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Laccase Enzymology in Relation to Lignocellulose Processing

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Ph.d. Dissertation
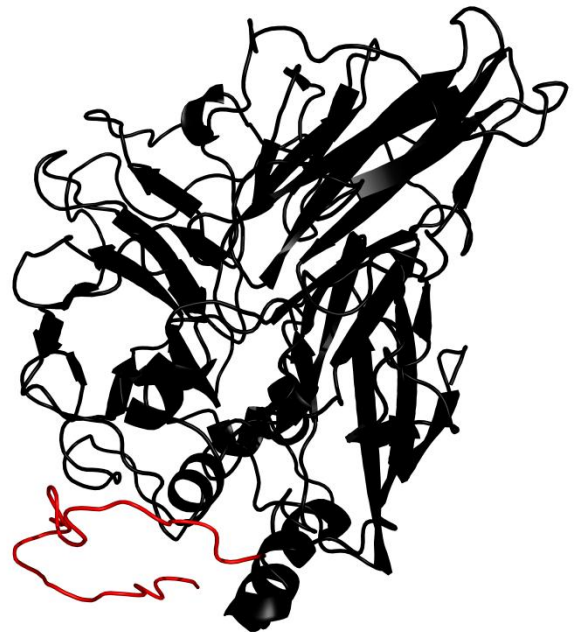
DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

PREFACE

This thesis is submitted in fulfillment of the requirements for the Doctorate (PhD) degree at the Technical University of Denmark (DTU). The work presented in this thesis was carried out from September 2008 until July 2012, at the Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering at the Technical University of Denmark. The work was accomplished under the supervision of Professor Anne S. Meyer and Professor Jørn D. Mikkelsen.

The PhD study was financed by the Danish Council for Strategic Research, project no. 2104-05-0017 and the Technical University of Denmark (DTU).

I would like to thank my supervisors, Anne and Jørn, for becoming their PhD student that memorable day in 2008. I am grateful for their support and the discussion-based input in polishing and shaping the scientist I became. Of all, I enjoyed the final stage of my PhD study the most, when research and non-work related laughter and joy (followed by a strong coffee intake), became one.

I would like to acknowledge Thomas Hvid Andersen from TERRANOL A/S for his professional assistance during DNA isolation and for sharing ideas and discussions also outside the scientific field. I would also like to thank Martin Willer and João Almeida for their friendship and help in handling toxic-compound-experiments for me while I was pregnant.

I want to thank all my colleagues at the Center of Bioprocess Engineering for creating a friendly and inspiring working environment. Special thanks go to Dayang Zaidel for her electrifying laughter and kind soul, Malwina Michalak for her down to earth perception of the world and scientific discussions, and Søren Brander for our laccase related discussions.

Na koniec chciałabym podziękować moim rodzicom i siostrze. Dziękuje za wszystkie, acz kosztowne rozmowy telefoniczne, które przez te lata wykonaliśmy i za nieskończenie wielkie wsparcie, szczególnie w tych "burzowych" chwilach.

Last, but not least, thanks to my fiancé, Stefan Neumeyer for his big support, encouragement, and taking care of Rasmus when I needed to spend my time on the PhD project, and to my little son Rasmus for bringing joy and happiness to my world.

Love of a family is life's greatest blessing

Unknown poet

Anna Sitarz

Lyngby, July 2012

Several studies have indicated that cellulase action on cellulose fibers and their conversion to glucose is inhibited by lignin and lignin-derived phenolic substances, which are released during the pretreatment of lignocellulosic biomass. A prerequisite for optimization of the cellulose-to-glucose conversion is to either get rid of the inhibitory substances or to alter them in a way, so they no longer decrease the action of cellulases.

The main focus in the present work was the investigation of the influence of the enzymes that are being expressed from the white-rot fungi when lignin was present in the cultivation broth, on the cellulase catalyzed hydrolysis of pretreated biomass, and to understand the mechanism of their action on phenolic substances.

