The Biochemical Characterization of *Thermus thermophilus* Large Laccase in the Context of Lignin Valorization

Bachelor thesis

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1. Declaration

Hereby I declare that I have compiled the thesis independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously presented for grading.

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*Thermus thermophilus*e suure lakaasi biokeemilised uuringud ligniini väärindamise kontekstis

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3. Contents

4. Abstract

Lignin is the second-most abundant component of plant biomass. It is a complex organic polymer that plays a key part in providing the structural support of most plants. The foremost commercial production of lignin comes from the paper making industry. The precursor of paper is lignocellulose, roughly a third of which is lignin. To date, most of it is burned as low-grade fuel, while only a small fraction of it gets valorized.

One of the main goals of current study is to find alternative solutions for lignin degradation. Utilization of lignin degrading enzymes is one of the possible avenues for lignocellulosic biomass conversion and lignin valorization.

Thermus thermophilus is a Gram-negative bacterium that is an extreme thermophile and has been found to be important in the degradation of organic materials in the thermogenic phase of composting. Laccases (LAC) are multicopper oxidases that oxidize a variety of phenolic substrates. They are classified as lignin-modifying enzymes since they take part in both formation and degradation of lignin.

In this study we focus on the large laccase (LLAC) from the bacteria *T. thermophilus* (*Tt*LLAC). We have successfully expressed and purified recombinant *Tt*LLAC protein. Kinetic parameters were determined for two classical laccase substrates. The enzyme retained the thermophilic properties of the bacterium and stayed fully active for at least 48 h at 70 °C. *Tt*LLAC mediated lignin degradation is demonstrated for pine, aspen, and barley straw organosolv lignins.

5. Annotatsioon

Ligniin on tselluloosi järel teine peamine taimse biomassi koostisosa. See on keerukas looduslik polümeer, mis on rakukesta oluline komponent ja tagab taimede struktuurse tugevuse. Enamus saadaval olevast ligniinist tuleb paberitööstustest. Paberitootmise tooraineks on lignotselluloos, millest umbes kolmandiku moodustab ligniin, mille peamiseks kasutusalaks on selle põletamine kütusena, kuid mida vähesel määral ka väärindatakse.

Antud töö üheks peamiseks eesmärgiks on leida alternatiivseid lahendusi ligniini väärindamisele. Üheks võimaluseks on kasutatada ligniini lagundavaid ensüüme, et viia läbi lignotselluloosse materjali teisendamine ja ligniini väärindamine.

Thermus thermophilus on Gram-negatiivne termofiilne bakter, mille puhul on näidatud selle olulist osalust orgaanilise materjali lagundamises komposteerimise termofiilses faasis. Lakaasid (LAC) on multi-vask oksüdaasid, mis oksüdeerivad erinevaid fenoolseid substraate. Nad osalevad nii ligniini sünteesis kui ka lagundamises ning seetõttu nimetatakse neid ligniini modifitseerivateks ensüümideks.

Käesolevas töös ekspresseeriti ja puhastati *T. thermophilus´*e suur lakaas (*Tt*LLAC), määrati selle ensüümi kineetilised parameetrid kahe klassikalise lakaasi substraadiga. Ensüüm säilitab bakteri termofiilsed omadused ja püsib aktiivsena 70 °C juures vähemalt 48 tundi. *Tt*LLAC vahendatud ligniini lagundamist näidati männipuidu, haavapuidu ja odrapõhu organosolv ligniinide puhul.

6. Abbreviations

ABTS – 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

BHT – butylated hydroxytoluene

Đ – dispersity

DMP - 2,6-dimethoxyphenol

DMSO – dimethyl sulfoxide

FPLC – fast protein liquid chromatography

GPC – gel permeation chromatography

HPLC – high performance liquid chromatography

IMAC – Immobilized metal affinity chromatography

IPTG - isopropyl-β-D-thiogalactopyranoside

LAC – laccase

LAC_2.9 – *Thermus* sp. 2.9 laccase

LB – Luria-Bertani medium

LLAC – large laccase

MQ water – water that has been purified using an ion exchange cartridge

NaOAc – sodium acetate

OD_{600} – Optic density at 600 nm

Rpm – revolutions per minute

SDS-PAGE - sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SEC – size exclusion chromatography

SLAC – small laccase

THF – tetrahydrofuran

Tris – tris (hydroxymethyl)aminomethane

Tris-HCl – tris (hydroxymethyl)aminomethane hydrochloride

*Tt*LLAC - *Thermus thermophilus* large laccase

7. Introduction

This study focuses on the research of an enzyme that utilizes phenolic compounds as its primary substrate. The enzyme in question is a large laccase (LLAC) produced from the bacteria *Thermus thermophilus* (*Tt*LLAC). It is a catalyst that performs sequential one-electron oxidations on phenolic substances. The main characteristic that makes this enzyme an attractive object of study is not only its ability to degrade compounds without producing toxic byproducts, but it also has the potential to be used industrially.

Lignin is a recalcitrant polymer component found in most plants, where it acts as an important structural element. The main industrial processes that produce lignin in large quantities are papermaking, production of hemicellulosic wood sugars and cellulosic ethanol. Currently, lignin is mostly burned as a low-grade fuel. The more sustainable solution would be to find cheap and effective methods that could make use of the valuable aromatic building blocks that lignin contains.

To achieve that, we must find means to degrade the lignin polymer into its monomeric subunits. Our research focuses on finding enzymes that would be efficient, cost-effective, and work under industrial conditions.

In nature there already exist organisms that can produce lignin degrading enzymes – most commonly, white rot fungi. However, fungal laccases cannot tolerate most industrial conditions and their production can be complicated. Although bacterial laccases are less effective compared to fungal ones, they tolerate higher temperatures, working in a broader pH range, and have increased tolerance to organic solvents and are therefore more suitable for industrial applications.

The focus of this thesis is the biochemical characterization of *Tt*LLAC in the context of effective and environmentally friendly degradation of lignin with the hypothesis being that a) the enzyme under study shows lignin modifying activity; and b) that it retains its catalytic activity even at elevated temperatures over extended periods of time.

8. Literature review

8.1 Lignin

Lignin is one of the most common biopolymers in nature. It is found mostly as a major structural component in plant cell walls (Eudes et al., 2014). It is comprised of three cross-linked monolignols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The overall amount of lignin varies from plant to plant, with its growth environment also playing a big role (Pollegioni et al., 2015).

Lignification is the process of monomers growing into macromolecules via free radical coupling. Due to its nature of not appearing as a regularly repeating macromolecular structure, it is hard to define the exact structure for lignin (Lu & Ralph, 2010).

Commercially, lignin is mostly treated as a waste product that is used as a source of heat (Pollegioni et al., 2015). It also is resistant to hydrolysis and is easily oxidized. Moreover, research shows that paper containing lignin is more susceptible to pollutant absorption and yellowing, making it undesirable in the paper-making industry (Menart et al., 2011).

