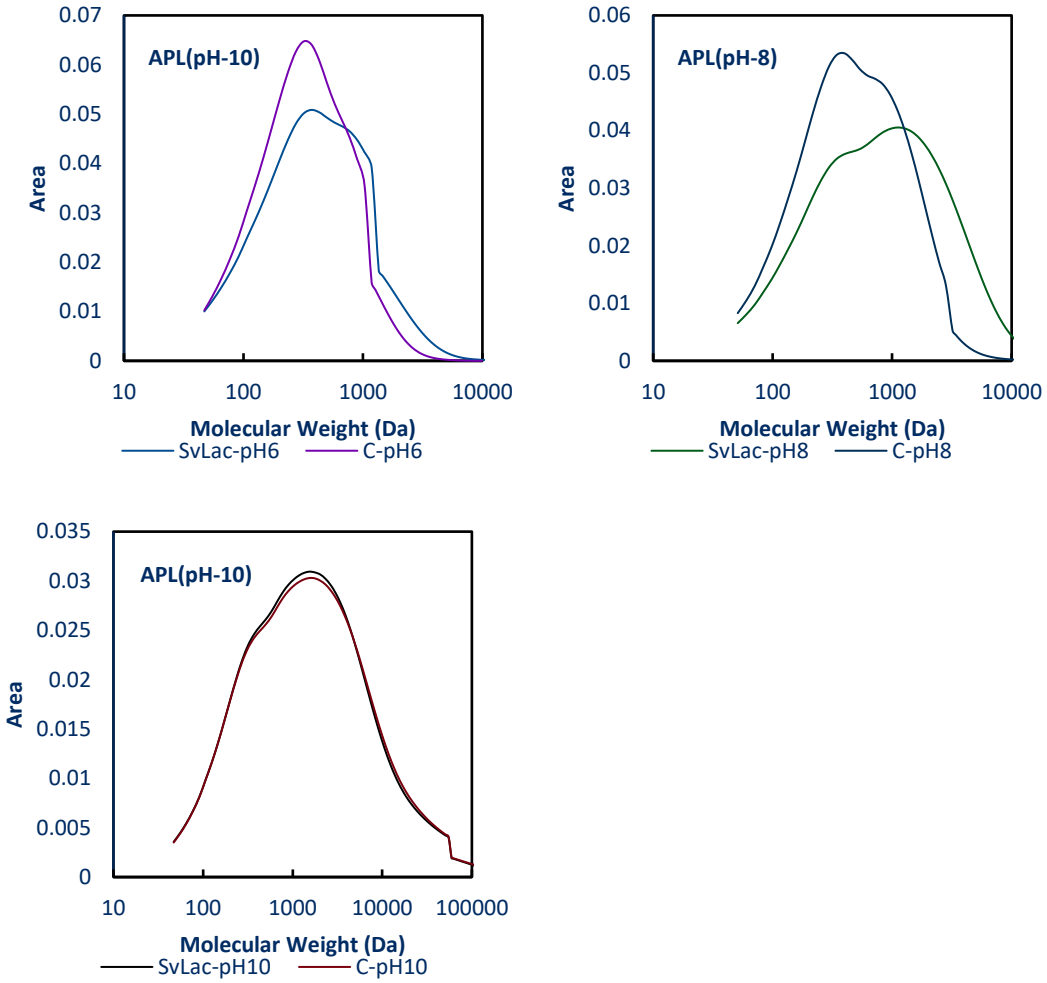

Figure 4. APL treated with SvLac at three different pH. 6 , pH 8, pH 10 in B&R buffer.

Table 1. Mn, Mw and PDI values of APL treated with AmLac(red), SvLac(green), Sclac(blue) and Control (black) at pH 6(a), 8(b), 10(c) in B&R and Tris-HCl(d) buffers.