In this thesis, 44 fungi from the genus *Alternaria, Fusarium, Memnoniella, Stemphylium, Ulocladium, Ganoderma, Trametes*, and *Polyporus* were evaluated for their ability to grow on lignocellulosic material, such as sugarcane bagasse – a competitive substrate for grain bioethanol. From this investigation, four white-rot fungi (*Ganoderma lucidum, Trametes versicolor, Polyporus brumalis, and Polyporus ciliatus*), were selected for the growth on lignin (lignin alkaline) and investigated for production of enzymes under such conditions (**Paper I**).

G. lucidum was found to produce high amounts of laccase which corresponded to its exceptional growth on lignocellulosic substrate and lignin. This observation led to a hypothesis that this particular laccase might act in a synergistic way with cellulase preparations and yield in higher cellulose-to-glucose catalyzed hydrolysis. To test this hypothesis the laccase-rich crude extract from *G. lucidum* was added to the cellulase catalyzed hydrolysis of cellulose from the pretreated sugarcane bagasse (**Paper I**). A positive outcome of this reaction, a 17% increase in the total glucose yields during cellulase catalyzed hydrolysis of cellulose, led to amplification of laccase gene and its expression in *Pichia pastoris* (**Paper II**). This approach was directed into obtaining a monocomponent laccase enzyme and to prove that the higher yields of cellulose-to-glucose conversion are partly due to the presence of laccase, and are not caused by the other proteins, present in the laccase-rich crude protein extract.

The addition of the laccase from *G. lucidum*, expressed in *P. pastoris* resulted in a total increase in the glucose yields by 20 and 33% depending on the cellulase cocktail preparation. This discovery is significant considering the fact that the cellulase cocktail preparations, namely Cellic®CTec1 and Cellic®CTec2, are improved in respect to phenolic-derived, and end-substrate inhibitors.

Additionally, the molecular dynamics simulations (MD) of the obtained amino acid sequence of the laccase from *G. lucidum* highlighted a potential mechanism of laccase detoxification of the cellulase-pretreated-biomass-derived inhibitors (**Paper II**).

The mechanism of laccase reaction on the phenolic substrates was further evaluated by the literature study of the reactions that take place in the catalytic pocket of this oxidoreductases and the structural alteration that can lead to a more robust, or completely inactive, laccase (**Review paper**).

Adskillige studier har vist, at cellulase katalyseret nedbrydning af cellulose til glucose inhiberes af lignin og lignin-afledte phenoliske stoffer, som frigives under forbehandling af lignocellulose-biomasse. En forudsætning for optimering af enzymkatalyseret cellulose-til-glucose konvertering er derfor enten at fjerne de inhibitoriske stoffer eller at ændre dem på en sådan måde, at de ikke inhiberer den cellulase katalyserede cellulose-nedbrydning.

Det primære fokus i det foreliggende PhD arbejde var at identificere et eller flere enzymer, der produceres af hvide rådsvampe under deres vækst på lignin, med det formål at undersøge, hvorvidt sådan enzymaktivitet kan booste cellulase katalyseret hydrolyse af forbehandlet biomasse, og desuden at forstå mekanismen for disse enzymers katalytiske oxidation af phenoliske stoffer.

I denne afhandling blev 44 svampe fra slægten *Alternaria, Fusarium, Memnoniella, Stemphylium, Ulocladium, Ganoderma, Trametes*, og *Polyporus* evalueret for deres evne til at vokse på lignocellulosemateriale, såsom sukkerrørsbagasse. Sukkerrørsbagasse er den fiberholdige masse, som er tilbage efter ekstraktion af sukker fra sukkerrør, og blev valgt som et lignocellulosesubstrat, idet det vurderes som et konkurrencedygtigt substrat til cellulose-baseret ethanol produktion. Fra denne undersøgelse blev fire hvide rådsvampe (*Ganoderma lucidum, Trametes versicolor, Polyporus brumalis*, og *Polyporus ciliatus*), udvalgt til vækst-studier på lignin (alkalisk lignin og lignocellulose) og undersøgt for produktion af phenol-oxidativ enzymaktivitet (**Paper I**).