Since lignin could be used as a renewable source of valuable aromatic components, it is imperative to find a way to degrade it. Some bacteria and white-rot fungi can use enzymes and catabolic pathways to do so. When it comes to enzymes, there are five major types of lignin degrading enzymes reported – lignin peroxidases, manganese peroxidases, versatile peroxidases, dye-decolorizing peroxidases, and laccases (Pollegioni et al., 2015).

8.2 Bacterial and fungal laccases

Multiple types of laccases can be found in the different domains of life - for example, fungi, plants, bacteria, and insects (Mehra et al., 2018). Most commonly they are isolated from two main groups – fungi and plants (Chauhan et al., 2017). With the advancement of biotechnology, bacterial laccases have also appeared in several recent studies (Choolaei et al., 2021; Panwar et al., 2022; Zhang et al., 2022).

With respect to fungal laccases, it has been noted that they do not always degrade lignin. Some *in vitro* studies suggest that while they catalyze degradation of high molecular weight compounds, while lower molecular lignins are often repolymerized. Usage of redox mediators or additional auxiliary enzymes can enhance their ability to degrade lignin. Thus, it can be concluded that *in vivo,* several types of enzymes work in synergy to degrade lignin (I. Solomon et al., 1996).

Bacterial laccases have multiple characteristics that aren't found in fungal laccases. Most notable are their stability in broad pH and temperature ranges. Adding to that, their production can be more cost effective and scalable, making them potentially a much better option for industrial use (Chauhan et al., 2017).

8.3 Laccases

Laccases (LACs), a part of the multicopper oxidase family, are enzymes that catalyze the oxidation of substrates with concomitant reduction of dioxygen to water (Figure1).

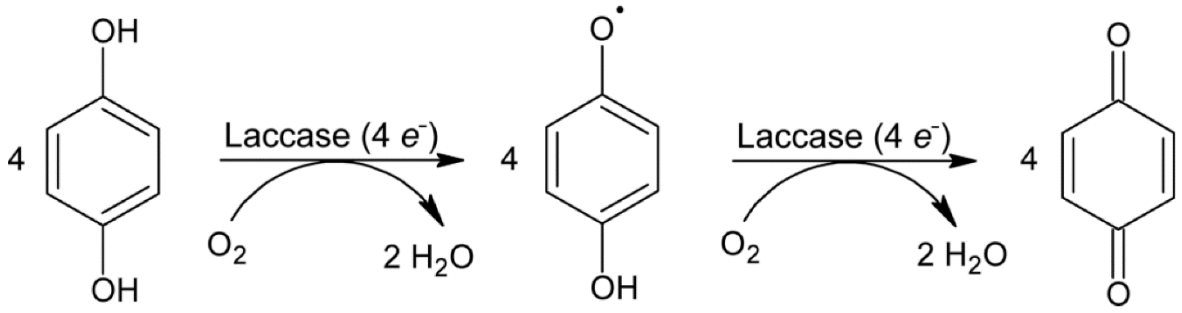

Figure 1. Enzymatic oxidation of the substrate (hydroquinone) by laccase (Sys et al., 2017).

They are considered lignin modifying due to their ability to both promote oxidative coupling during plant biomass lignification, while also being able to degrade that polymer (Jones & Solomon, 2015).

Large laccases (LLACs) contain 4 copper ions that are divided into 3 types. Type I copper only works as an electron transfer site and is coordinated by two His-, one Cys-residue, and an axial ligand that is a Met for plant and bacterial laccases or Leu/Phe residue for fungal laccases (Pardo & Camarero, 2015). Type II and III sites together form an ensemble that preforms the dioxygen reduction. Type II copper center has two His- and one hydroxide ligand. The type III copper site is binuclear, containing 2 copper ions. Each of those coppers has three His-ligands and they are connected through a hydroxide bridging ligand (Jones & Solomon, 2015).

There also exists a two-domain laccase form called the small laccase (SLAC). Compared to the three-domain laccase, it lacks the II domain, which enables the trinuclear cluster formation. Due to its absence, small laccases form the clusters with the interface of individual laccase monomers, requiring its oligomerization into a trimer (Chauhan et al., 2017).

8.4 Thermus thermophilus

Thermus thermophilus is a highly thermophilic Gram-negative bacterium isolated from hot spring in Japan. Due to its optimal growth temperature at 65 °C, which in some cases can even be close to 70-80 °C, it is an excellent source of thermozymes (OSHIMA & IMAHORI, 1974). Studies also show that this species has importance in the degradation of organic materials in composting (Beffa et al., 1996).

8.5 Enzyme kinetics

Enzymes are proteins that catalyze chemical reactions. They bind compounds, defined as substrates, to make new compounds, defined as products. The first scientists to note this process down in a mathematical sense were Leonor Michaelis and Maud Menten. They proposed that first the enzyme (E) and substrate (S) combine into an enzyme-substrate complex (ES). After the substrate turns into a product (P), the enzyme removes itself and regenerates into its native state, which is defined as follows: $E + S \Leftrightarrow ES \longrightarrow E + P$

For the reaction speed the constant v_0 is used (initial velocity), which shows the quantity of the product that has been made ($M \cdot s^{-1}$). V_{max} is used to mark the maximum speed that an enzyme can achieve and marks the point where all the enzyme molecules are saturated with substrate and are in the process of catalysis. In practice, V_{max} is never reached.

Michaelis constant, K_m, is the value of the substrate concentration where the reaction has reached half of V_{max}. Each enzyme has a characteristic constant value for a particular substrate. The higher this value is, the weaker the binding between substrate and enzyme and *vice versa*.

First order rate constant or turnover number (k_{cat}) is calculated by dividing V_{max} with the enzyme concentration. It describes the number of substrate molecules converted by the enzyme per unit time (s^{-1}).

Second order rate constant represents the best the effectiveness of an enzyme as a catalyst that takes the ratio of the kinetic constants – k_{cat}/K_m ($M^{-1} \cdot s^{-1}$), which considers the rate of production and the rate of formation for the enzyme-substrate complex (Tymoczko et al., 2016).

8.6 Protein purification using fast protein liquid chromatography

Fast protein liquid chromatography (FPLC), sometimes known as fast performance liquid chromatography, is a form of liquid chromatography. The system is designed for the separation and analysis of proteins and other biomolecules. It operates similarly to other forms of chromatography, in that mixtures are separated as a result of the components having different affinities to the mobile

and stationary phases. In contrast to its counterpart HPLC (High-performance liquid chromatography), the buffer pressure is usually low, typically les the 5 bar, but in comparison, the flow rate is higher (Pontis, 2017).

Immobilized Metal Affinity Chromatography (IMAC) is a method commonly used in the purification of proteins that contain a polyhistidine-tag. IMAC resins can be used in an FPLC system. It works due to the interaction between His residues and divalent metal ions, such as nickel, copper, or cobalt, that are immobilized on the solid phase in a chromatography column. The large number of histidines of the tagged protein interacts strongly with the metal bound to the solid phase ions and thus sequesters the protein from the mobile phase.

