Elucidation of Enzymes Suitable for Lignin Degradation

Ramljak, Sanja

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Sanja Ramljak

ELUCIDATION OF ENZYMES SUITABLE FOR LIGNIN DEGRADATION

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Istraživanje enzima prikladnih za razgradnju lignina

Sanja Ramljak, 0113140957

Sažetak: Kako bi se istražila enzimska razgradnja kraft lignina, određivana je aktivnost lakaze, najučinkovitijeg enzima u razgradnji lignina te aktivnosti tzv. "pomoćnih" enzima glukoza dehidrogenaze (GDH), celobioza dehidrogenaze (CDH) i litičke polisaharid monooksigenaze (LPMO). Proveden je uzgoj gljiva bijelog truljenja *Trametes versicolor* i *Phanerochaete chrysosporium* kako bi se usporedila njihova učinkovitost razgradnje lignina s učinkovitosti gore navedenih enzima. Lakaza se pokazala učinkovitom u razgradnji lignina, dok pomoćni enzimi ne djeluju na lignin, ali pospješuju depolimerizaciju lignoceluloznih sirovina. *Trametes versicolor* se pokazala učinkovitom u razgradnji lignina što se dovodi u vezu s proizvodnjom lakaze.

Ključne riječi: Kraft lignin, lakaza, *Trametes versicolor*, *Phanerochaete chrysosporium*

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Department of Process Engineering
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Co-supervisor: *Roland Ludwig*, PhD, associate prof.

ELUCIDATION OF ENZYMES SUITABLE FOR LIGNIN DEGRADATION

Sanja Ramljak, 0113140957

Summary: Enzymatic degradation of kraft lignin was investigated using purified fungal enzymes known to degrade lignin structures. The activity of laccase, the most frequent enzyme associated with ligninolysis and the activity of auxiliary redox enzymes such as glucose dehydrogenase (GDH), cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenase (LPMO) were tested. The effectiveness of these purified enzymes was compared with crude extracts obtained from fungal cultivations of the ligninolytic model fungi: *Trametes versicolor* and *Phanerochaete chrysosporium.* Laccase was proven to be the most efficient enzyme for lignin degradation. Auxiliary enzymes cooperate with other enzymes in order to enhance depolymerisation of lignocellulose. With observed high laccase activity, *Trametes versicolor* is found to be efficient in lignin degradation.

Key words: Kraft lignin, laccase, *Trametes versicolor*, *Phanerochaete chrysosporium*

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Contents

1. INTRODUCTION

Lignocellulosic raw materials are the most abundant biopolymers on Earth and therefore, critical for the development of ecologically and economically sustainable processes (Palonen, 2004). Efforts to obtain value added products from such renewable resources are subsummized in the "Biorefinery" concept. The overall aim is to achieve controlled and complete conversion of the individual constituents of plant biomass into energy-rich hydrocarbons that can be used to produce energy or chemicals. While this has been partly achieved for cellulosic and hemicellulosic building blocks of plant biomass, the abundant lignin fraction remains a largely untapped resource of raw materials. Attempts to valorize the lignin fraction are mainly hampered by its complex structure and resistance towards degradation.

The main driving force to improve the utilisation of these raw materials lies in the need to create renewable energy sources which do not affect the production of food or feed. Furthermore, the long-term goal is to reduce the use of fossil fuels and their harmful impact on the environment (Wyman et al., 2005; Lange 2007; Dashtban et al., 2009; Mäkelä et al., 2015).

Plant biomass, in particular agricultural and forestry wastes as well as the waste from the wood processing and food industries, have a great potential for biorefinery applications due to their global abundance and availability. Although this potential has been well recognised and partly exploited, the use of lignocellulosic materials is still challenging (Palonen, 2004; Wyman et al., 2005; Dashtban et al., 2009). The high physical and chemical stability of these plant cell walls is a major barrier for its economic utilization. This recalcitrance is the result of the complex composition of wood, characterized by the three most common components lignin, cellulose and hemicelluloses. Thus, the degradation of such raw materials is not only extremely technologically challenging but it is also economically demanding (Lewis et al., 1999; Palonen, 2004; Dashtban et al., 2009; 2010; Vanholme et al., 2010; Mäkelä et al., 2015; Kracher, 2016).

In nature, fungi and bacteria play a major role in the degradation of lignocellulosic raw materials. In order to adapt to different environmental niches, they have evolved a number of lignocellulolytic enzymes, which is reflected in their great taxonomic and phenotypic diversity. Cellulose and hemicellulose are degraded by both hydrolytic and oxidative enzymes and can be further used in various processes (Kracher, 2016). Lignin is a biopolymer composed of aromatic backbone building blocks. In addition to providing strength and protecting plants

from mechanical damage and pathogens, it also prevents the penetration of lignocellulolytic enzymes into the internal structure. The large and ubiquitous group of white-rot fungi produce so-called ligninases that are mainly responsible for lignin degradation. These enzymes are divided into two groups: phenol oxidase (laccase) and heme peroxidase (lignin peroxidase, manganese peroxidase, polyvalent peroxidase) (Malherbe and Cloete, 2002; Palonen, 2004; Pothiraj et al., 2006; Dashtban et al., 2009 ; 2010; Mäkelä et al., 2015).

Lignin, as the most difficult-to degrade component, limits the use of lignocellulosic raw materials. Controlled removal of lignin could greatly enhance the degradability of cellulose and hemicelluloses and, consequently, could boost their performance in bioprocesses such as bioethanol production (Christopher et al., 2014). Therefore, one of the goals of this study was to investigate the efficiency of enzymatic degradation using purified fungal enzymes that are known to degrade lignin structures. Thus the enzyme laccase, a frequent enzyme associated with ligninolysis, was used but also the effect of auxiliary redox enzymes such as glucose dehydrogenase (GDH), cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenase (LPMO) on lignin compounds was also tested. Furthermore, the effectiveness of these purified enzymes was compared with crude extracts obtained from fungal cultivations. To this end, the ligninolytic models *Trametes versicolor* and *Phanerochaete chrysosporium* were used.

2. THEORETICAL PART

2.1. LIGNOCELLULOSIC RAW MATERIALS

Lignocellulosic raw materials of technological relevance include biomass of woody plants such as agricultural and forestry waste and waste from the wood processing and food industries (Palonen, 2004; Wyman et al., 2005; Pothiraj et al., 2006; Dashtban et al., 2009; Mäkelä et al., 2015). Three major components, lignin (15 - 20%), cellulose (40 - 50%) and hemicellulose (25 - 35%) determine their unique properties. Lignocellulosic raw materials are characterized by a high mechanical strength and rigidity (Lange, 2007; Mäkelä et al., 2015). Proteins, terpene oils, fatty acids and their esters as well as inorganic substances (nitrogen, phosphorus and potassium compounds) can also be found in the lignocellulosic matrix (Lange, 2007; Dashtban et al., 2009). Depending on various genetic and environmental factors, the composition varies from raw material to raw material (Malherbe and Cloete, 2002; Palonen, 2004).

Cellulose is a linear biopolymer composed of D-glucose units. Molecules of D-glucopyranose within the chain are linked by β-1,4-glycosidic bonds, while hydroxyl groups of neighbouring cellulose strands form an extensive hydrogen bonds network with each other. These hydrogen bonds stabilize the structure of cellulose allowing the formation of a crystalline structure which constitutes 40-60% of the total cellulose. The crystalline fraction is interspersed by less-structured, amorphous regions (Malherbe and Cloete, 2002; Palonen, 2004; Wyman et al., 2005; Dashtban et al., 2009; 2010; Mäkelä et al., 2015).

Cellulose is tightly connected to a variety of heteropolysaccharides, collectively called hemicelluloses. Their composition depends on the type of plant, its developmental stage and tissue type. However, the most common carbohydrates that form hemicellulose backbones are D-mannose and D-xylose. The composition also includes D-glucose, D-galactose, L-arabinose as well as glucuronic-, acetic- and ferulic acid as well as mannans (Palonen, 2004; Wyman et al., 2005; Pothiraj et al., 2006; Dashtban et al., 2009; Mäkelä et al., 2015). The main hemicellulose backbone in soft wood is mannose with laterally linked units of glucose and galactose. Xylan chains are the backbone in hardwood and grass, of to which arabinose and glucuronic acid chains are linked laterally. The degree of polymerization of hemicellulose is usually in the range of 70 to 200 (Malherbe and Cloete, 2002; Palonen, 2004).

Lignin (lat. lignum-wood) is the second most abundant plant polymer, but it is the only biopolymer with an aromatic backbone. Formation of ester bonds between phenylpropane units results in a large, heterogenous three-dimensional structure. Precursors of phenylpropane units are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These precursors form *p*-hydroxyphenyl, guaiacyl or syringyl subunits within the lignin molecule, but the overall composition, varies from plant to plant. This unique polymer gives cells structural support, protects them from mechanical damage and pathogens, but also prevents the penetration of lignocellulolytic enzymes to the internal structure. Therefore, it is not surprising that the degradation of lignin is a major challenge. As such, lignin greatly limits the use of lignocellulosic raw materials (Lewis et al., 1999; Malherbe and Cloete, 2002; Palonen, 2004; Pothiraj et al., 2006; Dashtban et al. et al., 2009; 2010; Vanholme et al., 2010; Christopher et al., 2014; Mäkelä et al., 2015).

Currently, there are different procedures for lignin isolation in industry. Since there is no standard procedure for such process, characteristics of the obtained lignin extracts vary based on the isolation method. In order to discuss possible microbiological degradation, it is important to understand how these isolation methods affects the structure of lignin. For example, milled wood lignin obtained by Björkman's procedure has minor changes in the structure of lignin. On the other hand, kraft lignin (Figure 1), residual material of chemical pulping processes, is structurally highly modified. During the process almost three quarters of the hydroxyl groups become sulfonated (Lange et al., 2013).