Blandt disse blev *G. lucidum* identificeret som den bedste producent af laccase aktivitet, et resultat som stemte overens med denne organismes fremragende vækst på lignocellulose-substratet og lignin. Denne observation førte til den hypotese, at den særlige laccase, som *G. lucidum* producerer under disse vækstbetingelser, kan have synergistisk effekt på cellulase katalyseret nedbrydning af lignocellulose, og give højere udbytte i cellulose-til-glukose katalyseret hydrolyse. For at teste denne hypotese, blev det laccase-rige råekstrakt fra *G. lucidum* tilsat til cellulase katalyseret hydrolyse af cellulose i forbehandlet sukkerrørsbagasse (Paper I). Der blev opnået et positivt resultat af denne reaktion i form af en 17% stigning i det samlede glukose udbytte ved den cellulase katalyserede hydrolyse af cellulose. Det videre arbejde førte til succesfuld kloning og ekspression af det pågældende laccase gen fra *G. lucidum* i *P. pastoris* (**Paper II**). Denne fremgangsmåde blev valgt for at opnå produktion af monokomponent laccaseenzym, med henblik på at bevise, at det højere udbytte af cellulose-til-glucose omdannelse med det laccase-rige råekstrakt fra *G. lucidum* skyldtes tilstedeværelsen af laccase-enzymet, og ikke vat forårsaget af andre proteiner som var til stede i laccase-rige råekstrakt.

Tilsætning af laccase fra *G. lucidum*, udtrykt i *P. pastoris*, til cellulase-katalyseret nedbrydning af sukkerrørsbagasse resulterede i en samlet stigning i glucoseniveauer udbytter ved 20 og 33% afhængigt af cellulase-præparatet. Denne opdagelse er signifikant i betragtning af den kendsgerning, at de benyttede cellulasepræparater, nemlig Cellic ® CTec1 og Cellic ® CTec2, er de nuværende industrielle state-of-the art præparater.

Molekylære simuleringer (MD) af enzym-strukturen, baseret på den opnåede aminosyresekvens af laccasen fra *G. lucidum*, indikerede muligheden for docking af *p*-coumarinsyre i enzymets aktive site, dvs. at direkte interaktion med phenol er en potentiel mekanisme som kan forklare laccase-katalyseret afgiftning (via oxidation af phenol inhibitorer) under cellulase katalyseret nedbrydning af forbehandlet biomasse (**Paper II**).

Mekanismen for laccases reaktion på phenoliske substrater blev yderligere vurderet udfra en litteratur-baseret undersøgelse af de reaktioner, der finder sted i den katalytiske lomme af denne type oxidoreduktaser. Ligeledes blev de strukturelle ændringer, der kan føre til hhv. mere robuste eller inaktive laccaser kortlagt (**Review paper**).

The PhD thesis is based on the work presented in the following research papers:

I. Identification of a highly active laccase from a novel strain of *Ganoderma lucidum*.
    A.K. Sitarz, J.D. Mikkelsen, P. Højrup, A.S. Meyer
    Enzyme and Microbial Technology

II. A novel laccase from *Ganoderma lucidum*, recombinant enzyme expression, and the boosting of lignocellulose biomass degradation
    A.K. Sitarz, M. Łężyk, J.D. Mikkelsen, A.S. Meyer
    Applied and Microbial Technology

III. Structure, functionality, and tuning laccases for their industrial applications.
    A.K. Sitarz, J.D. Mikkelsen, A.S. Meyer
    Current Opinion in Biotechnology

The submission of the research papers has deliberately been held back due to the pending patent application

IV. Patent application
    Laccase from *Ganoderma lucidum* CBS229.93

TABLE OF CONTENT

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The main purpose of this chapter is to summarize the main hypothesis and objectives that were the basis for the PhD study. The main goals for each of the research papers are therefore presented below:

Paper I

Identification of a highly active laccase from a novel strain of *Ganoderma lucidum.*