A high concentration of imidazole is used to elute the histidine-tagged proteins, prior to which a low concentration of imidazole can be used to wash the resins from non-specifically bound proteins. This makes it a good method if high purity of a protein of interest is needed. It can be used to achieve higher purity in conjunction with other methods (Spriestersbach et al., 2015).

8.7 High-performance liquid chromatography method

HPLC or High-performance liquid chromatography is a method typically used to analyze a mixture. It separates each component from a mixture while helping to identify and quantify them. High-performance pumps are used to move the pressurized liquid, also referred to as the mobile phase, through a column that is filled with a solid stationary phase. Granular materials such as silica or polymer gels are usually used as the stationary phase. Dependent on the application, different materials are used in the column.

When using size exclusion chromatography (SEC) column, the stationary phase is porous, and the pores have defined sizes. Particles that have larger hydrodynamic radii, do not enter the pores, and exit the column first. On the other hand, particles with equal or smaller radii enter the pores and take longer to exit the column than the larger ones (Figure 2). The time it takes for a molecule to go through the column is known as retention time (Bhardwaj, 2015).

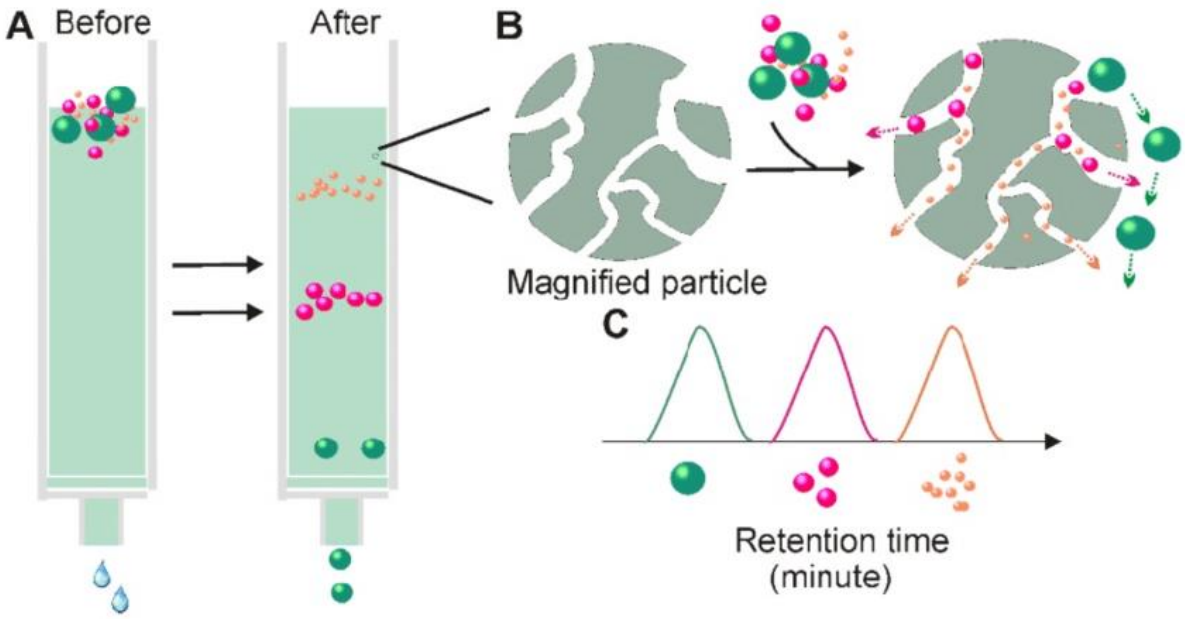

Figure 2. SEC - Size-Exclusion Chromatography principle (Yang et al., 2020).

8.8 Number- and weight-average molecular weight

The number average molecular weight (Mn) is an arithmetic mean that represents the total weight of the molecules divided by their number. It's a method used in determining the molecular weight of a polymer, giving the average molecular weights of individual macromolecules. It can be determined using different methods, e.g., gel permeation chromatography (GPC) and vapor pressure osmometry.

Weight-average molecular weight (Mw) is also used for determining the molecular weight of a polymer. It is the weight fraction of molecules in a polymer sample, giving the average of the molecular weights of the individual macromolecules in the polymer sample. Notable methods used in its determination are scattering methods like X-ray scattering and static light scattering, or by using of sedimentation velocity by centrifugation.

Polymer samples contain molecules of different sizes. Therefore, we cannot give the exact molecular weight of a polymer sample. Using different parameters, we can indicate the possible molecular weight. Number-average and weight-average molecular weight are two of the methods most used. Their main difference is that number-weight uses a mole fraction and weight-average uses the weight fraction of molecules in a polymer sample (Raja & Barron, 2019).

8.9 Dispersity

Dispersity (Đ) is the distribution of molecular mass in a polymer sample. It is calculated by dividing Mw by Mn and indicates the distribution of molecular weights in a polymer. If the polymer sample has molecules with the same size, shape, or weight, it is considered uniform and if the same characteristics are inconsistent then it is considered non-uniform. When a polymer is uniform, the dispersity value is 1, and the less uniform a polymer is, the greater its value.

$$Đ = Mw/Mn$$

It should be noted that the term “polydispersity index” (PDI or PI) was previously used and is still used to this day. But due to its misleading nature, IUPAC recommends the use of “dispersity” (Gilbert et al., 2009).

9. Aims of the study

Research goals:

1) Expression and purification of *Thermus thermophilus* large laccase,
2) Determination of its kinetic parameters
3) Temperature tolerance studies
4) Testing *Tt*LLAC activity towards lignin degradation

10. Materials and methods

10.1 Expression

Codon-optimized gene encoding *Thermus thermophilus* large laccase (*Tt*LLAC) (GenBank accession number: WP_014510825) was obtained pre-cloned in pET28a expression vector, carrying kanamycin resistance gene, N-terminal His_6-tag, and thrombin cleavage site (Twist Bioscience). The plasmid was then transformed into *E. coli* BL21 DE3 cells by electroporation. For the expression 2 x 3 ml of LB starter cultures, containing 30 μg/ml kanamycin, were inoculated and placed into an incubator to shake overnight at 37 °C, 180 rpm in aerated conditions. On the next day, 1 l of LB medium, containing 30 μg/ml kanamycin, was inoculated by adding the 2 x 3 ml of starter cultures. The bacteria were grown at 37 °C while shaking at 180 rpm until the optical density at 600 nm $OD_{600} \approx 0.6$. After about 5 h, the desired OD was reached, and the flask was placed on ice for cooling. The protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). In addition, 250 μM $CuCl_2$ solution was added to the medium for protein incorporation along with 4% glycerol to negate the copper toxicity. The expression was continued for 20 hours at 30 °C and 180 rpm.