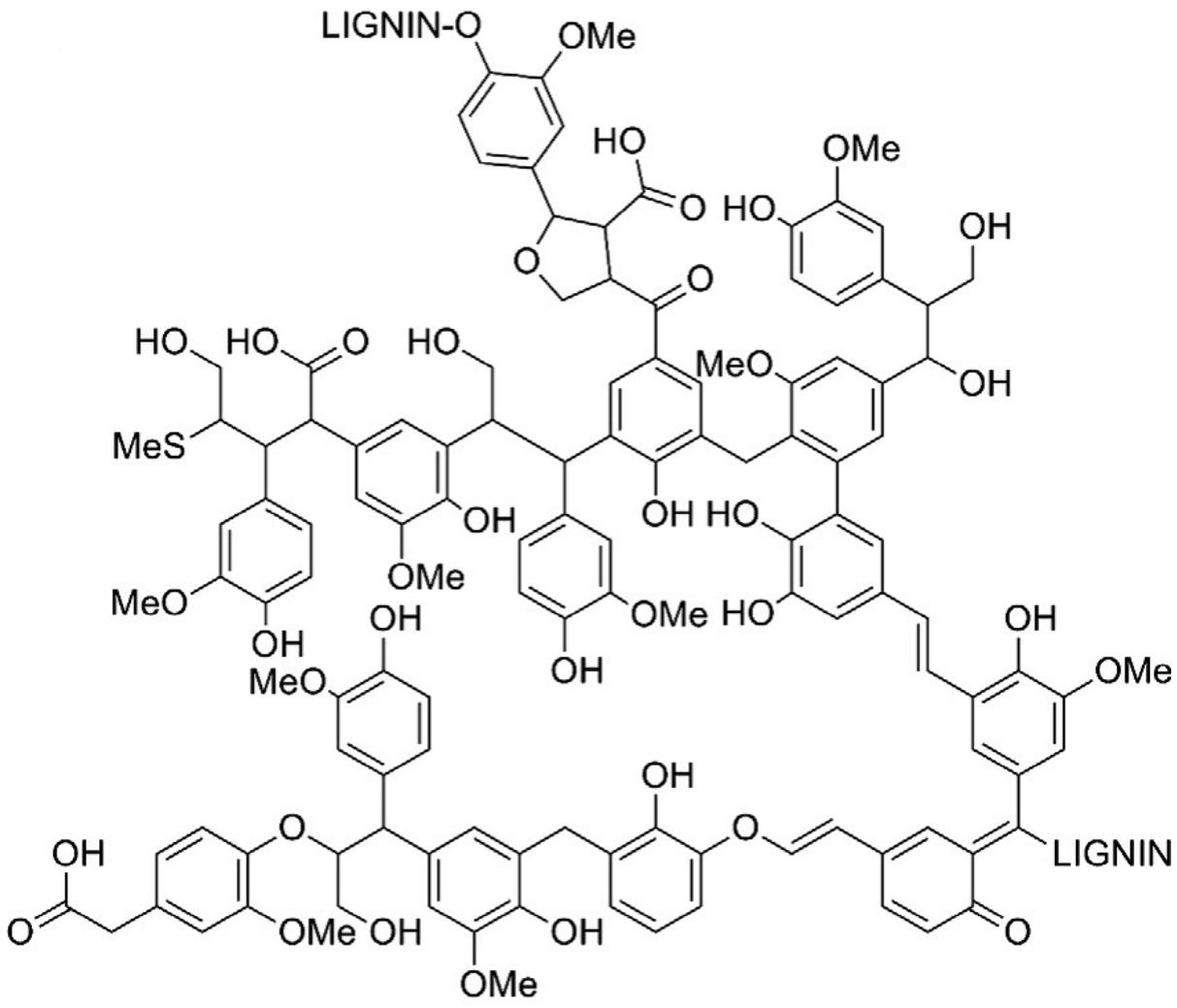

Figure 1. One possible substructure of kraft lignin (Lange et al, 2013).

2.2. FUNGI AND THEIR ROLE IN THE DEGRADATION OF LIGNOCELLULOSIC RAW MATERIALS

Fungi are among the most efficient degrader of plant biomass. Organisms of this large taxonomic group exhibit a number of different phenotypic features as a result of adaptation to environmental conditions. Thus, they secrete a number of unique and specific sets of enzyme systems that enables them to grow on a variety of lignocellulosic raw materials (Palonen, 2004; Mäkelä et al., 2015; Kracher, 2016).

Lignin-degrading fungi that produce the above mentioned enzymes typically include species from the class Ascomycota and Basidiomycota and several anaerobic species. Depending on their substrate specificity, fungi are classified as white-rot, brown-rot and dry-rot fungi. White-rot fungi are believed to be the only and most efficient lignin decomposers in our biosphere (ten Have and Teunissen, 2001; Pothiraj et al., 2006; Mäkelä et al., 2015). This group of fungi includes several hundred species from the class Basidiomycota and only a few species from the class of Ascomycota (Dashtban et al., 2010; Mäkelä et al., 2015; Sahadevan, 2016). Within this group, some species simultaneously degrade all three components of biomass, while others are selective toward lignin. For example, *Ceriporiopsis subvermispora, Phlebia spp., Physisporinus rivulosus, Dichomitus squalens* secrete lignin-selective enzymes, while *Trametes versicolor, Heterobasidium annosum, Phanerochaete chrysosporium*, and *Irpex lacteus* can, in addition to lignin, degrade cellulose and hemicellulose. *P. chrysosporium*, *Coriolus versicolor* and *T. versicolor* are believed to be the most effective lignin degrading fungi (Pothiraj et al., 2006; Dashtban et al., 2009; 2010). The enzymes produced by *Phlebia fascicularia, D. squalens, Daedalea flavida, Phlebia floridensis,* and *Phlebia radiata* have not been shown to be equally effective in the decomposition of industrial waste at high pH values. Generally, fungi prefer lower pH values (pH 4-7) for lignin decomposition, which corresponds to the natural growth conditions (Sahadevan, 2016).

White-rot fungi produce ligninases which are divided into two groups: phenol oxidase such as laccase and heme peroxidase such as lignin peroxidase, manganese peroxidase and polyphenol peroxidases. It is believed that these enzymes are too large to penetrate the interior of the dense network of lignocellulose. Therefore, enzymatic degradation is thought to be accomplished through small-molecule redox mediators that interact with the enzymes and penetrate preceded by a change in the tight structure of lignin. Thus, in addition to the mentioned enzymes, most of these fungi secrete other compounds necessary for the activity of these enzymes and to facilitate the efficient degradation of lignin (Malherbe and Cloete, 2002; Palonen, 2004; Pothiraj et al., 2006; Dashtban et al., 2009; 2010 ; Mäkelä et al., 2015). White-rot fungi owe their name to the unique bleaching effect during the degradation of lignocellulose. During this process, cellulose is hardly affected giving the decomposed matter a white color. ten Have and Teunissen (2001) listed parameters that affect the production of

ligninolytic enzymes, such as nutrient medium composition and growing growth conditions (temperature and pH). Studies with *P. chrysosporium* have shown that lignin degradation occurs during the secondary growth phase and is induced by a lack of nitrogen, carbon or sulphur. These results agree with the assumptions that fungi grow naturally under conditions with low nitrogen concentrations. On the other hand, research has shown that this does not apply for to all types of white rot fungi. The addition of an organic source of nitrogen source or amino acids did not cause a repressive effect on the production of ligninolytic enzymes in *Bjerkander adusta ssp*. and *C. versicolor.* In contrast, nitrogen-rich medium stimulated biomass growth and peroxidase production. Lignin degradation is enhanced in conditions with higher oxygen concentrations, in several ways. The increase in partial oxygen pressure results in higher production and activation of both ligninolytic enzymes and oxidases that produce H_2O_2. Cultivation of *P. chrysosporium* with aeration using pure oxygen resulted in higher lignin peroxidase activity, compared to the air aeration process. Such a significant effect of oxygen is expected since the production of hydrogen peroxide, which is crucial for peroxidase activity is enhanced under these conditions. Of course, many other parameters such as temperature, pH value, buffer composition and concentration of certain minerals affect the lignin degradation efficiency (ten Have and Teunissen, 2001).

2.2.1. Laccase

Laccases are copper-dependent polyphenol oxidases that are classified as benzenediol oxygen reductases (EC 1.10.3.2). Laccases catalyses the one electron oxidation of diverse phenolic and non-phenolic compounds, while oxygen is reduced to water through a 4-electron reduction. The active site of laccases contains four copper atoms, forming a T1-copper site and a trinuclear Cu-cluster (T2 Cu, T3α Cu and T3β Cu). Several functions of laccases are described in the literature. This enzyme participates in both, the degradation and the synthesis of lignin (ten Have and Teunissen, 2001; Periasamy et al., 2011; Christopher et al., 2014; Mäkelä et al., 2015). The oxidation reaction of various aromatic and non-aromatic compounds takes place by a mechanism in which radicals play a major role. When molecular oxygen is reduced to two molecules of water, free radicals are first formed, which further causes chain oxidation reactions. In this way, the formed mediators are released from the enzyme and reduce a wide range of high- redox potential substrates such as aminophenol, polyphenols, methoxy-

substituted monophenols, aryldiamines, thiols and aromatic amines, as well as some inorganic compounds. Therefore, so-called laccase mediator systems (LMS) are crucial for the lignin degradation, but also for its synthesis, morphogenesis, pathogenesis and detoxification (Palonen, 2004; Christopher et al., 2014; Mäkelä et al., 2015).

So far, laccases are best investigated in white rot fungi such as *Pleurotus ostreatus, Cerrena unicolor, T. versicolor, Trametes pubescens, P.radiata, Pycnoporus cinnabarinus* (Palonen, 2004; Christopher et al., 2014). Laccase production was also detected in liquid culture of some Ascomycetes, such as *Melanocarpus albomyces, Chaetomium thermophile, Magnaporthe grisea, Myrothecium verrucaria 24G-4 and Neurospora crassa*, as well as the bacteria of the genus *Bacillus* (Dashtban et al., 2010).

A wide range of laccase's substrates provides great opportunities for commercial application. Pulp bleaching, biosensors, biopolymer production, various applications in the textile and food industry are just some of the proposed applications. However, the biggest obstacle for the applications stated above, remains the required amount and the price of enzymes (Dashtban et al., 2010; Periasamy et al., 2011).

2.2.2. Lignin peroxidases (LiP)

Lignin peroxidases play an important role in lignin degradation. These glycoproteins contain heme as a prosthetic group. Lignin peroxidases catalyse the H_2O_2-dependent, oxidative depolymerisation reaction of a number of nonphenolic compounds in the lignin structure. In addition, they oxidize a wide range of phenolic compounds with redox potentials of up to 1.4 V vs. SHE (standard hydrogen electrode) (ten Have and Teunissen, 2001; Dashtban et al., 2010; Mäkelä et al., 2015).

During the multistep oxidation reaction of LiP, radicals, such as phenoxy radicals and veratryl alcohol cation radicals, are formed. The secondary metabolite, veratryl alcohol, is an ideal substrate for this enzyme. By forming radicals, the oxidation of otherwise difficult to oxidize substrates is enhanced. In the next reaction step, grouping or polymerization of the radicals, as well as decomposition of the side branches, demethylation, the reactions of addition and rearrangement occur. These reactions are not catalysed by the enzymes. What sets lignin peroxidases apart from other peroxidases is their unusually high redox potential. Thus, these

enzymes do not require the presence of redox mediators during the oxidation of nonphenolic aromatic compounds (ten Have and Teunissen, 2001; Dashtban et al., 2010; Mäkelä et al., 2015).

Crystallographic studies of this enzyme revealed a channel at the surface of the enzyme leading to the well-shelled heme. The channel size does not allow large polymers, such as lignin, to access the active site; however smaller lignin breakdown products, upon reaction with radicals, can bind to the enzyme and undergo catalysis (ten Have and Teunissen, 200; Dashtban et al., 2010).

Lignin peroxidase was first detected in *P. chrysosporium* and later identified in other species of the genus *Pleurotus*, as well as in *T. versicolor, Panus sp., P. coccineus, P. sanguineus, and Perenniporia medulla-panis* (Dashtban et al., 2010).

2.2.3. Manganese peroxidases (MnP)

Manganese peroxidases are glycoproteins, secreted as several isoforms with one molecule of heme. They also catalyse a peroxide-dependent reaction, but oxidize manganese (II) to manganese (III) which is then released from the enzyme as a chelate complex, either with oxalate or some other chelators. The resulting complex is actually a low molecular weight redox mediator that can diffuse easier into the lignocellulose than the enzyme. The chelated manganese complex can oxidize only phenolic compounds. For the oxidation of non-phenolic compounds, radicals are formed in the presence of other redox mediators. Oxalates and malonates are secondary mediators in the production of reactive radicals such as acetic acid radicals, peroxyl-, superoxide and formate radicals (ten Have and Teunissen, 2001; Mäkelä et al., 2015).