Laccases	Buffer	pH	Mn (Da)	Mw (Da)	PDI
AmLac	B&R	6	200	389	1.94
		8	357	1122	3.14
		10	302	791	2.61
	Tris-HCl	7.75	256	599	2.34
SvLac	B&R	6	303	980	3.23
		8	347	1210	3.48
		10	404	1709	4.23
	Tris-HCl	7.75	194	388	2.00
ScLac	B&R	6	347	1985	5.71
		8	393	2512	6.3
		10	468	3472	7.4
	Tris-HCl	7.75	200	409	3.04
Control	B&R	6	245	550	2.24
		8	262	615	2.34
		10	288	708	2.45
	Tris-HCl	7.75	194	392	2.02

Table 2. Mn, Mw and PDI values of HL treated AmLac(red), ScLac(green), Svlac(blue) and Control (black) at pH 6(a), 8(b), 10(c) in B&R and Tris-HCl(d) buffers.

Laccases	Buffer	pH	Mn (Da)	Mw (Da)	PDI
AmLac	B&R	6	213	728	3.4
		8	244	691	2.8
		10	268	856	3.1
	Tris-HCl	7.75	241	665	2.7
ScLac	B&R	6	241	1624	6.7
		8	245	1205	4.9
		10	265	1322	4.9
	Tris-HCl	7.75	253	1594	6.3
SvLac	B&R	6	232	1020	4.4
		8	238	950	3.9
		10	258	958	3.7
	Tris-HCl	7.75	246	871	3.5
Control	B&R	6	192	469	2.4
		8	208	437	2.0
		10	215	492	2.2
	Tris-HCl	7.75	211	448	2.1