10.2 Lysis and preparations for chromatography

To extract the cells from the medium, it was centrifuged at 4°C, 4000 rpm for 15 minutes (Du Pont, Sorvall RC 28S). The excess liquid was discarded, and the biomass resuspended in 30 ml of lysis buffer (lysis buffer: 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole pH 7.5). The cells were disrupted using sonication at 30% cycle for 15 min, 200 W of power (Bandelin, Sonoplus GM 2200). Lastly, to clear the lysate, the disrupted cells were centrifuged for 45 min at 35000 g, 4°C (Beckman Coulter, Avanti J-E Centrifuge).

10.3 Nickel affinity chromatography and His-tag cleavage

All the liquid chromatography steps were performed using an ÄKTA Pure 25 instrument (GE Healthcare). The cell lysate was applied onto a nickel affinity chromatography column (HisTrap FF 5 ml, GE Healthcare). The nonspecifically bound proteins were washed off using 10% elution buffer (elution buffer: 20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole pH 7.5). The *Tt*LLAC was eluted with a linear gradient of 10-100% of elution buffer covering 20 column volumes. Absorbance was monitored at 280 nm.

All spectrometry measurements were done using Shimadzu UV-2700 instrument. The protein concentration was measured at 280 nm (ε=46410 $M^{-1} \cdot cm^{-1}$, calculated using Expasy ProtParam) and the amount of thrombin needed for His-tag removal was calculated at 2 units per 1 mg of protein. The thrombin was added to the protein solution and left at 4 °C overnight.

10.4 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

10 µl of protein sample was mixed with 2x Laemmli buffer and heated at 98 °C for 5 minutes. The 12% sodium dodecyl sulfate (SDS) polyacrylamide gel was made and soaked into 1x SDS buffer (25 mM Tris, 192 mM glycine, 0,1% SDS pH 8.3). 15 µl of sample and 10 µl of protein ladder (Thermo Scientific™ PageRuler Broad Range Unstained Protein Ladder) was applied to the gel. Electrophoresis was performed using 25 mA as a constant value. After the electrophoresis the gel was soaked into Coomassie Brilliant Blue R-250 staining solution, it was warmed up in a microwave oven and left on a tilting mixer for 20 minutes. The extra color was removed by incubating the gel in destaining solution, containing 40% ethanol and 10% acetic acid. Lanes containing pure *Tt*LLAC protein were identified.

10.5 Buffer exchange and concentration

For buffer exchange, the HiPrep 26/10 Desalting column (GE Healthcare) was used and a buffer containing 20 mM Tris-HCl pH 7.5 and 100 mM NaCl. The fractions containing the enzyme were pooled together. The sample volume was reduced using membrane-concentrator Vivaspin Turbo 15 (Sartorius) with molecular weight cut off 10 kDa. The protein solution was centrifuged at 4000 g, 4°C (Universal 32 R centrifuge, Hettich) until desired concentration.

10.6 Enzyme kinetics

All of the spectrophotometric measurements for the determination of kinetic constants were carried out with a Shimadzu UV-2700 spectrophotometer using 1 ml plastic cuvettes (Greiner Bio-One). For the kinetic characterization, substrates 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP) were used. Oxidation of ABTS was monitored at 420 nm and the oxidation of DMP was measured at 470 nm. pH optima for each substrate were determined. For the determination of kinetic parameters for ABTS, the buffer solution contained 0.25-5 mM ABTS, 0.5 µM *Tt*LLAC, 75 µM $CuCl_2$, 50 mM sodium acetate (NaOAc) pH 5.0. For DMP the buffer solution contained 0.025-1 mM DMP, 0.5 µM *Tt*LLAC, 75 mM $CuCl_2$, 50 mM Hepes pH 7.0. The results are presented as mean values of three individual experiments. The analysis and graphs were done using the Origin program (OriginPro 2019).

Afterwards, the most efficient concentrations were used in temperature tolerance tests. The enzyme was incubated at 85 °C. During allotted points of time, it was diluted into a solution, determined by the previous tests, and the residual activity was measured.

10.7 Enzymatic treatment of lignins

Organosolv (dioxane) extraction of lignin from pine, aspen, and barley straw biomasses was performed by Kristiina Leiman as described in (Jõul et al., 2022). For lignin stock solution, 20 mg/ml of organosolv lignin powder was dissolved in methanol. For enzymatic treatment, lignin solutions were diluted into aqueous buffers at pH 7, 8, and 9 with a final concentration of 2 mg/ml, containing 10 μM *Tt*LLAC. For pH 7, 50 mM Hepes buffer was used, for pH 8, 50 mM Tris-HCl buffer was used and for pH 9, 50 mM glycine buffer was used. The mixtures were incubated for 48 h at 42 °C on a shaker (180 rpm). After incubation the samples were centrifuged for 10 min 8000 rpm (Hettich) and supernatant was removed. The pellets were lyophilized (Labogene, Scanvac Coolsafe) and solubilized in tetrahydrofuran (THF). The samples were centrifuged for 10 min at 13000 rpm at room temperature (Heraeus) and 20 μl of supernatant was injected into HPLC (Shimadzu, Prominence LC-20AT) size exclusion chromatography column (MesoPore, Agilent). The mobile phase was tetrahydrofuran stabilized with 250 ppm of butylated hydroxytoluene (BHT). The data was analyzed using LabSolutions application (Shimadzu, LabSolutions 2021).

11. Results

11.1 *Tt*LLAC expression and purification

The His-tagged enzyme was expressed in *E. coli* BL21 cells using 250 µM $CuCl_2$ for protein incorporation and the extracted biomass was disrupted using sonication. The lysate was loaded on a nickel-affinity chromatography column. Nonspecifically bound proteins were removed from the column using 10 % elution buffer. *Tt*LLAC, that was well bound to the column, and was eluted using an elution buffer gradient from 10% to 100%. Fractions 4-10, containing the enzyme, were collected, in total 35 ml (Figure 3), for further purification. The concentration of the protein solution was 28.9 µM, containing 52.6 mg of protein. 100 units of thrombin was added to cleave the His tags.