Hypotheses

❖ The hypothesis for **Paper I** was that if white-rot fungi are capable of growing and destroying lignin, then they must produce enzymes that are responsible for lignin modification or its degradation. Most probably, such enzymes catalyze the redox reactions

❖ The enzymes involved in the lignin degradation or detoxification of the fungal growth environment from the growth limiting factors are suspected to be expressed during the organism cultivation on lignin (LA – lignin alkaline) and/or lignin containing lignocellulosic materials (SCB – sugarcane bagasse)

❖ These enzymes may therefore be useful in boosting lignocellulose degradation during cellulase catalyzed hydrolysis, by either directly modifying the lignin to allow better access for the cellulases, or – more likely – by catalysis of lignin derived phenols to lesser inhibitory compounds. As the result of that enzymatic action (cellulase plus laccase) we expect to obtain a higher release of monosaccharides which could be used in the bioethanol production

Objectives

❖ To test the hypotheses, forty fungal strains (described in detail in **Paper I**), selected on *a priori* knowledge of their lignocellulosic habitat conditions, would be evaluated for their ability to perform growth when LA and/or SCB were added to the cultivation medium in order to stimulate their lignin modifying enzyme production

❖ The proteins released during the fungal growth would be evaluated for their activity to oxidize phenolic and/or phenolic-derived substrates (that the lignin structure is built of)

❖ The protein that shows activity towards the tested phenolic-derived substrates would be analyzed for its amino acid sequence (MALDI-TOF/MS analysis) and identified to which class of oxidizing enzymes it belongs (lignin peroxidase, manganese peroxidase, laccase) by amino acid sequential blasting in the protein database

Paper II

A novel laccase from *Ganoderma lucidum*, recombinant enzyme expression, and the boosting of lignocellulose biomass degradation.

Hypotheses

❖ The cellulase action during hydrolysis of cellulose is retarded by lignin or inhibitory lignin derivatives (see section; *2.1.3.1 Fate of lignin – factors limiting the cellulolytic hydrolysis of biomass*). We expect that the enzymatic modification of these inhibitory substances by enzymes produced by white-rot fungi would boost cellulase catalyzed cellulose degradation

❖ In other words, the addition of the laccase-rich protein extract from *G. lucidum* to the cellulase catalyzed hydrolysis of the pretreated SCB would enhance the yields of released glucose **(Paper I),** and therefore we expect that a laccase activity is responsible for this phenomenon. The mechanism of obtaining increased glucose yields may follow either detoxification of the pretreated material, lignin degradation to smaller compounds that will not bind cellulases nonspecifically, or polymerization of small components to larger ones that will no longer be able to inactivate cellulases

Objectives

❖ To clone the laccase gene from *G. lucidum* and express it under the AOX1 promoter in a high copy number

❖ To add the expressed in *P. pastoris* laccase from *G. lucidum* to the same pretreated lignocellulose preparation as described in **Paper I**, and evaluate its effect on the overall glucose yields released during cellulase catalyzed hydrolysis of lignocellulose

❖ To evaluate the optimal temperature and pH conditions for obtaining maximal yields of glucose

❖ To perform molecular dynamic (MD) simulations of the cloned and expressed in *P. pastoris* laccase (LacGL1) with the most dominant phenolic inhibitor present in the steam-exploded hydrolysates of the sugarcane bagasse

- ❖ To evaluate the structural changes in the substrate binding site of the LacGL1 laccase in comparison to a well known and studied laccase model structure from *Trametes versicolor,* and to propose a hypothetical mechanism for the increased total glucose yields during cellulase catalyzed hydrolysis of the pretreated SCB

Research Review

Structure, functionality, and tuning laccases for their industrial applications.

Hypotheses

- ❖ Laccases are suspected to take part in the delignification/detoxification process of the wood (see section; *3.2 Lignin modifying enzymes – why focus on laccases?*). Therefore, if the 3D model structure of the expressed (*G. lucidum*) laccase shows structural similarity to the well studied laccase from *Trametes versicolor* then it could indicate that the oxidative catalysis is comparatively good for the *G. lucidum* enzyme.