So far, manganese peroxidases were found to be produced mainly by basidiomycetes, such as *P. chrysosporium, Panus tigrinus, Lenzites betulinus, Phanerochaete flavido-alba, Agaricus bisporus, Bjerkandera* sp. and *Nematoloma frowardii* (Dashtban et al., 2010) and species of the genus *Pleurotus* (Salame et al., 2013). Manganese peroxidases have several potential applications in biotechnology. Earlier research showed that the presence of this enzyme increased the efficiency of dye decolourization processes. Later, it was shown that this enzyme is mainly responsible for this process. Furthermore, manganese peroxidases also play a major

role in the bio-bleaching of so-called kraft lignin. The main drawback in the application of this enzyme are the lack of an efficient recombinant expression system (Dashtban et al., 2010).

2.2.4. Versatile peroxidase, (VP)

Polyvalent peroxidases are glycoproteins of various properties, capable of oxidizing wide range of substrates. What distinguishes these enzymes from other peroxidases is their biooxidative ability, i.e. these enzymes can oxidize both manganese and phenolic and non-phenolic compounds. High redox potential compounds including several artificial dyes are also substrates of these enzymes (Dashtban et al., 2010; Mäkelä et al., 2015).

A possibility to oxidize broad range of substrates is a consequence of their hybrid structure with multiple substrate binding sites. Namely, this is the reason for superiority of these enzymes compared to other peroxidases. The reaction mechanism is similar to the reaction mechanism catalysed by manganese peroxidase. Manganese (II) is oxidized to manganese (III), which is released from the enzymes and as such can diffuse into the lignocellulose structure and oxidize phenolic compounds. The heme molecule is hidden inside the enzyme but is connected to the surface of the enzyme via two tunnels on the surface of the enzyme. The first tunnel is common to all peroxidases, while the second tunnel is characteristic for manganese and polyvalent peroxidases. This second tunnel is the proposed site of manganese oxidation (Dashtban et al., 2010; Mäkelä et al., 2015).

Given the similarities with manganese peroxidase, some authors classify these two enzymes into the same group (Salame et al., 2013), while others describe them as separate groups of enzymes (ten Have and Teunissen, 2001; Dashtban et al., 2010; Mäkelä et al., 2015). These enzymes were first detected in species of the genus *Pleurotus (*ten Have and Teunissen, 2001). Regarding the above described features of polyvalent peroxidases, these enzymes may have the greatest potential for application in various bioprocesses. The primary drawback in industry applications is the limited availability of the enzyme. Like manganese peroxidase, there is currently a lack of efficient expression systems that can produce VP with high titers. The main answer to this problem is the application of recombinant technology. Not only does this technology provide the possibility of expression in well-known hosts that grow significantly faster than the native producer organisms, but the possibility of improving the properties of enzymes as well (Salame et al., 2013).

2.2.5. Auxiliary enzymes

Lignin degradation is also significantly enhanced by the so-called auxiliary enzymes. These enzymes do not act directly on lignocellulosic polymers, but they cooperate with other enzymes in order to enhance the depolymerisation of lignocellulose. These enzymes belong to diverse groups which include members of the large family of FAD-dependent GMC (glucose–methanol–choline family) oxidoreductases such as cellobiose dehydrogenase (CDH), glucose oxidoreductases (glucose dehydrogenase (GDH) and glucose oxidase), aryl-alcohol oxidase, alcohol (methanol) oxidase, and pyranose oxidoreductases. Reaction products resulting from the reduction of oxygen by oxidases (H_2O_2) as well as from the reduction of quinones by dehydrogenases (hydroquinones) are involved in reactions catalysed by lignin-degrading enzymes. For example, H_2O_2 is the electron acceptor in peroxidase reactions. One of the most described enzymes involved in lignocellulose degradation is the flavocytochrome cellobiose dehydrogenase (CDH). This enzyme has two separate domains: a dehydrogenase domain (DH) with a non-covalently bound FAD in the active site and the non-catalytic cytochrome domain containing a heme *b*. Its *in vivo* function has been debated since its discovery in 1974. The most accepted theory earlier was that CDH degrades lignocellulosic biomass in a Fenton type reaction by generating highly reactive hydroxyl radicals. In such reaction, the oxidation of carbohydrate substrates (cellobiose) at the DH domain generates electrons which reduce one electron acceptors such as Fe(III) via the heme *b* domain. In the presence of hydrogen peroxide and at low pH values (<4), these reduced species generate highly reactive hydroxyl radicals which randomly oxidise lignocellulose. Besides the ability to reduce Fe^{3+} ions, recent research has shown that CDH is an efficient reductase for the fungal copper-dependent lytic polysaccharide monooxygenases (LPMO), which oxidatively cleave cellulose and various hemicelluloses. The reductive activation of LPMOs by CDH involves the initial 2-electron reduction of the FAD. The generated $FADH_2$ is re-oxidation via single electron transfer events to CDH's cytochrome domain, which interacts with the LPMO active site. These electrons from CDH reduce the type-II copper centre in the LPMO active site from Cu(II) to Cu(I), which initiates LPMO activity. Reduced LPMO than catalyzes the oxidative degradation of all major

polysaccharides. Figure 2 summarises “Auxiliary Enzyme Activities (AA)” and their roles in lignocellulose degradation (Levasseur et al., 2013; Sützl et al., 2018).

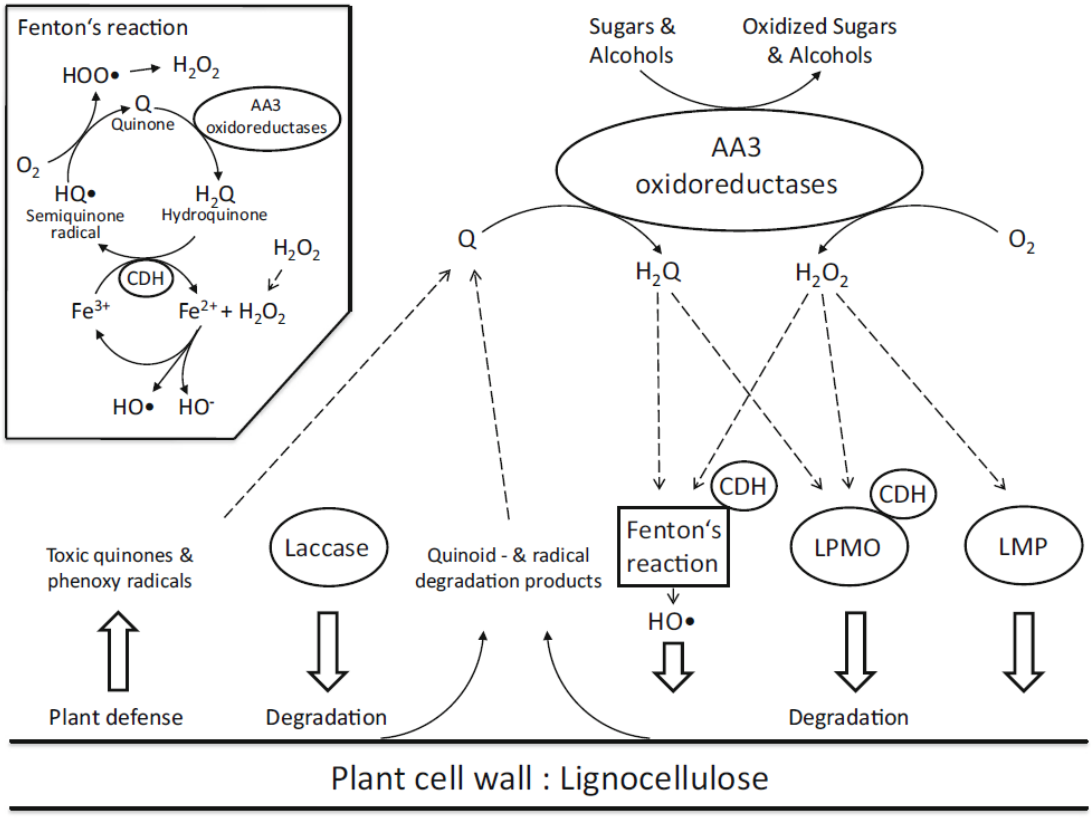

Figure 2. AA enzymes involved in lignocellulose degradation (adapted from Sützl et al., 2018).

3. EXPERIMENTAL PART

3.1. AIMS

This thesis aims to investigate the degradation of lignin by fungal enzymes. To this end, a set of purified fungal enzymes derived from potent lignin-degrading fungi were used. These include the aforementioned auxiliary enzymes, purified laccase and oxidoreductases such as GDH, CDH and LPMO. To date, the action and possible synergy of these enzymes on lignin components remains poorly understood. To gain further insight into the role of these enzymes, whole cell extracts from selected fungal strains known for their ability to degrade lignin were used. These include the supernatants from the strains *Trametes versicolor* and *Phanerochaete chrysosporium*.

3.2. MATERIALS AND METHODS

3.2.1. Enzymes

For this work the following enzymes were used:
Cellobiose dehydrogenase (CDH)
Laccase (Lac)
Lytic polysaccharide monooxygenase (LPMO)
Glucose dehydrogenase (GDH)

All enzymes used in this study were provided by the Biocatalysis and Biosensing Research Group, at the Department of Food Science and Technology, BOKU–University of Natural Resources and Life Sciences, (Vienna, Austria) in a homogenous and purified form.

3.2.2. Strains

For the purposes of this project the following strains were cultivated:
Trametes versicolor DSM6401
Phanerochaete chrysosporium DSM 6909
The strains was obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

3.2.3. Chemicals

The list of chemicals is shown in the Table 1.

Table 1. List of chemicals

Chemical	Manufacturer
α-Cellulose	Merck, Germany
Acetic acid	Fluka, Switzerland
2.2'-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	Fluka, Switzerland
Acetonitrile, HPLC-grade	Merck, Germany
Ammonium nitrate	Merck, Germany
Aurintricarboxylic acid ammonium salt, ATA	Merck, Germany
Bradford reagent	Sigma, USA
Boric acid, ≥ 99%	Sigma, USA
Calcium chloride	Sigma, USA
Cellulose, microcrystalline	Merck, Germany
D(+)-cellobiose	Fluka, Switzerland
Citric acid	Sigma, USA
Cobalt(II)chloride hexahydrate	Sigma, USA
Copper(II)sulphate	Sigma, USA
Cytochrome *c* from equine heart	Sigma, USA
Disodium hydrogen phosphate dihydrate	Fluka, Switzerland
EDTA (Ethylenediaminetetraacetic acid)	Sigma, USA
α-D(+)- glucose monohydrate	Roth, Germany
Hydrogen peroxide, 30%	Fluka, Switzerland
Iron(II)chloride	Sigma, USA
Iron(II)sulfate heptahydrate	Sigma, USA
D(+)-lactose monohydrate	Fluka, Switzerland
Kraft Lignin	/
Magnesium sulfate	Sigma, USA

Manganese(II)sulphate monohydrate	Fluka Switzerland
Manganese(II)chloride	Sigma, USA
Methanol, HPLC-grade	Merck, Germany
Potassium chloride	Fluka, Switzerland
Potassium dihydrogen phosphate	Roth, Germany
Potassium hydroxide	Fluka, Switzerland
Phosphoric acid	Sigma, USA
Sodium chloride	Roth, Germany
Sodium hydroxide	Fluka, Switzerland
Sodium molybdate dihydrate	Fluka, Switzerland
Syringol	Sigma, USA
Tartaric acid	Roth, Germany
Yeast extract	Sigma, USA
Zinc sulfate heptahydrate	Sigma, USA
2,5-dimetoxi-o-benzoquinone	Sigma, USA

3.2.4. Solution and buffers

The composition of solutions and buffers used:

50 mM NaOH

50% NaOH 2.61 mL L^{-1}

100 mM NaCl

2.922 g of NaCl was dissolved in total volume of 500 mL

50 mM Potassium phosphate buffer (KPP) pH 6.0

Potassium dihydrogen phosphate 3.40 g per 500 mL

The salt was dissolved in 300 mL of distilled water and the pH adjusted to 6.0 with KOH. Afterwards, the solution was filled up to 500 mL with distilled water.