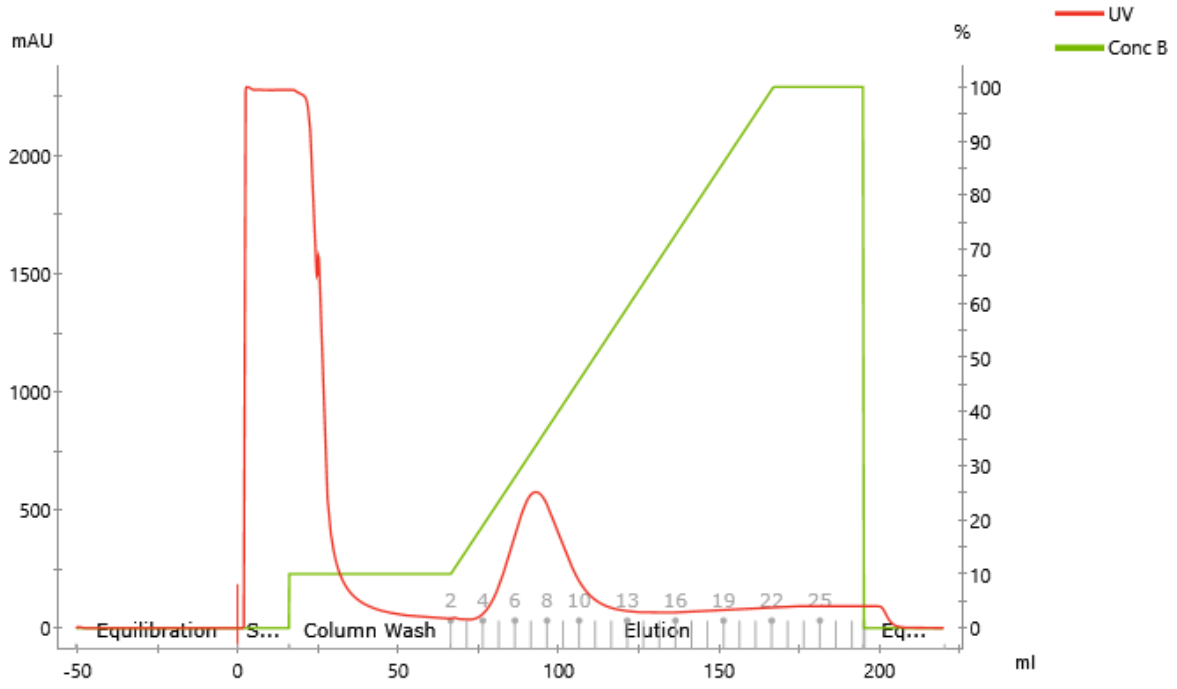

Figure 3. Purification of *Tt*LLAC with nickel-affinity chromatography. Fractions 4-10 were chosen for further purification. Buffers: 20 mM Tris-HCl 0.5 M NaCl 5 mM imidazole pH 7.5; 20 mM Tris-HCl 0.5 M NaCl 0.5 M imidazole pH 7.5 (Buffer B). Column: HisTrap FF 5 ml.

Samples for SDS-PAGE were taken from each fraction and from the pooled fraction before and after thrombin treatment. Compared to the theoretical molecular weight, it was confirmed that the protein indeed moved near the expected 50 kDa marker (Figure 4). There was also a considerable band near the 100 kDa marker. Since it is twice the molecular weight, it is reasonable to believe that dimerization

had occurred. Albeit SDS-PAGE is a non-native method, dimer bands are commonly described even for the trimeric bacterial small laccase (Machczynski et al., 2004).

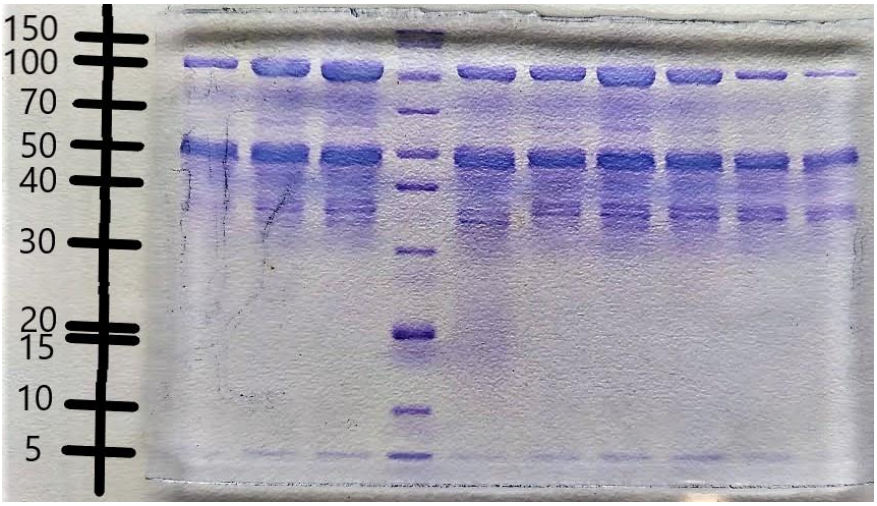

Figure 4. SDS-PAGE of *Tt*LLAC. From the left, Lane 1-3 – Fractions 4-6, Lane 4 – Protein Ladder, Lane 5 – Cleaved protein sample, Lane 6 - Uncleaved protein sample, Lane 7-10 - Fractions 7-10.

To remove the extra salt and imidazole, and to separate the His_6-tags from the cleaved protein sample, the desalting step was done using HiPrep 26/10 Desalting column with a 20 mM Tris-HCl buffer pH 7.5 containing 100 mM NaCl (Figure 5).

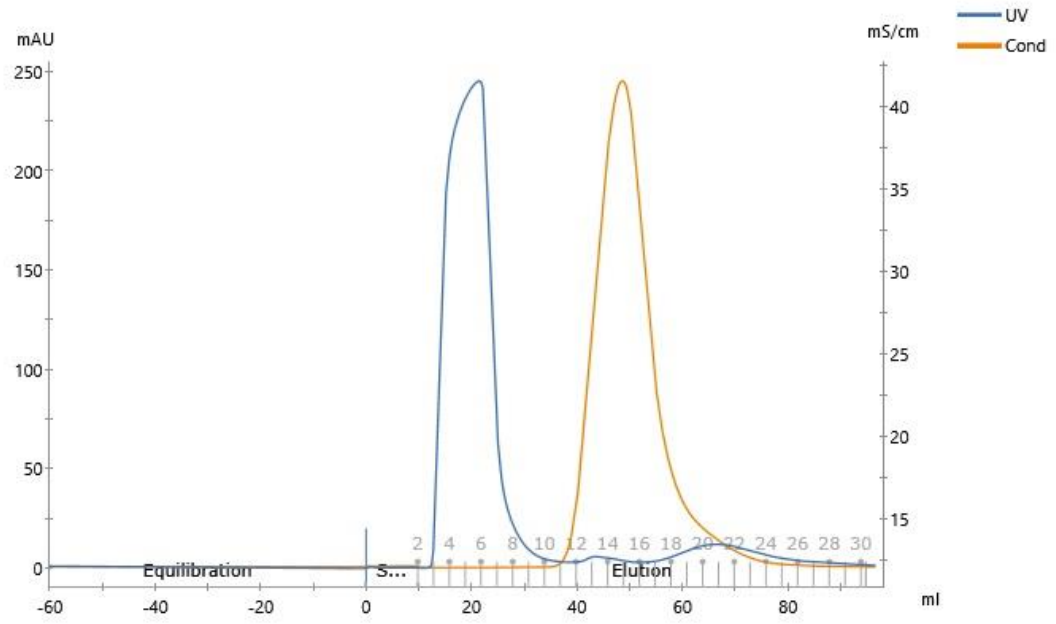

Figure 5. Desalting of *Tt*LLAC. Column: HiPrep 26/10. Buffers: 20 mM Tris-HCl pH 7.5; 20 mM Tris-HCl 1 M NaCl pH 7.5 (buffer B, 10%). Fractions 3-7 were collected.