- ❖ The exact mechanism of laccase action in lignin degradation/depolymerization is not known. Therefore, by trying to understand in detail the structural features and the step-by-step catalytic mechanism, it would be easier to interpret the obtained results (**Paper I** and **Paper II**) and the possible unique features of *G. lucidum laccase* (**Review paper)**.

Objectives

- ❖ To understand the mechanism and specific sub-reactions that take place in the catalytic pocket of laccases (reduction of dioxygen to water) and how that process/mechanism can influence or contribute to lignin depolymerization

- ❖ To understand how the electrons, that are abstracted from phenoxy radicals, migrate within the copper centers that are responsible for catalysis (T1-Cys-His-T3β pathway)

- ❖ To understand what happens to the substrate once it is being oxidized by laccases (oxidation of phenolic and non-phenolic substrates)

- ❖ To focus on the laccase features, such as the redox potential that define how good the laccase is in potential degradation/detoxification of lignin

- ❖ To focus on laccase structural regions that improve enzyme's performance, such as the substrate binding loops, amino acids that coordinate to the T1 and T2/T3 Cu sites

- ❖ To focus on laccase structural regions that decrease or even totally inactivate the enzyme, such as the C-terminal region

- ❖ To understand the structural features of laccases (fold and copper binding) and their potential role in lignin depolymerization/degradation processes

INTRODUCTION

The biofuel production from the lignocellulosic material is an alternative to the rising demands for oil in the transportation sector, especially in the USA and Asia (India, China). The need for higher ethanol production is caused by an increase number of vehicles per citizen, booming infrastructure, high prices for the depleting fossil oil, and the desire of the politicians to become independent of the foreign oil suppliers (Edenhofer, 2011).

The world fuel production by feedstock ranges from 61% for sugar crops (e.g. sugarcane bagasse, sugar beet) and 39% for grains (e.g. corn), with an area distribution as depicted in Fig. 1.

Figure 1. The world's fuel ethanol production prognosis for 2013 (Berg and Licht 2010, Ministry of Economy, Trade and Industry, Japan).

The biofuel production sector uses lignocellulosic materials to produce ethanol. However, the nature of the lignocellulose is complex and resistant to the enzymatic attack. Therefore, the lignocellulosic biomass needs to be subjected to pretreatment. Pretreatment is a process where the structure of lignin, cellulose and hemicellulose is altered, in order to increase the surface area of the material, leading the same to an easier access to the cellulose and hemicellulose fibers. These fibers can, in turn, be hydrolyzed by cellulases to monosaccharides. Glucose, and now also xylose, are the two most important carbon sources for microbial fermentation where the metabolic end product is ethanol.

The main obstacle to the cellulolytic conversion of cellulose to glucose is the presence of lignin and lignin-derived phenolics that are released after pretreatment and which tend to retard the enzymatic conversion (Palonen et al. 2004; Selig et al. 2007). Despite the significant progress, recently been made with respect to improving the cellulolytic enzyme blends and minimizing the

cellulolytic enzyme adsorption to lignin by development of integrated pretreatment systems for removing the lignin from pretreated biomass, there is a surprising scarcity of work on enzymatic modifications of lignin and lignin-derived compounds to improve lignocellulose processes (Koo et al. 2012; Yang et al. 2012).

In nature, there exist microorganisms that can efficiently degrade lignin in wood. These microorganisms, namely white-rot fungi which are proven to be the best lignin degraders (Kawase 1962), can completely devoid wood of lignin. Their enzymes could therefore be of a big value for further improvements of the cellulolytic enzymes blends for the biofuel production and make the cellulose-to-glucose conversion less laborious.

COMPOSITION OF LIGNOCELLULOSIC MATERIAL

In a process of understand the lignin degradation mechanism and the obstacles that the microorganisms need to overcome in order to get the access to the plant polysaccharides, one needs to learn and be aware of the structure of the lignocellulosic material.