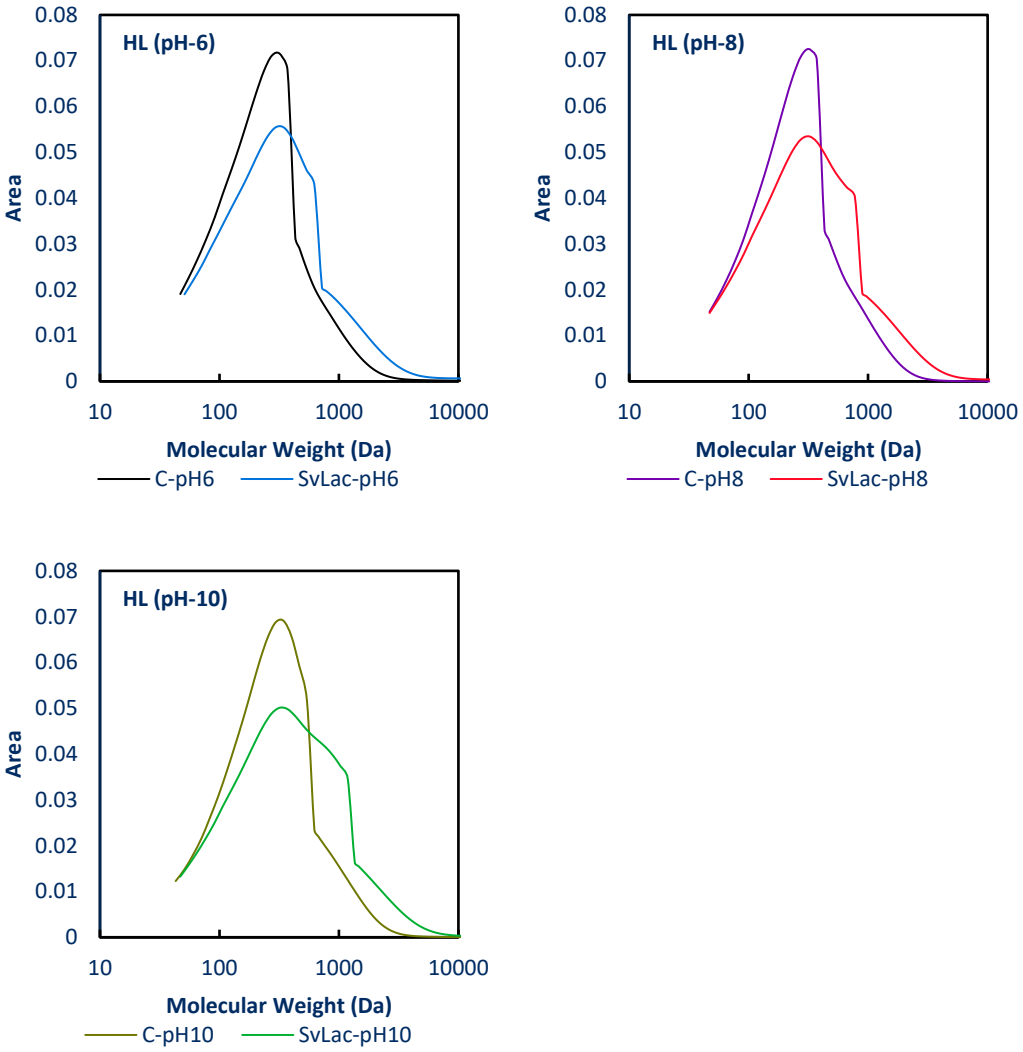

Figure 5. HL treated with SvLac at three different pH. 6 , pH 8, pH 10 in B&R buffer.

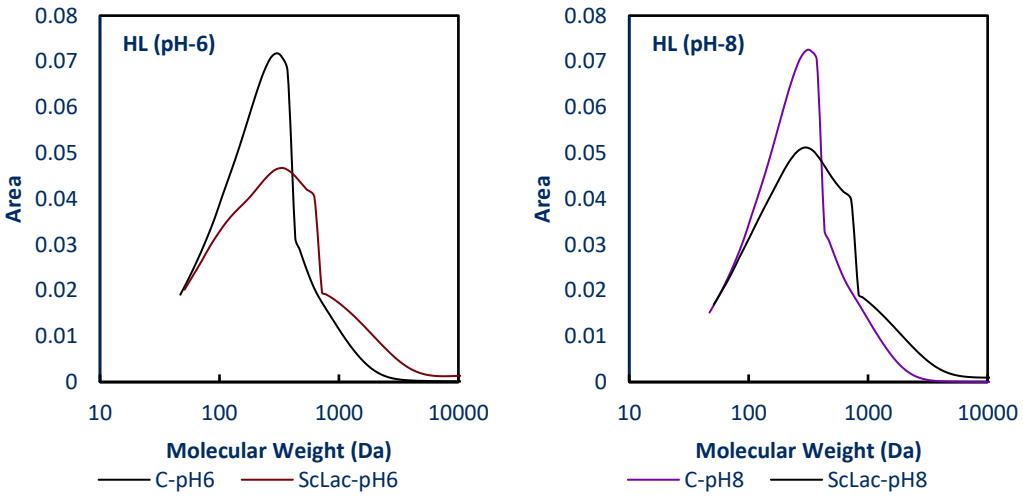

Figure 6. HL treated with ScLac at two different pH. 6 , pH 8 in B&R buffer.