100 mM sodium-acetate buffer (pH 4)

3 g or 2.86 ml of acetic acid (99%) was dissolved in 400 mL of distilled water and titrated with NaOH to pH 4. Afterwards, the solution was filled up to 500 mL with distilled water.

50 mM sodium tartrate buffer (pH 4.5)

3.75 g of tartaric acid was was dissolved in 400 mL of distilled water and titrated with NaOH to pH 4.5. Afterwards, the solution was filled up to 500 mL with distilled water.

10mM 2,5-dimetoxi-o-benzoquinone

In order to get 10 mM solution of above stated redox mediator, 84 mg was dissolved in a total volume of 50 mL deionized water.

50 mM Glucose solution

Glucose: 495 mg L^{-1}

In order to obtain a glucose solution with the desired concentration the corresponding amount was dissolved in deionized water.

300 mM Lactose solution

Lactose 10.81 g L^{-1}

Corresponding amount was dissolved in deionized water.

50 mM Cellobiose solution

Cellobiose: 17.1 g L^{-1}

In order to obtain a cellobiose solution with the desired concentration the corresponding amount was dissolved in deionized water to a final volume of 50 mL.

1M $FeCl_2$

0.1988 g of $FeCl_2$ was dissolved in total volume of 1 mL deionized water.

1M $MnCl_2$

0.1258 g of $MnCl_2$ was dissolved in total volume of 1 mL deionized water.

5 mM $MgSO_4$

84.5 mg of $MgSO_4$ was dissolved in 100 mL distilled water.

1 M Hydrogen peroxide solution

113.4 mL of 30% H_2O_2 solution were diluted in one litre of buffer solution.

10 mM Hydrogen peroxide solution

20.3 µL of 30% H_2O_2 were diluted in 20 mL of distilled water. The solution was freshly prepared prior to all measurements.

Bradford reagent

10 mL of Bio-Rad protein assay dye reagent concentrate was diluted in 40 mL of high quality distilled water and filtered through a paper filter.

1 mM Cytochrome c solution

Stock solutions were prepared by dissolving 3.1, 6.2 or 12.4 mg of cytochrome *c* in 0.25, 0.5 or 1 mL of distilled water, respectively. The stock solutions were stored at 4 °C in the dark for up to three days.

10 mM 2,2'azino-bis-[3-ethylbenzthiazoline-6-sulfonate] ABTS

10.3 mg of the ABTS reagent was dissolved in 2 mL distilled water.

10 mM Syringol solution

1.54 mg of syringol was dissolved in 100 mL of distilled water.

Trace element solution

$ZnSO_4 \cdot 7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnCl_2 \cdot 4H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.5 g
$CoCl_2 \cdot 6H_2O$	0.17 g
$CuSO_4 \cdot 5H_2O$	0.16 g
$NaMoO_4 \cdot 2H_2O$	0.15 g
Na_2EDTA	0.5 g

EDTA was dissolved before the addition of other salts. These were dissolved in 80 mL of distilled water. The pH value was adjusted to 8.0 with KOH and the solution filled up to 100 mL with distilled water.

3.2.5. Culture media

Culture media plates:

Composition:

dextrose	20.0 g L^{-1}
potato extract	4.0 g L^{-1}
agar-agar	15 g L^{-1}
KH_2PO_4	1.2 g L^{-1}

The medium was adjusted to pH 5.5 with H_3PO_4 prior autoclaving.

Liquid culture media

Media of the following composition was used:

α-cellulose	10 g L^{-1}
yeast extract	10 g L^{-1}
$MgSO_4$	1 g L^{-1}
KH_2PO_4	1.2 g L^{-1}
KCl	0.6 g L^{-1}
Trace element solution	0.3 mL L^{-1} (the composition is given in 3.2.4.)

The medium was adjusted to pH 5.5 with H_3PO_4 and the flasks were autoclaved at 121 °C for 20 min.

3.2.6. Equipment

During this work the following equipment was used (except for consumables):

- Analytical balance (AW-4202, Sartorius group, Germany)
- Autoclave (VarioClav Classic, Thermo Fisher Scientific, USA)
- Centrifuge (Avanti J-26 XP, Beckman Coulter Inc., USA)
- Centrifuge (Centrifuge 5810 R, Eppendorf, Germany)
- Digital dry bath (Accublock, Labnet International, USA)
- Filters Milipak-40 Gamma Gold' 0.22 µm (Merck, Germany)
- HPLC (Summit, Thermo Fisher Scientific, USA)
  - precolumn and column – SEC-300 Acclaim 5 µm, analytical 4.6 × 300 mm
  - Photodiode Array (PDA) detector
- Shaking incubators (Unitron, Infors HT, Schwitzerland)
- Laminar flow cabinet (Safe 2020, Thermo Fisher Scientific, USA)
- Magnetic stirrer (RCT basic, IKA, Germany)
- Peristaltic pump (Ismatec, Germany)
- pH meter (744, Metrohm, Switzerland)
- Spectrophotometer (DU 800, Beckman Coulter Inc., USA)
- Spectrophotometer (Lambda 35 UV/VIS, Perkin Elmer, USA)
- Table centrifuge (Centrifuge 5415 R, Eppendorf, Germany)
- Thermoshaker (Eppendorf, Germany)
- Technical balance (AW-224, Sartorious group, Germany)
- Ultrasonic Probe Sonicator (Bandelin, Germany)
- Ultrasonic bath (Sonorex super, Bandelin, Germany)
- UV/VIS spectrophotometer (Lambda 35, Perkin Elmer, USA)
- Vivaflow 50 Laboratory cross flow cassette (Sartorius, Germany)
- Vortex (Vortex genie 2, Scientific Industries, USA)
- Water bath (TW 12, Julabo, Germany)

- Water bath (F10, Julabo, Germany)

3.2.7. Methods and experimental design

Preparation of lignin samples

Lignin samples were prepared by dissolving 300 mg of kraft lignin in 50 mM NaOH in a total volume of 30 mL. The final concentration of kraft lignin was 10 mg mL^{-1}. The solution was treated with an ultrasonic probe sonicator for a total of 54 min. Undissolved particles were removed by centrifugation for 5 min at 4000 rpm and the sample solution adjusted with CH_3COOH to pH 4.0. The kraft lignin solution was stored at 4 °C in a light-protected flask.

Reactions set up

In order to evaluate the activity of the enzymes on the lignin, a set of conversion reactions were conducted. Reactions are listed in Table 2, given below. Prior to the experiment, the concentration of the enzymes were determined using the Bradford protein assay.

Table 2. Reaction set up

1	LIGNIN + GDH (0.5 µM) + glucose (5 mM)
2	LIGNIN + GDH (0.5 µM)
3	LIGNIN + CDH (0.5 µM) + cellobiose (5mM)
4	LIGNIN + CDH (0.5 µM)
5	LIGNIN + CDH (0.5 µM) + cellobiose (5 mM) + $FeCl_2$ (50 mM)
6	LIGNIN + CDH (0.5 µM) + $FeCl_2$ (50mM)
7	LIGNIN + CDH (0.5 µM) + cellobiose (5 mM) + $MnCl_2$ (50 mM)
8	LIGNIN + CDH (0.5 µM) + $MnCl_2$ (50 mM)
9	LIGNIN + H_2O_2 (50 mM)
10	LIGNIN + H_2O_2 (1 mM)
11	LIGNIN + H_2O_2 (50 µM)
12	LIGNIN + H_2O_2 (1 mM) + $FeCl_2$ (50 µM)
13	LIGNIN + H_2O_2 (1 mM) + $MnCl_2$ (50 µM)
14	LIGNIN + $FeCl_2$ (50 mM)
15	LIGNIN + $MnCl_2$ (50 mM)
16	LIGNIN + Laccase (0.5 µM)

17	LIGNIN + LPMO (0.5 µM)
18	LIGNIN + LPMO (0.5 µM) + cellulose (10 mg)
19	LIGNIN + cellulose (10 mg)
20	LIGNIN + LPMO (0.5 µM) + CDH (0.5 µM) + cellulose (10 mg)
21	LIGNIN + LPMO (0.5 µM) + cellulose (10 mg) + cellobiose (5 mM)
22	LIGNIN + LPMO (0.5 µM)
23	LIGNIN + LPMO (0.5 µM) + 2,5-RM (50 µM)
24	LIGNIN + LPMO (0.5 µM) + 2,5-RM (50 µM) + cellulose (10 mg)
25	LIGNIN + Laccase (0.5 µM) + 2,5-RM (50 µM)

*RM=2,5-dimetoxy-1,4-benzoquinone-redox mediator

The reactions were conducted in 1.5 mL reaction tubes which contained holes in the lid to allow for oxygen transfer into the tube. Each vial contained 500 µL of lignin solution, which was treated as described above.

The reaction tubes were incubated on a rotary shaker operated at 145 rpm and at 30 °C for 24 h. Samples were analyzed using a UV/VIS spectrophotometer.

Inoculum preparation

Fungal inocula were prepared by growing all strains at 30 °C on the plates with above described composition for 6-7 days. Four fungal mycelial plugs (ca. 1 cm^2) were extracted from each agar plate under sterile conditions for inoculation.

Submerged cultivation

Fungi were cultivated in 300 mL unbaffled shaking flasks containing 150 mL of medium (for composition see 3.2.5.). Flasks were incubated on a rotary shaker at 145 rpm and 30 °C. For each fungus, two flasks were inoculated for comparison. To one flask 10 mg of lignin was added. At chosen time intervals (4, 6, 11, 12 days), samples of 1 mL were withdrawn from the flasks and cellulose and mycelium separated by centrifugation at 10.000 rpm for 5 min at room temperature.

The supernatants obtained after sampling were analysed for protein concentration and enzyme activity according to the assays previously described.

Harvesting and concentration of crude extracts

The crude extracts with the highest protein concentration and enzyme activities were used for the following experiments and were harvested.

Flasks were centrifuged in 500 mL autoclaved plastic bottles (6000 rpm, 4 °C for 20 min). The supernatant was transferred to sterile glass bottles under sterile conditions. The collected supernatants were sterile filtrated using a 'Milipak-40 Gamma Gold' 0.22 µm disposable filter system connected to a BVP-Ismatec standard peristaltic pump.

Crude extracts were concentrated to approximately 50 mL using a VivaFlow cross-flow cassette with a cut-off of 50 kDa. In order to ensure that no enzymes were lost during this procedure, the permeate was constantly monitored for proteins using the Bradford protein assay. To further concentrate the sample solutions, the crude extracts were centrifuged for 10 min at 4000 rpm in Amicon centrifugal filters with a cut-off of 10 kDa. During this step, the sample solution was rebuffered to 100 mM Na-acetate, pH 5.0. The waste was measured for any remaining proteins that might have been flushed through. The concentrate was stored at 4 °C until further use.