2.1   The Plant Cell Layers

The plant cell wall consists of several layers (Fig. 2.1 A), which include; middle lamella (ML), primary wall (P), inner layer of the secondary wall (S_1), middle layer of the secondary wall (S_2), inner layer of the secondary wall (S_3), and wart layer (W) (Sjöström, 1993). These walls provide mechanical strength, but also have ability to expand allowing the plant cells to grow and divide.
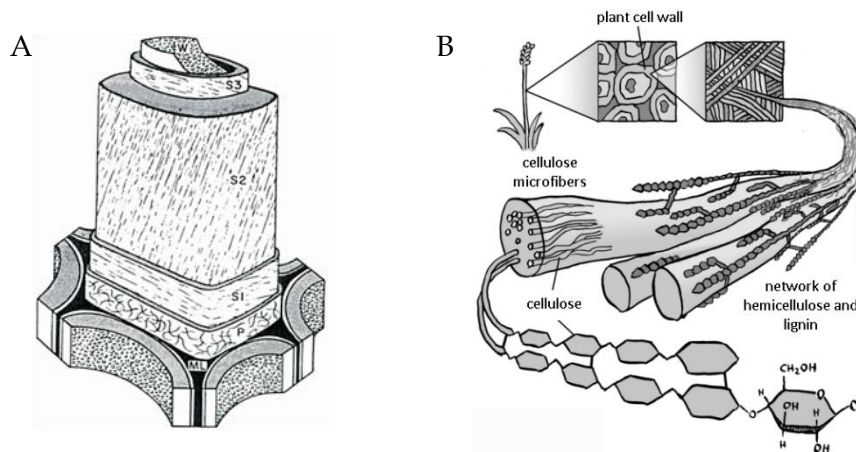
Figure 2.1 Representation of the plant cell walls; A) Wood cell structure showing the middle lamella (ML), the primary cell wall (P), and the secondary cell wall comprising of three layers (S_1, S_2, S_3) (Côté, 1967); B) The primary cell wall with cellulose, hemicellulose and fibers, embedded in lignin (Rosgaard et al. 2005).

The *middle lamella*'s function is to cement two adjoining cells of the cell wall together. Early in the growth development of the plant, it mainly contains pectic substances which eventually become highly lignified (Alberts et al. 2008). The *primary wall* is generally thin (0.1-0.2 μm), flexible, and extensible layer formed while the cell is growing. It mainly consists of cellulose, hemicellulose and protein and is completely embedded in lignin (Fig. 2.1 B). The *secondary wall* is a thick layer formed inside the primary wall, after the cell is fully grown. It is build of three layers namely S_1, S_2, and S_3 (as described above), which consist mostly of cellulose microfibrils wounded around the plant cell wall in a neatly organized matrix of hemicellulose and lignin with different orientations in each layer (Sjöström, 1993; Krogh, 2008). The warty layer is a thin amorphous membrane located in the inner surface of the cell wall in all softwoods and hardwoods,

containing deposits of still unknown, but characteristic for each species, composition (Sjöström, 1993).

2.1.1   Cellulose

Cellulose is a linear polymer of D-glucose monomers, linked by β-1,4-glucosidic bonds in which every second residue is rotated 180° around its longitudinal direction (Fig. 2.1.1 A). The linearity of the cellulose chain is stabilized through inter- and intramolecular hydrogen bonds (Fig. 2.1.1 A) (Gardner and Blackwell, 1974). Typically, 36 cellulose chains, with 10.000 D-glucose molecules each, having the same direction but a different starting and ending point, assemble in one microfibril (5-15 nm in diameter) (Krogh, 2008). The microfibril is greatly stabilized through inter- and intramolecular hydrogen bonds, which prevents cellulose from the microbial degradation and penetration by as small molecules as water (Lynd et al. 2002). From the structural point of view, microfibrils are heterogeneous in structure and comprise of highly ordered (crystalline) regions, which are alternated by less ordered (amorphous) regions (Sjöström, 1993). The amorphous regions of cellulose also have weaker intrermolecular hydrogen bonds. Microfibrils build up fibrils and finally fibers (Fig. 2.1.1 B). In addition to crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks, twists of microfibrils or voids such as surface micropores, large pits, and capillaries (Lynd et al. 2002).