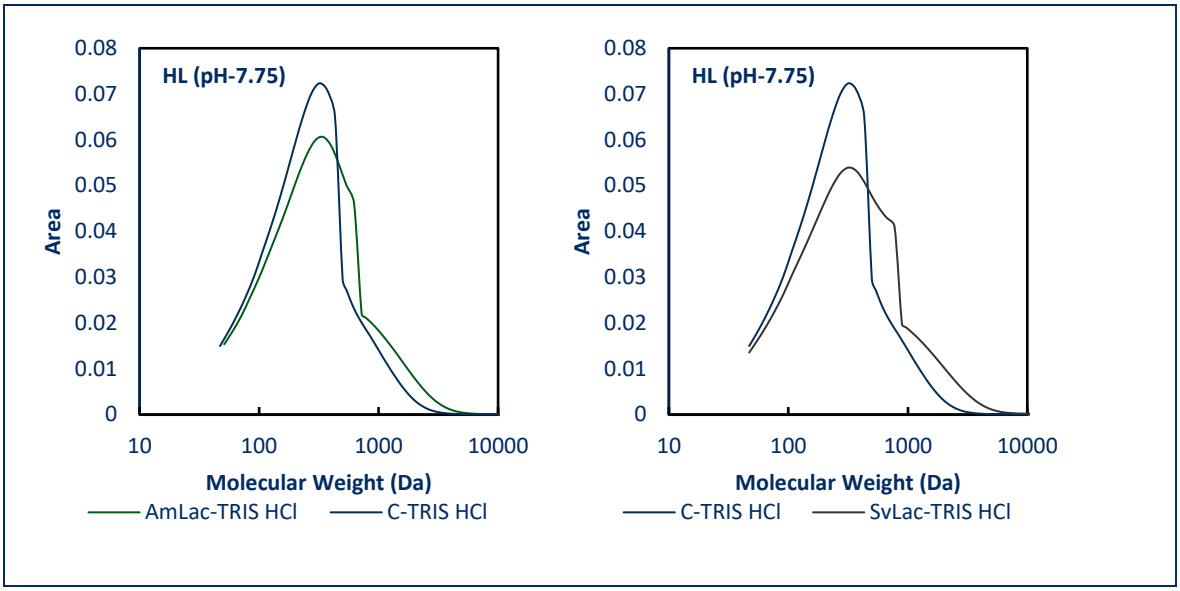

Figure 7. HL treated with AmLac and SvLac in Tris-HCl buffer.

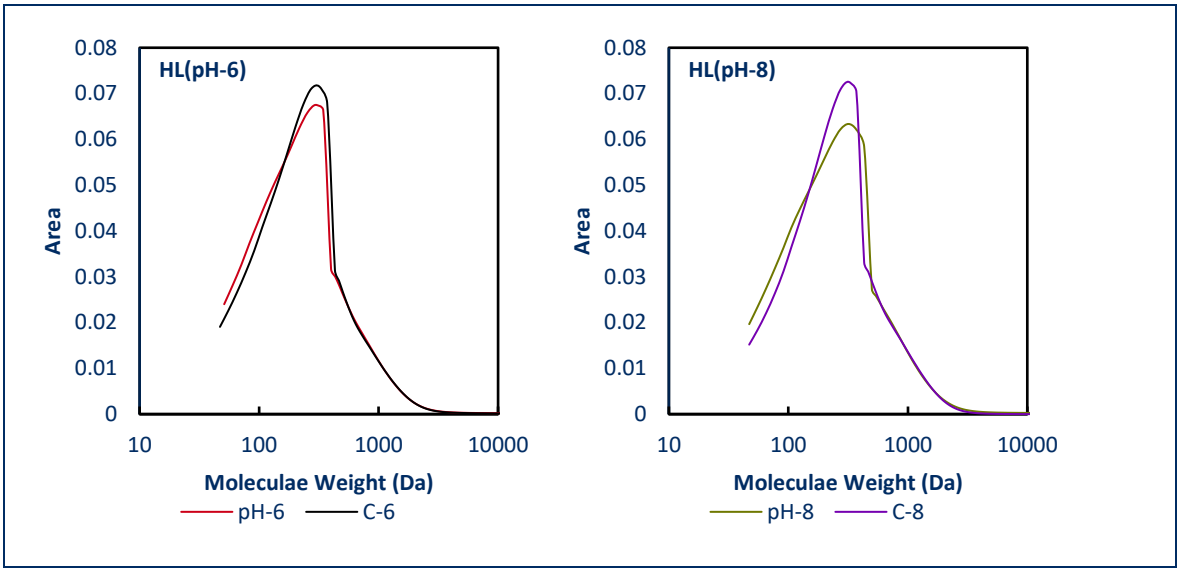

Figure 8. De- and Repolymerization of HL with DYP enzyme in B&R buffer

Table 3. Mn, Mw and PDI values of HL treated with ScLac (blueat pH 6, 8, 10 in B&R and Tris-HCl buffers.