After desalting, the protein sample was concentrated. The absorption of the enzyme-solution was measured at 280 nm to determine the protein concentration using the Lambert-Beer law and extinction coefficient ε=46410 $M^{-1}\cdot cm^{-1}$, calculated based on protein sequence using ProtParam web tool (https://web.expasy.org/protparam/). The protein concentration was 227 µM or 11.82 mg/ml as the Mw of *Tt*LLAC is 52,001.27 Da. To use the fresh enzyme throughout experiments, the protein solution was frozen as small beads using liquid nitrogen and stored at -80 °C.

11.2 Kinetics

For the kinetic characterization of *Tt*LLAC two substrates were used: ABTS and DMP. The experiments were conducted at room temperature. To monitor for the oxidation of ABTS we used an extinction coefficient of 36000 $M^{-1}\cdot cm^{-1}$ and for DMP we used 14800 $M^{-1}\cdot cm^{-1}$ (Majumdar et al., 2014).

11.2.1 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

The oxidation of ABTS was measured at 420 nm. First, it was tested if additional Cu is needed for enzyme's activity. It was concluded that adding 75 µM $CuCl_2$ to the reaction buffer yielded the highest

activity of the enzyme. Following that, the optimal pH for ABTS oxidation was confirmed to be 5.0 (pH profile data not shown).

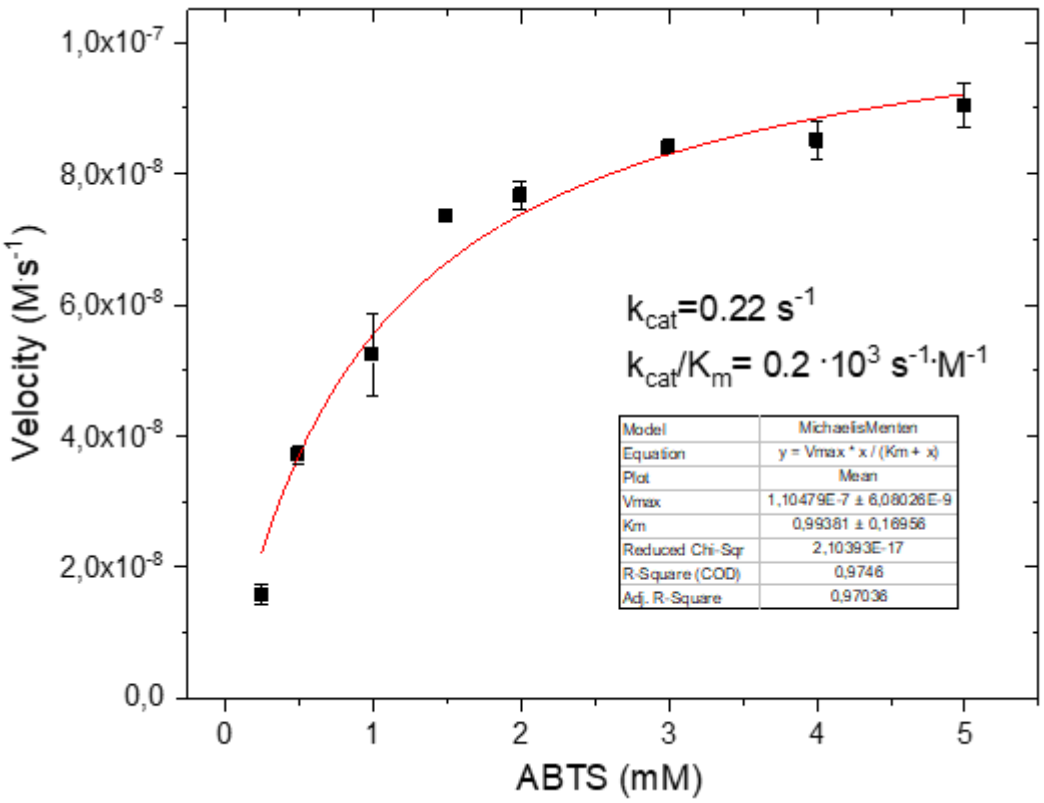

Figure 6. *Tt*LLAC kinetics with ABTS. Solution: 0.5 µM *Tt*LLAC, 75 µM $CuCl_2$, 50 mM NaOAc pH 5.0, ABTS 0.25-5 mM. The results are mean values of three individual experiments, standard deviations are shown as error bars.

The kinetic values for ABTS based reaction came out as follows: k_{cat}=0.22 s^{-1}, k_{cat}/K_m=0.2×10^3 s^{-1}·M^{-1} and K_m=0.99 M (Figure 6).

11.2.2 2,6-dimethoxyphenol

The oxidation of DMP was measured at 470 nm. The optimal pH for DMP oxidation was determined to be 7.0 (pH profile data not shown).

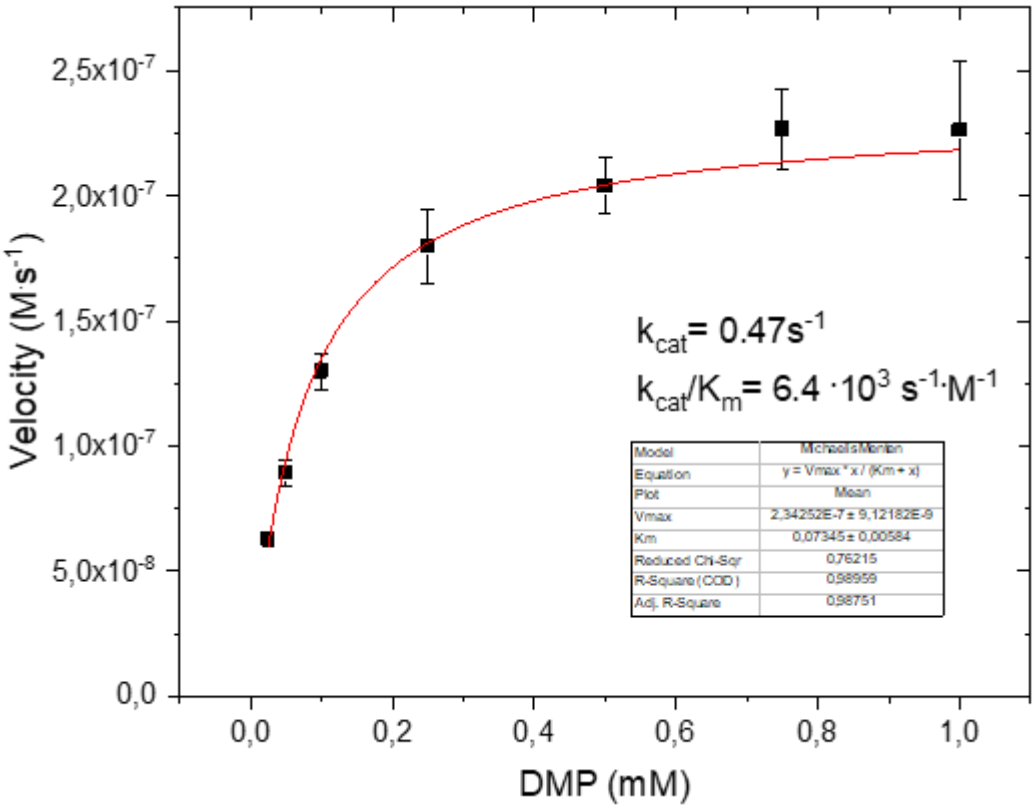

Figure 7. *Tt*LLAC kinetics with DMP. Solution: 0.5 µM *Tt*LLAC, 75 µM $CuCl_2$, 50 mM Hepes pH 7.0, DMP 0.025-1 mM. The results are mean values of three individual experiments, standard deviations are shown as error bars.