In general, cellulose gives rigidity, but to confer the rigidity, e.g. to a stem, there is a need for other materials that can stick and glue the polysaccharides together. The stickness is caused by hemicellulose and the glue is lignin (Krogh 2008).
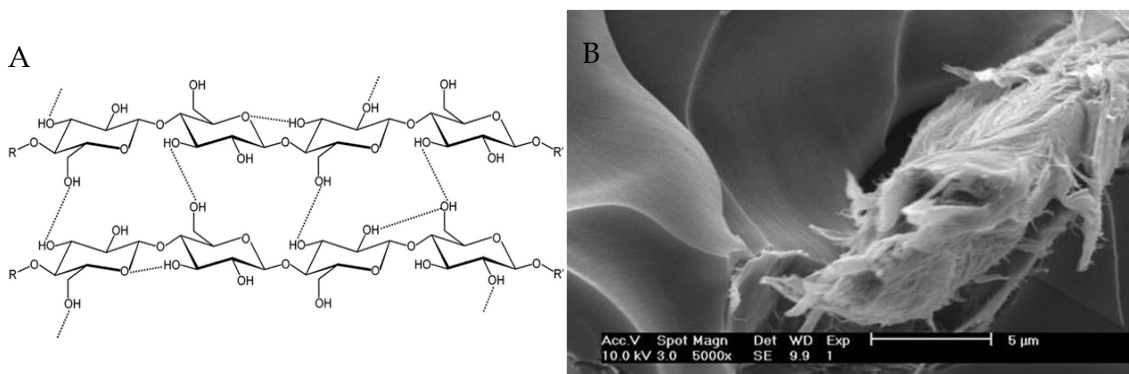
Figure 2.1.1 A) Inter- and intramolecular hydrogen bonds in cellulose; B) SEM image of the cellulose fibers, available at http://www.personal.psu.edu/tjr5043/extened%20text/index.html

2.1.2   Hemicellulose

Hemicellulose, in contrast to cellulose, is a heterogeneous polysaccharide and functions as an interlock to the cellulose microfibrils. Hemicellulose can be a branched chain polysaccharide of both different hexoses and pentoses. The hexose monomers are mainly D-glucose, D-mannose, D-galactose, and the pentose monomers are mainly D-xylose and L-arabinose (Krogh, 2008).

Another monomeric component that is also present in the hemicellulose is uronic acid (Sjöström, 1993). The hemicelluloses from different types of wood, e.g. softwood and hardwood, differ both in structure and amount (Table 2.1.2). In hardwood (angiosperm tree), e.g. birch, beech, poplar, aspen or oak, the predominant hemicellulose type is glucuronoxylan (O-acetyl-4-O-methylglucurono-β-D-xylan). On the other hand, softwood (gymnosperm tree) e.g. spruce, pine or fir is mostly (20%) composed of galactoglucomannan (Sjöström, 1993). In the gras family (poaceae), e.g. rice, wheat, oat and switchgrass is mainly glucoarabinoxylan as in hardwood, but in contrast the substituting glucuronic acid is not methylated (Carpita, 1996). For a more detailed description of grass hemicellulose (Carpita, 1996) and hardwood and softwood celluloses (Sjöström, 1993).