Laccases	Buffer	pH	Mn (Da)	Mw (Da)	PDI
Dyp	B&R	6	191	546	2.85
		8	196	670	3.41
		10	215	761	3.54
	Tris-HCl	7.75	203	542	3.15
Control	B&R	6	196	406	2.07
		8	207	440	2.12
		10	228	489	2.14
	Tris-HCl	7.75	216	463	2.14

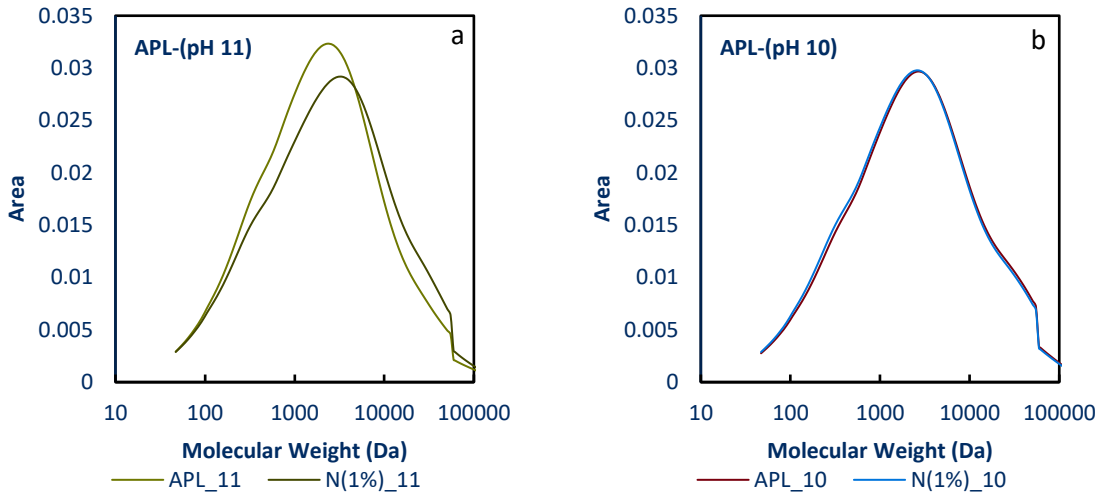

Figure 9. APL with Sclac in 1% NaOH solution at pH 11(a) and 10(b).

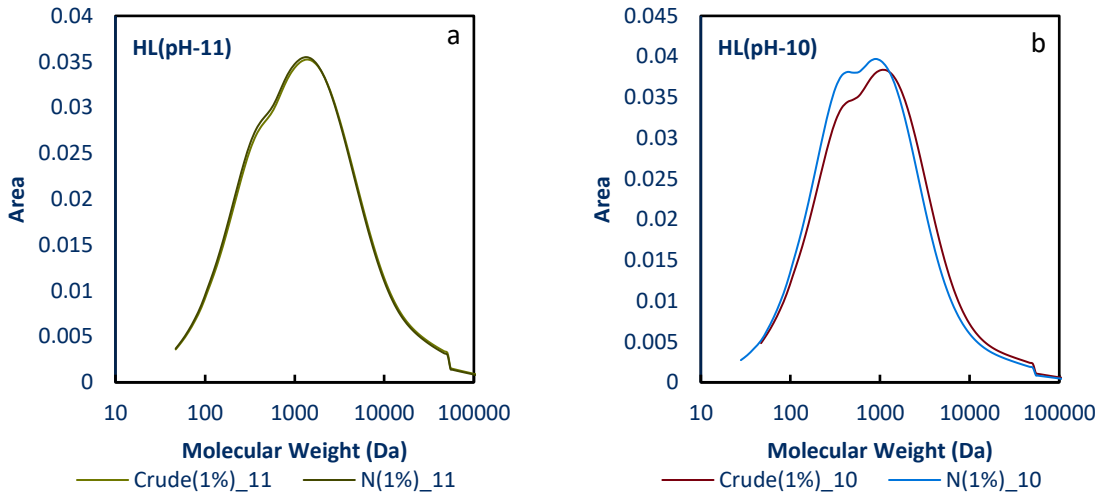

Figure 10. HL with ScLac in 1% NaOH solution at pH 11(a) and 10(b).