Large scale conversion experiment

In order to obtain a lignin solution with a concentration of 10 mg/mL, 2 grams of kraft lignin was added to 180 mL of 50 mM NaOH and left in an ultrasonic water bath overnight at room temperature. The solution was then cleared by centrifugation for 10 min at 4000 rpm and adjusted to pH 5.0 with 2M CH_3COOH.

The conversion of lignin experiment for 48 h as follows:

Enzyme reactions were initiated by adding of 2.5 mg/mL of crude extract to 10 mL of the lignin solution in a 50 mL falcon tube. In order to ensure aerobic conditions necessary for enzymatic activity, but to avoid the entry of contaminants during the reaction, the tubes were covered with an O_2-permeable membrane. The detailed conditions of the reactions are given below.

Table 3. Reaction conditions

Reaction 1	10 mL lignin solution + *Trametes versicolor* crude extract (2.5 mg protein)
Reaction 2	10 mL lignin solution + 10 mg cellulose + *T. versicolor* crude extract (2.5 mg protein)
Reaction 3	10 mL lignin solution + *Phanerochaete chrysosporium* crude extract (2.5 mg protein)
Reaction 4	10 mL lignin solution + 10 mg cellulose + *P. chrysosporium* crude extract (2.5 mg protein)

The tubes were kept on a rotary shaker at 140 rpm and 30 °C. The samples were taken at 0, 3, 6, 12, 24 and 48 h. In order to remove proteins and to stop the reaction, samples were filtered through centrifugal filters with a cut-off of 10 kDa. The protein-free flow-through was subjected to biochemical analyses.

In order to compare the activity of crude extracts with the activity of isolated enzymes, the following reactions were conducted:

Reaction 1: 10 mL lignin solution + GDH (1 mg mL^{-1}) + glucose (1 mg mL^{-1})

Reaction 2: 10 mL lignin solution + laccase (1 mg mL^{-1})

Reaction 3: 10 mL lignin solution + laccase (1 mg mL^{-1}) + GDH (1 mg mL^{-1}) + glucose (1 mg mL^{-1})

Lignin degradation analysis

All samples for both conversion experiments were analyzed using size exclusion chromatography (SEC).

The conditions for SEC were as follows:

mobile phase	100 mM NaCl in 50 mM KH_2PO_4 buffer (pH 6.0)
sample volume	100 µL
injection volume	20 µL
flow rate	0.35 mL min^{-1}
temp.	25 °C
pressure	62 bar
Run time	30 min per sample (plus 15 min calibration).

Lignin solution was used as reference material.

3.2.8. Analytical methods

Bradford protein assay

Based on the absorbance maximum shift of the Coomassie Brilliant Blue G-250 dye from 465 nm to 595 nm when it binds to proteins in acidic solution (Bradford, 1975), this colorimetric assay is widely used for protein concentration measurement. Absorbance was measured with a Beckmann DU-800 spectrophotometer. The measurement was performed as follows: 600 µL of the Bradford reagent was added to 15 µL of the protein sample. After incubation in the dark at room temperature for 15 min, absorbance of the samples was measured at 595 nm. Protein concentrations of unknown samples were calculated by comparison to a linear standard curve generated with bovine serum albumin (BSA).

Laccase activity assay

The laccase activity assay is based on the change in absorbance at 420 nm upon oxidation of ABTS (ε_{420}=36 mM^{-1} cm^{-1}). All reactions were carried out in 100 mM sodium-acetate buffer (pH 4) (Michal et al., 1983) and were performed as follows: 100 µL of ABTS solution were mixed with 880 µL Na-acetate buffer and incubated for 20 min at 30 °C. Reactions were initiated by addition of 20 µL properly diluted clear culture supernatant. The measurement time was set to 3 min. One unit of laccase activity is defined as the amount of protein that oxidizes 1 µmol of ABTS per minute.

Manganese peroxidase activity assay

The MnP assay is based on the oxidation of phenols in the presence of dicarboxylic acid chelators or α-hydroxy acids. In this reaction, the active center of MnP is initially oxidized by H_2O_2. Reduction to the resting state of the enzyme is achieved by two successive one-electron transfers with the consecutive reduction of Mn(II) to Mn(III). Mn(III) is readily complexed by chelating agents such as tartrate. The Mn(III)-tartrate complex is an efficient oxidant which oxidizes syringol (2,6-dimethoxyphenol), yielding a reddish-brown color (Wariishi et al., 1992). The reaction mixture contained the following: 100 µL of 10 mM syringol solution, 100 µL of 5

mM $MnSO_4$, 10 µL of 10 mM H_2O_2 solution, 770 µL of 50 mM sodium tartrate buffer (pH 4.5). After incubation for 20 min at 30 °C in a water bath, 20 µL of the clear sample supernatant was added to above described mixture. The absorbance was measured at 469 nm for 5 min (ε_{469} = 27.5 mM^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of protein that converts 1 µmol of syringol per minute.

Cellobiose dehydrogenase (CDH) activity assay

CDH activity was measured spectrophotometrically using cytochrome *c* (cyt *c,* ε_{520} = 19.6 mM^{-1} cm^{-1}). In this assay, CDH´s natural substrate cellobiose is replaced by lactose, since the latter does not lead to substrate inhibition. The reaction is based on the detection of the reduced (ferric) cyt *c* (the electron acceptor) resulting in a colour change from orange to pink (Baminger et al., 2001). Cyt c is reduced by the heme *b*-containing domain of CDH; thus, the assay allows measuring the activity of the FAD and heme *b* -containing holoenzyme. The reaction mixture contained: 20 µL of 1 mM cyt *c* solution, 100 µL of 300 mM lactose solution, 860 µL of 100 mM Na-acetate buffer (pH 4.0) and 20 µL of the diluted sample. Prior to adding the sample, the mixture was incubated for 20 min at 30 °C. The assay was run for 3 min. One unit of enzyme activity is defined as the amount of protein that reduces 1 µmol of cyt *c* per minute.

Data analysis

The following software programs were used for data analysis: HPLC Chromeleon 7 software Microsoft Excel 2013 (Microsoft Excel 2013, Redmond, Washington, SAD).

4. RESULTS AND DISSCUSION

4.1. LIGNIN CONVERSION WITH ENZYMES

UV/VIS spectra of different reaction set ups, described in chapter 3.2.7. are shown in the figures 3-7.

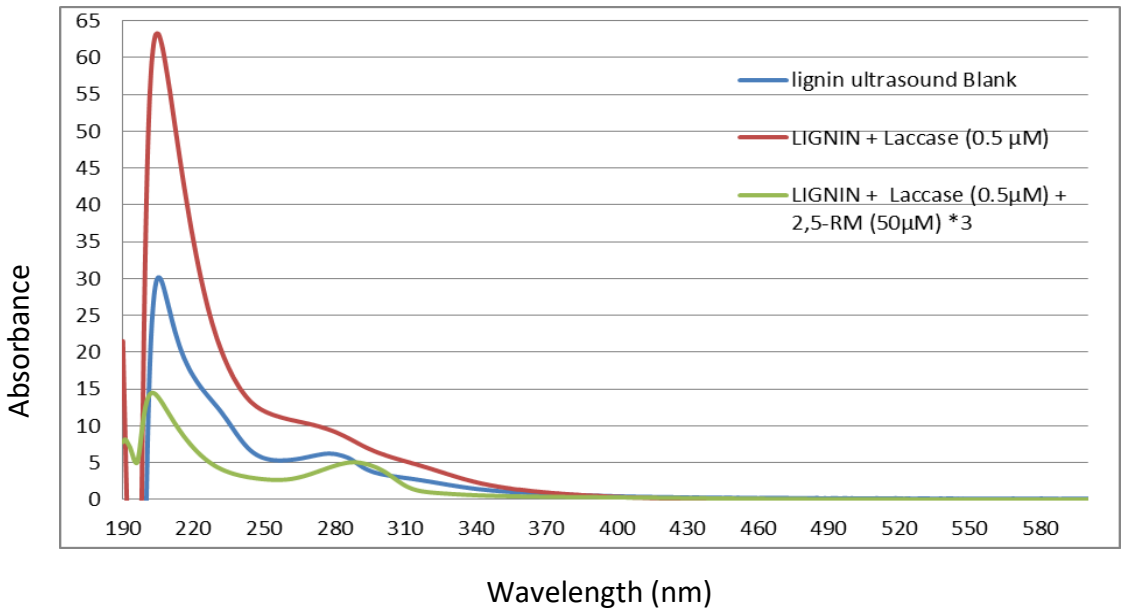

Figure 3. Absorbance of lignin solution itself (blue); in reaction with laccase (red) and in reaction with laccase and redox mediator (RM) (green)

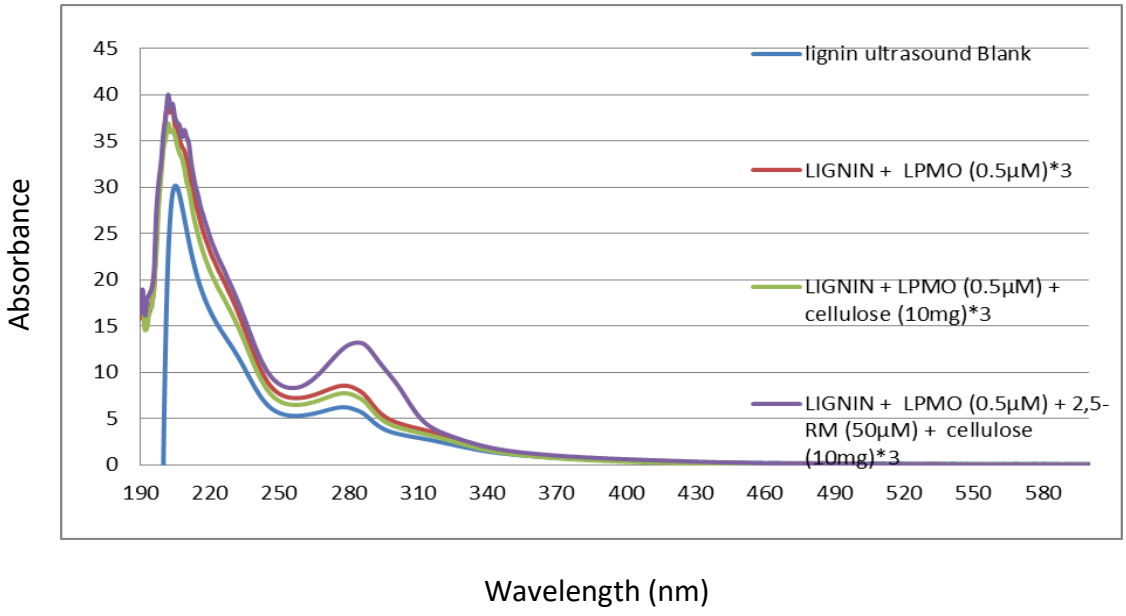

Figure 4. Absorbance of lignin solution itself (blue); in reaction with LPMO (red); in reaction with LPMO and cellulose (green); and in reaction with LPMO, cellulose and redox mediator RM (purple)