For DMP the kinetic values were: k_{cat}=0.47 s^{-1}, k_{cat}/K_m=6.4×10^3 $s^{-1}{\cdot}M^{-1}$ and K_m=0.07 M (Figure 7).

11.3 Thermal tolerance of *Tt*LLAC

Going forward it was decided to use ABTS as the main substrate for the temperature testing. 100 µM enzyme was incubated at 85 °C in 20 mM Tris-HCl, 0.1 M NaCl pH 7.5. At different time points the enzyme was diluted into solution containing 2 mM ABTS, 75 µM $CuCl_2$, 50 mM NaOAc pH 5.0 at 42 °C and residual activity was measured. Final concentration of the enzyme for activity measurements was

1 μM. During the observations, the peak activity was found at the 4-hour incubation mark. Subsequently, it had a gradual drop in activity, falling to around 50% of the maximum enzyme activity after 24 hours and lower than 10% at the 48-hour mark. It is interesting to note that the enzyme seemingly required a high-temperature treatment before reaching maximum activity (Figure 8).

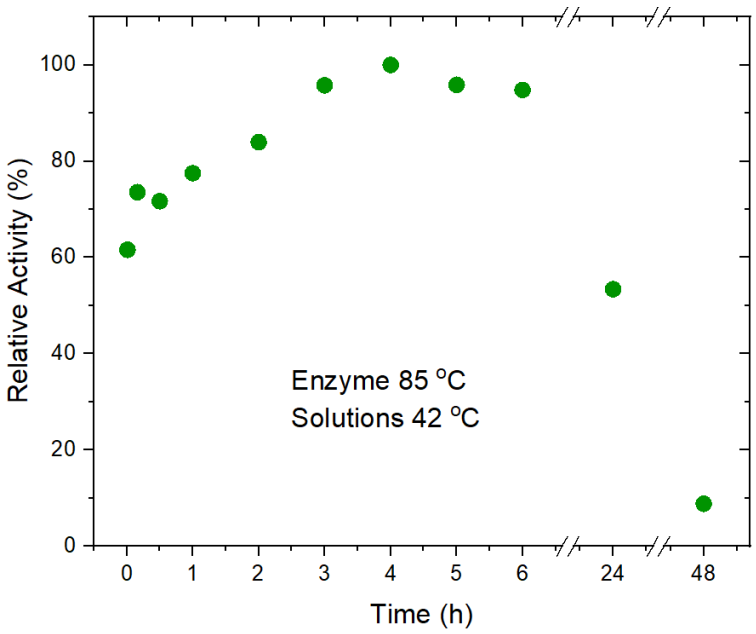

Figure 8. Thermal tolerance of *Tt*LLAC. *Tt*LLAC was incubated at 85 °C. At different time-points the residual activity was measured. Conditions: 1 μM *Tt*LLAC, 2 mM ABTS, 75 μM $CuCl_2$, pH 5.0. Solutions were incubated and activity measured at 42 °C. The results are mean values of three individual experiments.

11.4 Lignin modification

Pine, aspen and barley straw lignin solutions were prepared and incubated with *Tt*LLAC in aqueous buffer solutions at different pH-s. After the incubation, samples were centrifuged, supernatant

removed, and the pellet analyzed using HPLC. Control samples, without the enzymatic treatment, were prepared and treated the same way (Figures 9, 10, and 11).

The HPLC chromatograms have all been y-axis normalized to achieve a better understanding of them qualitatively and comparatively.

Using Shimadzu LabSolutions software, the Mn and Mw values were calculated from HPLC chromatograms. Đ was calculated based on the equation Đ = Mw/Mn (Table 1).

The results appeared as follows:

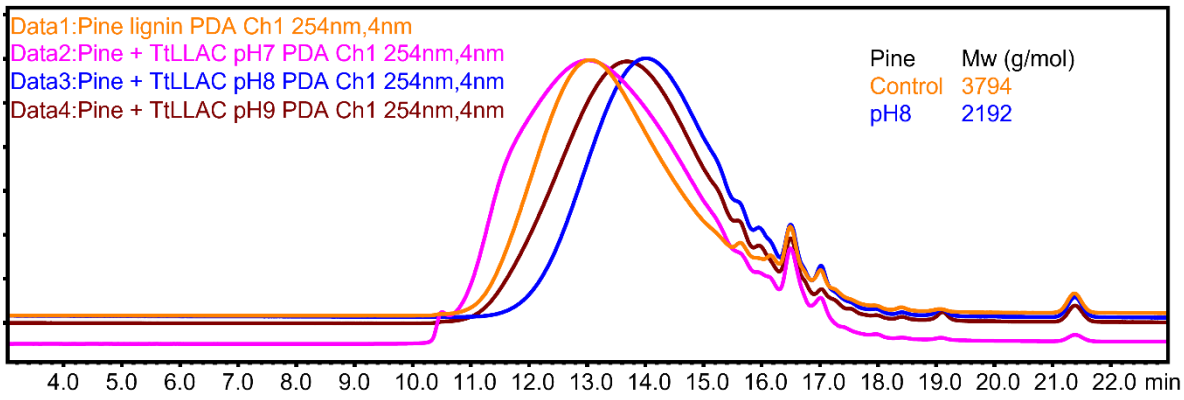

Figure 9. HPLC analysis of pine lignin treated with *Tt*LLAC.