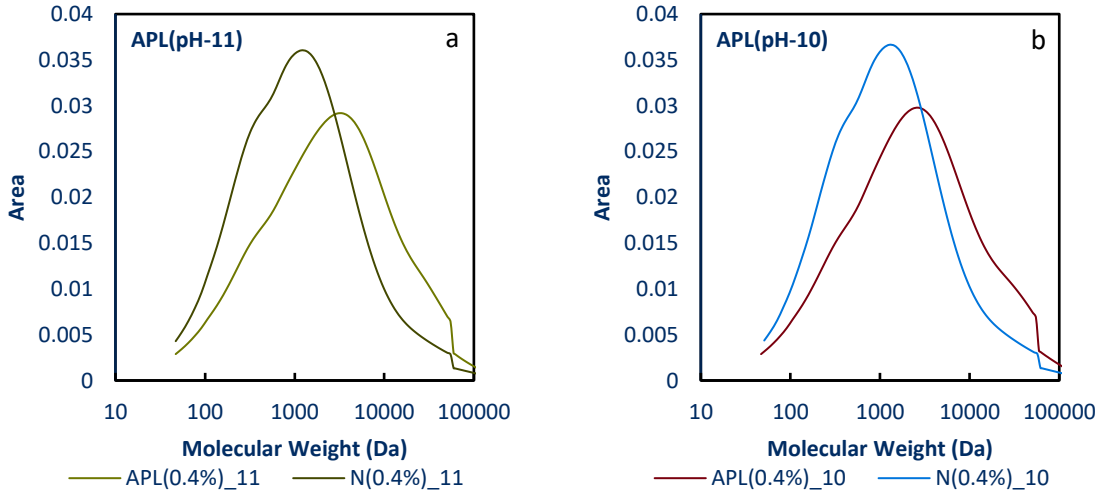

Figure 11. APL with ScLac in 0.4% NaOH solution at pH 11(a) and 10(b).

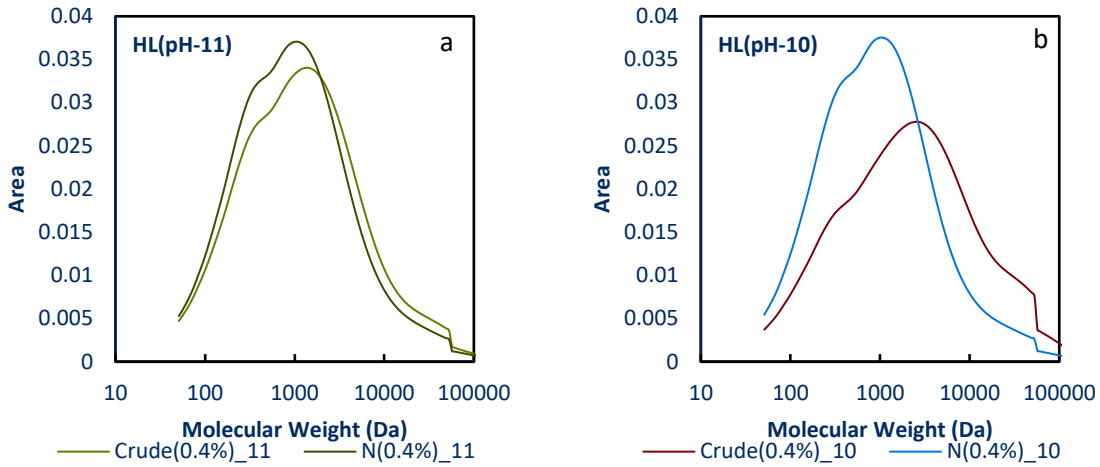

Figure 12. HL with Sclac in 0.4% NaOH solution at pH 11(a) and 10(b).

Table 4. Mn, Mw and PDI values of APL and HL with ScLac at 1% and 0.4% NaOH solution where APL(red), HL(green) and Control (black) at pH 12, 11, 10 and 9.