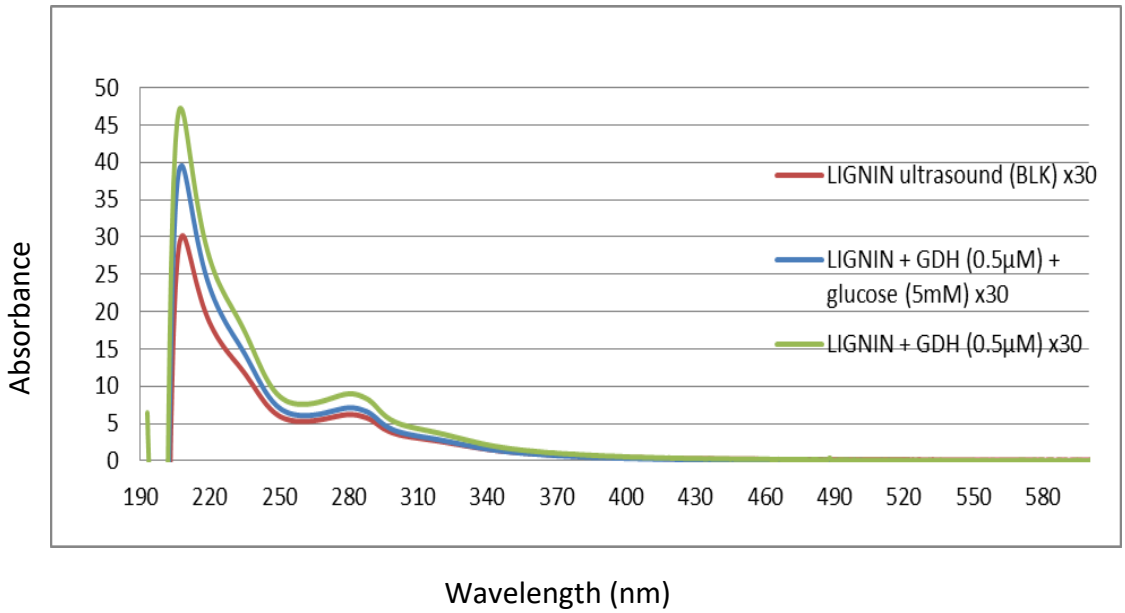

Figure 5. Absorbance of lignin solution itself (blk-blank, shown in red); in reaction with GDH (green); in reaction with GDH and glucose (blue)

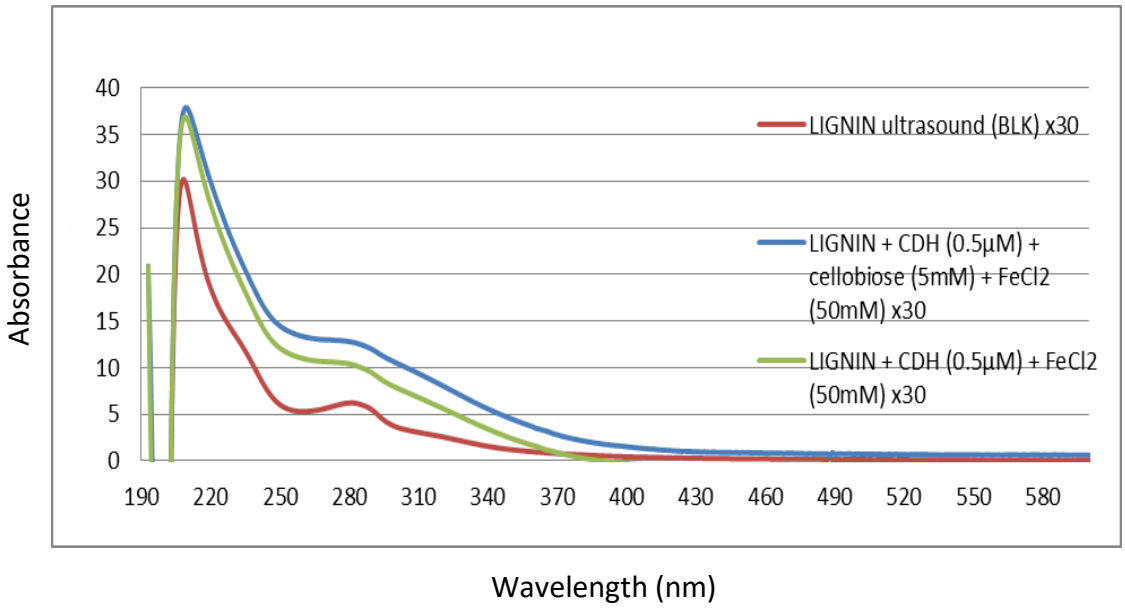

Figure 6. Absorbance of lignin solution itself (blk-blank, shown in red); in reaction with CDH and cellobiose (blue); in reaction with CDH. $FeCl_2$ and cellobiose (green)

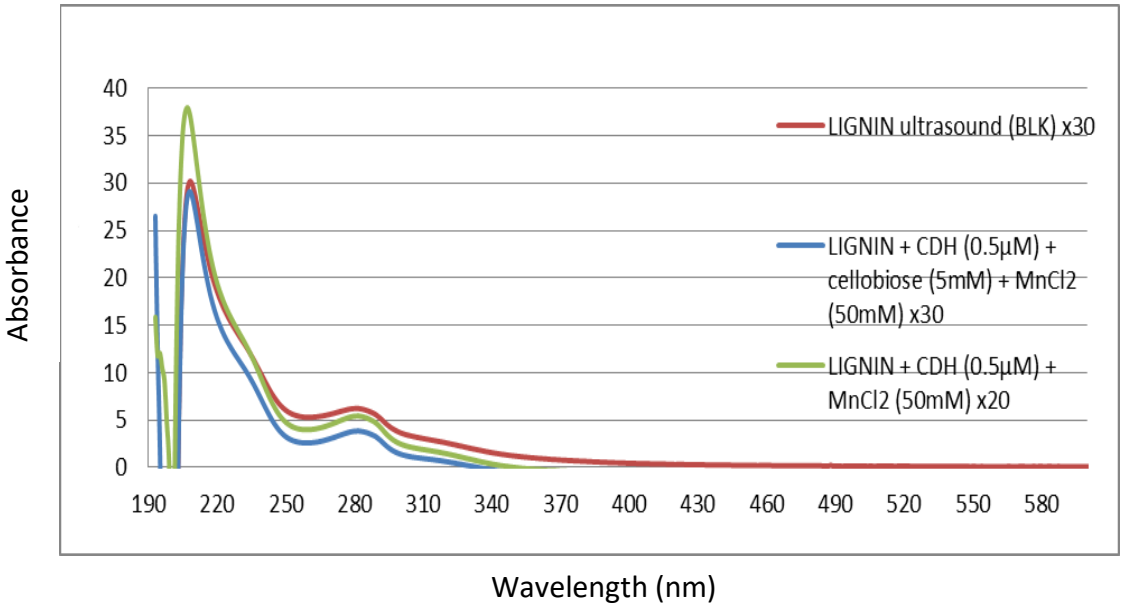

Figure 7. Absorbance of lignin solution itself (blk-blank, shown in red); in reaction with CDH, cellobiose and $MnCl_2$ (blue); in reaction with CDH and $MnCl_2$ (green)

4.2. LARGE SCALE LIGNIN CONVERSION

In order to compare activity of enzymes with the activity of crude extracts from white rot fungi *T. versicolor* and *P. chrysosporium*, set of experiments as described in chapter 3.2.7. was conducted.

In the tables 4-7, protein concentrations as well as enzymes activities during cultivation are given.

Table 4. Protein concentration during cultivation (results are shown as average value ± standard deviation)

	Concentration [mg/mL]				
Days	**0**	**4**	**6**	**11**	**12**
***T. versicolor* (CELL)**	0	0.0117 ± 0.00127	0.1294 ± 0.00304	0.1007 ± 0.00438	0.0676 ± 0.00035
***T. versicolor* (CELL + lignin)**	0	0.0087 ± 0.00438	0.1113 ± 0.00552	0.1120 ± 0.01004	0.0832 ± 0.01202
***P. chrysosporium* (CELL)**	0	0.0019 ± 0.00085	0.0318 ± 0.00247	0.2943 ± 0.06732	0.2855 ± 0.07715
***P. chrysosporium* (CELL + lignin)**	0	0.0117 ± 0.00587	0.0365 ± 0.00905	0.2746 ± 0.09793	0.2761 ± 0.02885

Table 5. Results of the ABTS assay for determining laccase activity (results are shown as average value ± standard deviation)

	Activity [U/mL]				
Days	**0**	**4**	**6**	**11**	**12**
***T. versicolor* (CELL)**	0	0.0002 ± 0.00025	0.0211 ± 0.00066	0.0186 ± 0.00328	0.0140 ± 0.00271
***T. versicolor* (CELL + lignin)**	0	0	0.0074 ± 0.00098	0.0292 ± 0.00279	0.0168 ± 0.01025
***P. chrysosporium* (CELL)**	0	0	0	0	0
***P. chrysosporium* (CELL + lignin)**	0	0	0	0	0

Table 6. Results of the MnP activity during cultivation (results are shown as average value ± standard deviation)

	Activity [U/mL]				
Days	**0**	**4**	**6**	**11**	**12**
***T. versicolor* (CELL)**	0	0.0046 ± 0.00067	0.0177 ± 0.00050	0.0179 ± 0.00058	0.0128 ± 0.00217
***T. versicolor* (CELL + lignin)**	0	0.0019 ± 0.00067	0.0093 ± 0.00025	0.0307 ± 0.00953	0.0188 ± 0.00150
***P. chrysosporium* (CELL)**	0	0	0	0.0073 ± 0.00635	0.0067 ± 0.00593
***P. chrysosporium* (CELL + lignin)**	0	0	0	0.0688 ± 0.07972	0.0588 ± 0.07680

Table 7. Results of the CDH activity during cultivation (results are shown as average value ± standard deviation)

	Activity [U/mL]				
Days	**0**	**4**	**6**	**11**	**12**
***T. versicolor* (CELL)**	0	0	0.0040 ± 0.00018	0	0
***T. versicolor* (CELL + lignin)**	0	0	0.0112 ± 0.00288	0.0014 ± 0.00198	0.0004 ± 0.00054
***P. chrysosporium* (CELL)**	0	0.0003	0.0098 ± 0.00523	0.8977 ± 0.37775	0.9394 ± 0.28922
***P. chrysosporium* (CELL + lignin)**	0	0.0060	0.0408 ± 0.02344	0.7338 ± 0.58511	0.8884 ± 0.30437

In order to additionaly visibly observe the degradation of lignin the samples from both conversion experiments with enzymes and conversion with crude extracts were ran on HPLC. In the figures 8-13, chromatograms of different reaction set ups described in chapter 3.2.7. are given.