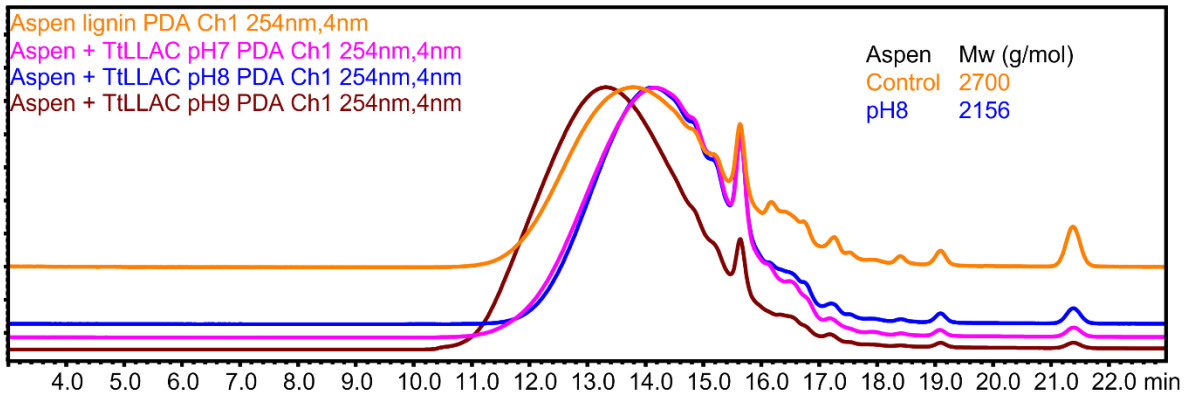

Figure 10. HPLC analysis of aspen lignin treated with *Tt*LLAC.

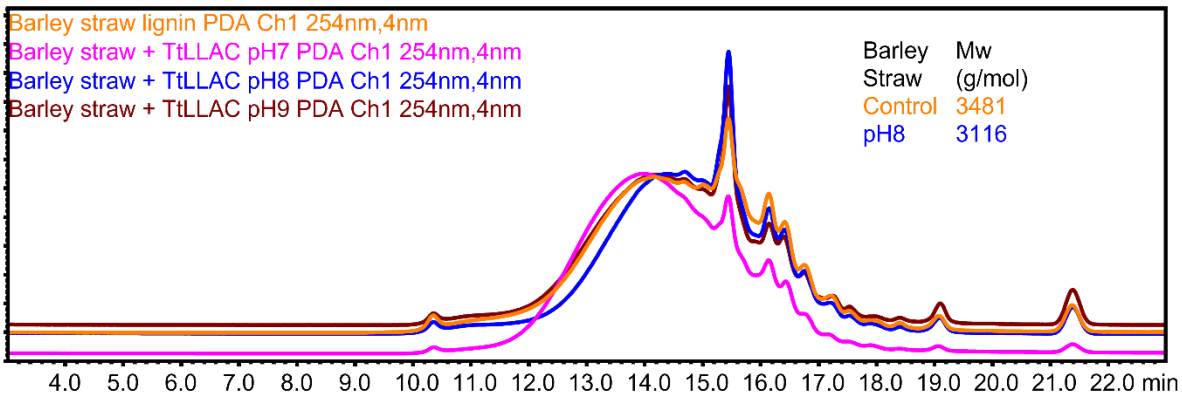

Figure 11. HPLC analysis of barley straw lignin treated with *Tt*LLAC.

Table 1. Mw and Mn values for different pine, aspen and barley straw lignin samples. Values were calculated using Shimadzu LabSolutions postrun analysis software.

PINE	Mn	Mw	Đ
Control	2430	3794	1,56
pH7	2533	4734	1,87
pH8	1712	2278	1,33
pH9	1996	3085	1,55
ASPEN	Mn	Mw	Đ
Control	1901	2700	1,42
pH7	1714	2386	1,39
pH8	1665	2156	1,29
pH9	2316	3717	1,60
BARLEY STRAW	Mn	Mw	Đ
Control	2376	3481	1,47
pH7	1926	2720	1,41
pH8	1748	2559	1,46
pH9	1866	2800	1,50

Based on the HPLC results, the change in molecular weight was used to determine whether degradation or polymerization had occurred. If compared to the control, the sample had a lower

molecular weight, then degradation had occurred. Inversely, if the result was higher than the control, then it is a sign of polymerization. Using that as a base, it can be said that *Tt*LLAC was able to degrade pine lignin at pH 8 and 9, whereas at pH 7, polymerization occurred (Figure 9). Aspen lignin was degraded at pH-s 7 and 8, while polymerization was detected al pH 9 (Figure 10). Barley straw lignin degradation occurred at pH 8, whereas at pH 7 and 9 no remarkable change was detected compared to the control (Figure 11). To conclude, it can be said that *Tt*LLAC was able to degrade all tested lignins at pH 8.

12. Discussion

Lignin is the second most common plant-based polymer found in nature. For a long time, it was considered as an unwanted side-product in papermaking. The problem resides in its highly recalcitrant structure, which makes it a difficult material to process. Biological alternatives exist for its degradation, such as fungal and bacterial peroxidases and laccases. Although fungal enzymes might be better lignin oxidizers, bacterial enzymes are preferred for industrial use due to their ability to function in harsh processing environments. For the industrial processing of lignin, bacterial enzymes from thermophilic organisms make an excellent research subjects.

Looking at the results for expression and purification of *Thermus thermophilus* large laccase (*Tt*LLAC) in this study, it can be said that the conditions used were successful in producing an active enzyme. The nickel affinity chromatogram (Figure 3) shows that the His-tagged protein bound successfully to the column and the following SDS-page (Figure 4) confirms that the protein was the desired one due to it being in the right size category: 50 kDa for the monomer and a possible dimer at 100 kDa.

It was confirmed that additional copper in the reaction mixture yielded higher enzyme activity, similar effect was described for *Thermus* sp. 2.9 laccase (LAC_2.9) (Navas et al., 2019).

The catalytic activity for *Tt*LLAC was confirmed (Figure 6 and Figure 7) with a higher efficiency for the phenolic substrate DMP. Similar results, higher efficiency for DMP, was also shown for *Thermus* sp. laccase LAC_2.9 (Navas et al., 2019). In addition, it can be surmised that the protein retained the thermal resistance characteristics of the *Thermus thermophilus* sp. During the tests for temperature tolerance, it maintained half of its activity after a 24 h incubation at 85 °C (Figure 8). In contrast, LAC_2.9, retained 80% of its activity after 6 hours at 80 °C and 16 hours at 70 °C (Navas et al., 2019).

In terms of lignin degradation, the results show that the organosolv lignins (pine, aspen and barley straw), that were treated with *Tt*LLAC at pH 8, had a lower molecular weight than the starting material (Table 1). This points that the enzyme was able to degrade the tested organosolv lignins. Treatment at pH 7 and pH 9 had diverse results depending on the lignin type - some showing degradation and some polymerization. Comparably, partial delignification of woody-eucalyptus biomass was reported for LAC_2.9 treated samples but only with the addition of a mediator (Navas et al., 2019).

In conclusion, *Tt*LLAC retained activity at elevated temperatures, making it favorable for potential industrial use, and was able to degrade organosolv lignins from different biomasses.

13. Conclusion

This research revealed several notable characteristics in the kinetic activity and degradation of organosolv lignins by *Tt*LLAC:

1) *Tt*LLAC expression and purification was successful.
2) *Tt*LLAC had higher catalytic efficiency for phenolic substrate DMP.
3) *Tt*LLAC maintained half of its activity after being 24 h at 85 °C.
4) *Tt*LLAC was able to degrade pine, aspen, and barley straw lignins at pH 8.

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