Lignin	Solution	pH	Mn (Da)	Mw (Da)	PDI
APL	1%	9	650	6990	12.74
		10	861	7831	12.64
		11	864	6977	15.01
		12	808	6864	14.18
HL	1%	9	526	6886	11.75
		10	840	7071	14.36
		11	830	6970	15.02
		12	728	6597	15.25
Control	1%	9	656	8709	11.27
		10	572	6677	11.66
		11	562	6831	11.15
		12	560	6573	11.74
APL	0.4%	9	532	5232	11.83
		10	522	6879	13.22
		11	563	6630	11.78
		12	535	5675	11.92
HL	0.4%	9	570	6711	11.78
		10	576	6992	12.14
		11	463	5945	11.68
		12	338	5752	11.70
Control	0.4%	9	460	5086	11.06
		10	481	5696	11.84
		11	475	5676	11.95
		12	472	5580	11.83

Table 5. Mn, Mw and PDI values of HL(red) and APL(blue) in presence of mediator with Dyp in B&R (pH 6, 8, 10), NaOH solution and control (black).

Lignin	Laccases	Buffer	pH	Mn (Da)	Mw (Da)	PDI
APL	Dyp	B&R	6	260	661	2.54
			8	258	671	2.59
			10	266	670	4.3
		0.4%NaOH	10	224	846	5.1
HL	Dyp	B&R	6	426	506	8.49
			8	435	549	11.60
			10	428	626	8.46
APL	Control	B&R	6	275	435	4.5
			8	534	455	7.8
			10	535	430	8.8
		0.4%NaOH	10	527	560	11.8
HL	Control	B&R	6	255	335	4.5
			8	434	555	7.8
			10	635	630	8.8

Table 6. Mn, Mw and PDI values of HL in NaOH solution.

Time	Mn(Da)	Mw(Da)	PDI
S	142	957	6.76
S+E	129	897	6.96
1H	133	899	6.78
3H	153	1052	8.17
5H	193	1084	10.79
22H	253	1100	17.96

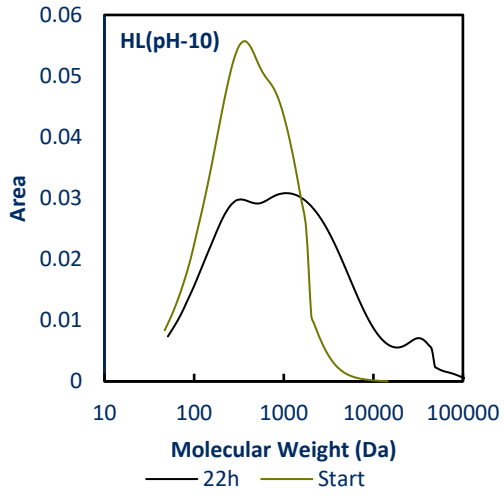

Figure 13. HL treated with L371 in NaOH solution at pH 10.

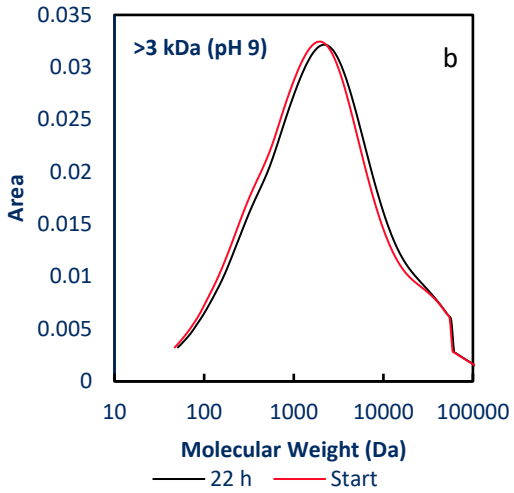

Figure 14. >3kDa fractions treated with L371 in NaOH solution at pH 10.

Table 7. Mn, Mw and PDI values treated >3kDa with L371 fractions in NaOH solution at pH 10.

Time	Mn (Da)	Mw (Da)	PDI
S	148	218	1.47
S+E	150	220	1.5
1H	150	230	1.5
3H	160	250	1.6
5H	160	280	1.7
22H	300	290	1.8

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