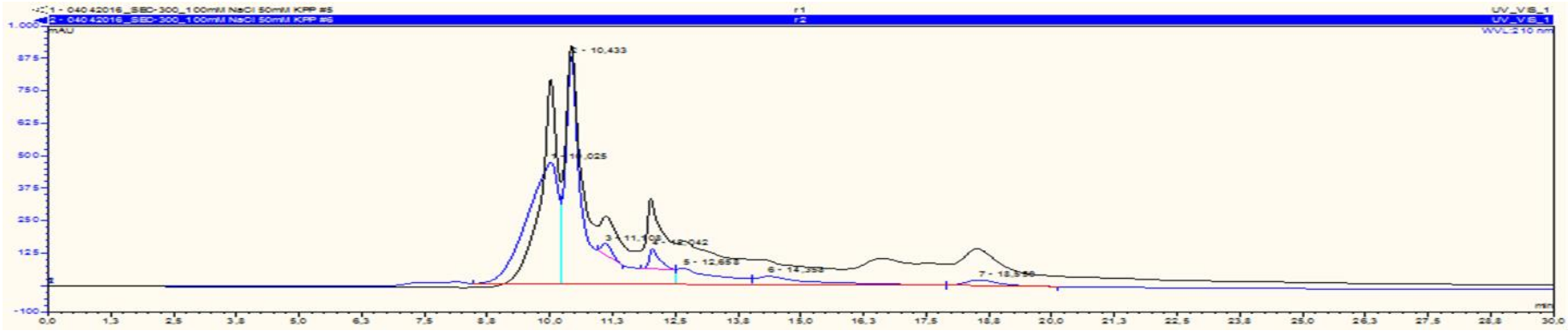

Figure 8. SEC chromatogram of 10 mL lignin solution + *T. versicolor* crude extract. The reference material, lignin solution itself, is shown in black.

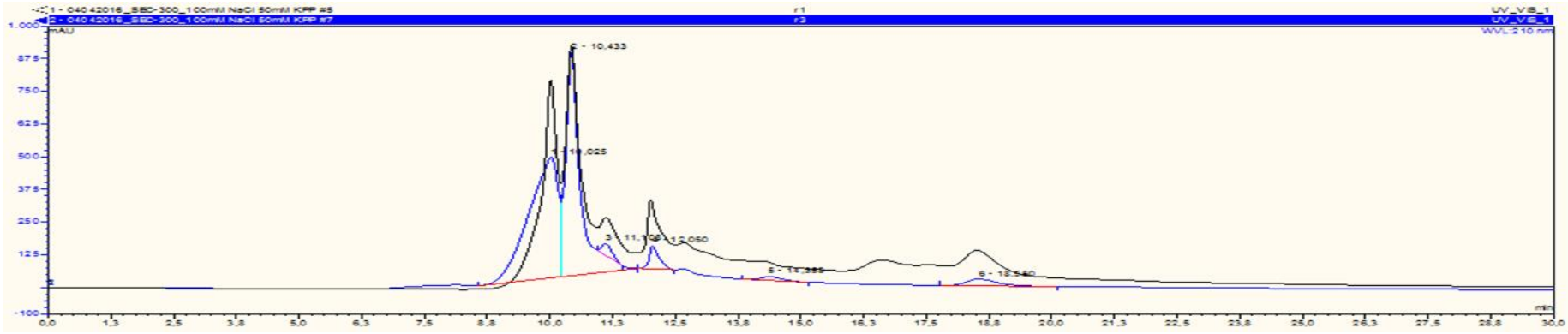

Figure 9. SEC chromatogram of 10 mL lignin solution + 10 mg cellulose + *T. versicolor* crude extract. The reference material, lignin solution itself, is shown in black.

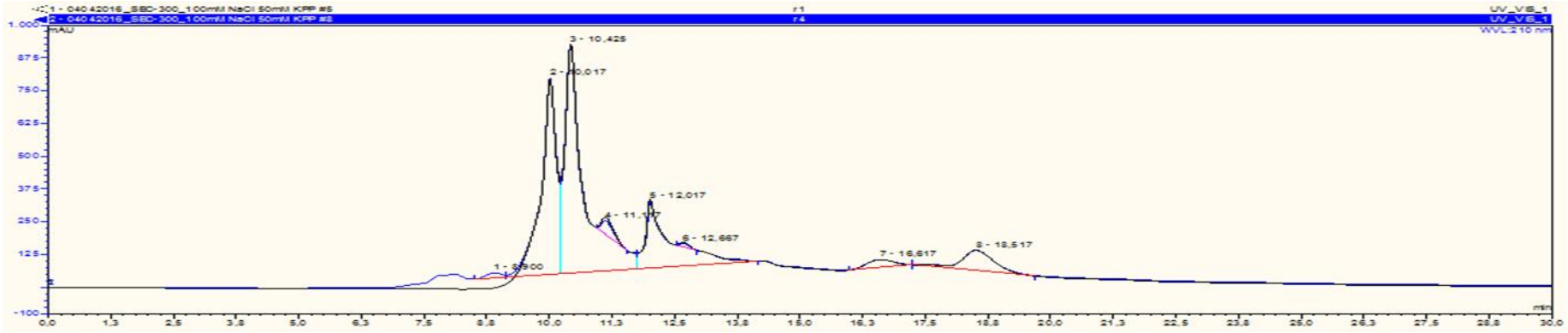

Figure 10. SEC chromatogram of 10 mL lignin solution + *P. chrysosporium* crude extract. The reference material, lignin solution itself, is shown in black.

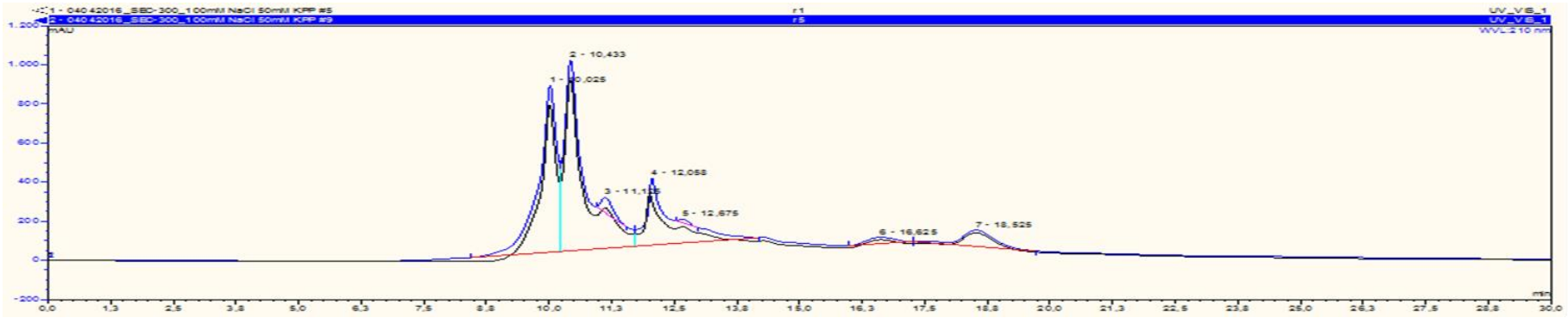

Figure 11. SEC chromatogram of 10 mL lignin solution + 10 mg cellulose + *P. chrysosporium* crude extract. The reference material, lignin solution itself, is shown in black.

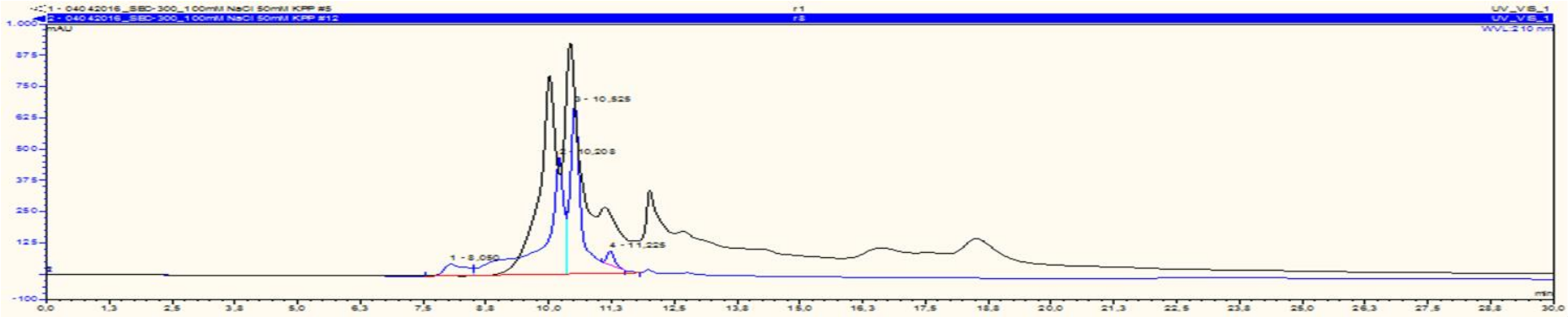

Figure 12. SEC chromatogram of 10 mL lignin solution +laccase. The reference material, lignin solution itself, is shown in black.

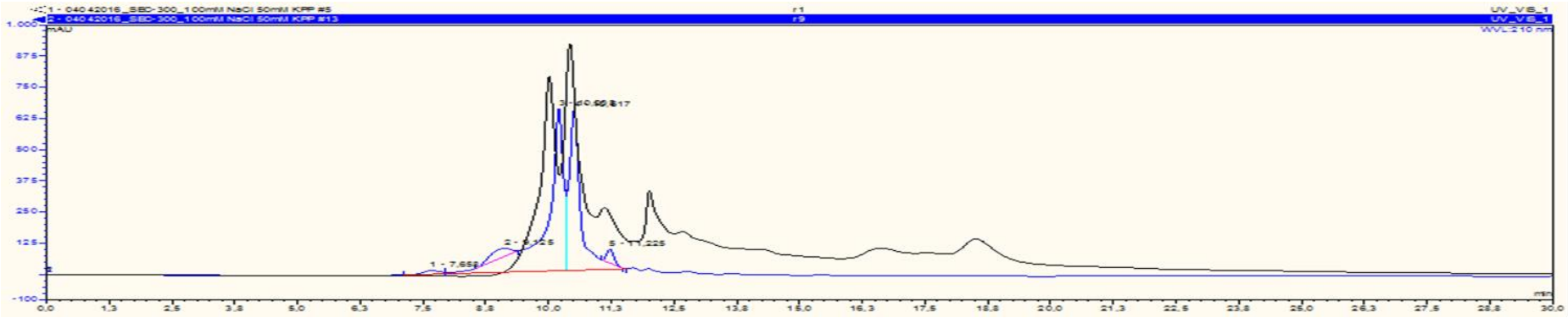

Figure 13. SEC chromatogram of 10 mL lignin solution + laccase + GDH + glucose. The reference material, lignin solution itself, is shown in black.

4.3. DISCUSSION

The potential of lignocellulosic materials as a source of raw materials for numerous bioprocesses is recognized, but their usage is still challenging. As described in chapter 2.1., lignin notably limits the use of those widespread materials (Palonen, 2004; Wyman et al., 2005; Dashtban et al., 2009). Kraft lignin, the residual material of chemical pulping processes, was used for the purposes of this research.

An important point in the research was to properly dissolve the lignin and to obtain a sample solution with reproducible quality. To this end, incubation of the lignin in 50 mM NaOH proved to be the most efficient strategy, which corresponds to data reported in the literature (Lange et al., 2013). However, it has to be noted that ultrasound treatment was used to improve the dissolution process.

Initially, the activity of isolated enzymes commonly associated with lignin degradation was tested. Laccases are considered to be among the most efficient enzymes in ligninolytic fungi. Their especially broad substrate spectrum allows them to act on a variety of different lignin compounds. Here, kraft lignin incubated with laccase was investigated by UV/VIS spectroscopy. Figure 3 shows a UV/VIS spectrum of untreated lignin. The absorbance in the near- and far UV range is due to the specific absorbances of the individual non-conjugated phenolic groups present in lignin. Absorbance maxima were observed at 280 nm and 200 nm, which is in agreement with data reported for other technical lignins. Incubation of lignin with laccase led to pronounced peak shifts and a general increase in absorbance. Absorbance increase is probably the consequence of different polymerization reactions. This is supported by the fact that during the trial, precipitation was observed. This is in accordance with the literature; laccase is involved not only in lignin degradation but also different polymerization reactions in lignin synthesis (ten Have and Teunissen, 2001; Periasamy et al., 2011; Christopher et al., 2014; Mäkelä et al., 2015; Abdelaziz and Hulteberg, 2016). While it is not possible to identify individual products using UV/VIS spectroscopy, these observations are indicative for extensive oxidation reactions. It is well known that laccases can greatly extend their product scope through the use of diffusible redox mediators, which can also oxidize non-phenolic lignin constituents. As stated in the literature, when molecular oxygen is reduced to two molecules

of water, free radicals are first formed, which further cause chain oxidation reactions. In this way, the formed mediators are released from the enzyme and reduce a wide range of high redox potential substrates (Palonen, 2004; Christopher et al., 2014; Mäkelä et al., 2015). Since it was unclear whether the processed kraft lignin contains low molecular weight compounds that can potentially act as redox mediators for laccase, the artificial redox mediator 2,5-dimetoxy-1,4-benzoquinone was added to the reaction. In presence of the mediator, the UV/VIS spectrum showed pronounced changes. The absorbance around 200 nm decreased significantly, while a new spectroscopic maximum at 290 nm was observed (Figure 3, green line). Biodegradation of lignin with laccase-mediator systems may facilitate different modes of redox reactions, including electron transfer, radical hydrogen atom transfer and ionic mechanisms (Christopher et al., 2014). Without further product analysis, the exact product profile of the reaction under investigation remains unclear. However, it was suggested that during laccase-mediator reactions both polymerisation and depolymerisation reactions compete, which may, at least partly, explain the loss of absorbance in the sample. From this experiment, it can be concluded that laccase from *T.versicolor* efficiently oxidised kraft lignin, and that an external redox mediator is needed to enhance the reaction.

Next, the interaction of kraft lignin with lytic polysaccharide monooxygenase 9C (LPMO9C) from the Ascomycete *N. crassa* was tested. Fungal LPMOs are copper-containing enzymes known to oxidatively degrade cellulose and hemicelluloses. In order to get activated, LPMOs require external electrons and an oxygen containing cosubstrate (O_2 or H_2O_2) (Chylenski et al., 2019). It is well-known that small- and medium molecular weight lignin compounds can serve as electron donors for the LPMO reaction (Westereng et al., 2015). In addition, it was shown that a number of soluble phenolic lignin constituents can be recycled (reduced) by members of the GMC oxidoreductase enzymes once they are consumed by LPMO (Kracher et al., 2016). Such a mechanism greatly enhances the availability of these electron donors, and may improve the performance of LPMO. In the following, the effect of LPMO treatment on the kraft lignin was tested. Incubation of the lignin with LPMO9C alone led to subtle changes in the intensity of the UV/VIS spectrum of the lignin (Figure 4, red curve). No apparent peak shifts could be observed. However, it has been also shown that LPMOs may require only substoichiometric amounts of electrons, and may undergo several catalytic cycles per electron obtained. Thus, it is not expected that LPMO can modify (oxidise) a significant amount of the lignin unless a

suitable substrate is provided that would guarantee constant consumption of electrons by the LPMO. Addition of cellulose to this reaction, however, did not lead to notable changes in the UV/vis spectrum. In the following, a redox mediator 2,5-dimetoxy-1,4-benzoquinone that was previously shown to interact with the LPMO9C was added to the reaction. Under these conditions, there was observed an increase in the lignin absorbance and a peak shift from 280 to 285 nm. In view of these data, it can be concluded that a redox mediator is required to facilitate efficient electron shuttling between the lignin and the LPMO. Also, the presence of substrate is essential and provides an electron sink for the LPMO reaction.

Oxidoreductases of the GMC family are frequently associated with lignin degrading enzymes (Levasseur et al., 2013; Sützl et al., 2018). These enzymes use sugar substrates released during the degradation of cellulose to produce hydrogen peroxide. Hydrogen peroxide is a co-substrate for many lignin degrading enzymes, including lignin-, manganese- or versatile peroxidases and LPMOs. Here, we set out to test whether these enzymes can also modify lignin. Glucose dehydrogenase from *Glomerella cingulata* oxidises glucose while reducing the FAD cofactor to $FADH_2$. In the reductive half reaction, a number of quinones are reduced in an oxygen-independent reaction to reoxidize the flavin cofactor (Sygmund et al., 2011). As can be seen in Figure 5, GDH itself was inefficient in lignin degradation. The presence of glucose had only a marginal impact on the absorbance, which slightly increased. Therefore, it can be concluded that either the lignin did not serve as an electron acceptor of the GDH, or that this reaction was not sufficient to modulate the lignin structure and composition. CDH is a redox enzyme that harbours both an FAD and a heme *b*, both of which are located on separate domains (Tan et al., 2015). CDH is the proposed native interaction partner of LPMOs. CDH reduces the LPMO active site through the transfer of single electrons to the copper active site. CDH is also among the few enzymes capable of direct electron transfer to macroscopic electron acceptors such as electrode surfaces (Scheiblbrandner and Ludwig, 2020). CDH alone did not exert any observable effect on the kraft lignin, as evidenced in Figure 6. CDH does not degrade lignin which corresponds to later. However, another proposed function of CDH suggested a participation in a Fenton-type chemistry, which creates highly reactive hydroxyl radicals through the reduction of ferric compounds. The generated ferrous species can react with hydrogen peroxide to produce highly reactive hydroxyl radicals. These radicals are potent oxidants and randomly attack the lignocellulose structure. At low pH, CDH is capable of

reducing Fe(II), while simultaneously providing low amounts of hydrogen peroxide (Kracher et al., 2020). Here, we added $FeCl_2$ to the CDH reaction to induce the formation of hydroxyl radicals. This approach resulted in a general increase in absorbance of the lignin fraction, indicating polymerization reactions (Figure 7). Of note, this effect was also observed in the absence of the CDH substrate cellobiose. It is feasible that the lignin itself could reduce the CDH to induce a Fenton-type reaction. In presence of the cellobiose substrate, the observed increase of absorbance was much more pronounced. Figure 7 shows that the replacement of $FeCl_2$ by $MnCl_2$ did not induce the same effect. There is no significant difference between graphs, suggesting that the CDH could not efficiently reduce the manganese ion under the tested conditions.

In order to compare the activity of the investigated enzymes with the activity of crude extracts from the native lignin degrader *T. versicolor* and *P. chrysosporium,* these strains were grown in submerged cultures using cellulose as the main source of carbon. In order to induce the expression and secretion of enzymes involved in lignin degradation, kraft lignin was added to the cultivation in a different set of experiments. The cultures were sampled for total protein, manganese peroxidase, laccase and CDH activity. Total soluble proteins throughout the cultivation reached a maximum of 139 mg L^{-1} for *T. versicolor* and 294 mg L^{-1} for *P. chrysosporium*. The addition of lignin did not cause notable changes in the protein concentration throughout the cultivation. The growth of *T. ver*sicolor was significantly faster than those of *P. chrysosporium.* Maximal protein concentrations in the supernatant of *T. versicolor* were reached after 6 days, compared to 11 days for *P. chrysosporium.* The activity of lignin degrading enzymes was low (below 100 U mL^{-1}) for both fungi and under all tested culture conditions. The presence of lignin in the growth medium had no apparent influence on the concentration of ligninases. Despite the lower concentrations of secreted protein, *T. versicolor* produced more lignin degrading enzymes than *P. chrysosporium.* A notable difference between the two fungi is the absence of laccase in the supernatant of *P. chrysosporium.* In contrast, the latter fungus produced high amounts of CDH, which is involved in the degradation of cellulose. After 11 days of cultivation, 890 U L^{-1} of CDH activity were detected in the supernatant of *P. chrysosporium*. In view of the low measured enzyme concentrations, an optimization approach would be required to obtain higher enzyme yields. A feasible strategy could be to vary the nitrogen/carbon ratio in the cultivation medium, to

increase the cellulose concentration or to test different inducer. However, this was beyond the scope of this thesis. All cultivations were stopped after 12 days and supernatants harvested by centrifugation. Since the overall protein concentrations were low, sample solutions were concentrated in order to minimize the amount of crude extract that had to be used in subsequent lignin conversion experiments. Set of reactions was conducted (see chapter 3.2.7.). In order to visibly observe the possible lignin degradation, the samples for both conversion experiments, the soluble lignin fractions were resolved by size exclusion chromatography on an HPLC system.

Figure 8 shows SEC chromatograms of kraft lignin (black) and kraft lignin after treatment with *T. versicolor* supernatant. Kraft lignin showed prominent peaks eluting at 10 min, and minor peaks eluting at 16-20 min. This indicates that a large fractions of the lignin had a high molecular weight. There is a visible decrease in intensity in the largest and smallest fractions of the lignin upon incubation with of *T. versicolor* supernatant (blue). This indicates that enzymes in the *T. versicolor* supernatant attack both small and high molecular weight components in the lignin fraction. *T. versicolor,* which also produced laccase, is believed to be one of the most effective fungus in the lignin degradation (Pothiraj et al., 2006; Dashtban et al., 2009; 2010).

Although *P. chrysosporium* is also believed to be efficient in lignin degradation, we did not observe a comparable activity of its supernatant on lignin. During the cultivation of *P. chrysosporium* in presence of lignin, higher MnP activity was observed; however, no significant difference can be seen between the reference chromatogram of kraft lignin and the peaks obtained from the reaction with *P. chrysosporium* (Figure 9 and 10).

Lastly, we also tested the effect of laccase treatment of Kraft lignin using SEC. Figure 12 confirms the high efficiency of lignin degradation by laccase. Especially the fractions of Kraft lignin with the smallest molecular weight (elution times between 11 and 20 min) completely disappeared, why pronounced peak shifts in the largest fractions were observed. This indicates that, under the conditions tested, polymerisation reactions that consume small molecular weight components dominated over degradation reactions. Addition of GDH to enhance the recycling of small molecular weight compounds had little or no impact on the reaction, as is shown in Figure 13. The stated corresponds to results obtained during the first conversion experiment.

5. CONCLUSIONS

Based on the obtained results in this thesis, the following can be concluded:

1. Laccase was the most efficient enzyme for the degradation of kraft lignin. Addition of low amounts of the redox mediator 2,5-dimetoxy-1,4-benzoquinone to the reaction contributed significantly to laccase's activity and modified the UV/VIS spectrum. Addition of different redox mediators or redox mediator concentrations may allow to modulate the specificity of the laccase, which would be interesting in technological applications. However, it has to be noted that thorough product analysis is required to determine which lignin constituents are oxidized or polymerized by the laccase.

2. In comparison, other auxiliary enzymes barely act on lignocellulosic polymers, but they cooperate with other enzymes in order to enhance depolymerisation of lignocellulose. However, when using CDH in combination with Fe (II) the spectral features of the lignin changed notably, indicating oxidation reaction. The UV/VIS profiles of laccase-modified and CDH-modified kraft lignin fraction were notably different, suggesting that these enzymes generate different product profiles. In case of laccase, competition of polymerisation/depolymerisation reactions may dominate the reaction, whereas the CDH-derived hydroxyl radicals are supposed to oxidise lignin randomly.

3. The white-rot fungus *T. versicolor* is efficient in lignin degradation, which is probably the result of the high observed laccase activity.

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