Table 2.1.2 The major hemicellulose components (Sjöström, 1993)

Hemicellulose type	Occurrence	Amount (%of wood)	Units	Composition Molar ratio	Linkage
Gallactoglucomannan	Softwood	5-8	β-D-Man$p$ β-D-Glc$p$ α-D-Gal$p$ Acetyl	3 1 1 1	1→4 1→4 1→6
(Galacto)glucomannan	Softwood	10-15	β-D-Man$p$ β-D-Glc$p$ α-D-Gal$p$ Acetyl	4 1 0.1 1	1→4 1→4 1→6
Arabinoglucuronoxylan	Softwood	7-10	β-D-Xyl$p$ 4-O-Me-α-D-Glc$p$A α-L-Ara$f$	10 2 1.3 6	1→4 1→2 1→3 1→3, 1→6
Glucuronoxylan	Hardwood	15-30	β-D-Xyl$p$ 4-O-Me-α-D-Glc$p$A Acetyl	10 1 7	1→4 1→2
Glucomannan	Hardwood	2-5	β-D-Man$p$ β-D-Glc$p$	1-2 1	1→4 1→4

β-D-Man$p$: β-Mannopyranose, β-D-Glc$p$: β-D-Glucopyranose, α-D-Gal$p$: β-D-Galactopyranose, β-D-Xyl$p$: β-D-Xylopyranose, 4-O-Me-α-D-Glc$p$A: 4-O-Methyl-α-D-Glucuronic Acid, α-L-Ara$f$: α-Arabinofuranose,

2.1.3   Lignin

Lignin derives from a Latin word *lignum* which means wood. Lignin is an integral part of secondary cell walls and its main function is to strengthen and conduct the water in the plant stems. Due to its hydrophobic nature and crosslinking with polysaccharides, lignin is a barrier for water to access the cell walls. Lignin is a heterogeneous biopolymer build of three phenylpropane units (Fig. 2.1.3); guaiacyl (from the precursor coniferyl alcohol), syringyl (from the precursor sinapyl alcohol), and *p*-hydroxyphenyl (from the precursor *p*-coumaryl alcohol).
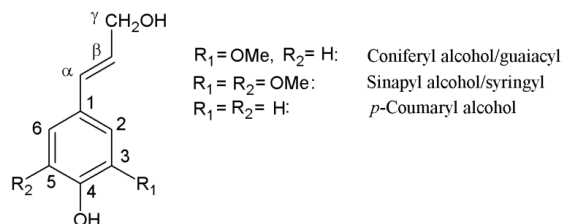
Figure 2.1.3 Schematic representation of three main monolignols forming a three-dimensional network of lignin.

Those three building blocks of lignin are generated from D-glucose through complex reactions catalyzed by various enzymes (Sjöström, 1993) and their amount differs significantly between hardwood, softwood and grasses. For example, there are 98% of guaiacyl units in softwood, *Cycas revoluta*, and 88% in *Podocarpus macrophyllus*. Softwood is almost completely devoid of syringyl units and instead contains *p*-hydroxyphenylunits (Gross, 1980). In contrast to softwood, hardwood contains a higher amount of syringyl units, whose content can vary from 40% (beech) to 84% (Eucalyptus globulus) (Choi et al. 2001; Pinto et al. 2005). The syringyl unit has two methoxy groups on the phenyl ring, whereas guaiacyl has one that confers different chemical properties and reactivity. One theory is that guaiacyl lignin keeps the lignin-cellulose more densely packed that syringyl lignin, which might be the reason for restricted hydrolysis of cellulose (Krogh, 2008). The radical coupling of the monomeric phenylropane units yield in a complex irregular matrix (Fig. 2.1.4) where the single phenylpropane units are joined together by variety of linkages. The model lignin presented by Adler (1977) consists of 16 phenylpropane units and it represents only a fragment of the lignin matrix (Sjöström, 1993). The high variability in the molecular structure of lignin is to a large degree due to the different resonance structures of the phenoxy radicals (Durbeej et al. 2003). The linkage type in lignin differs significantly in softwoods and hardwoods (Table 2.1.3), however, the most common linkages are β-O-4, β-5, β-β, β-1, 5-5, and 5-O-4 (Fig. 2.1.4; Table 2.1.3).

Lignin has also been reported to bind to hemicellulose and probably also cellulose in the so-called lingo-cellulose- or lignin-carbohydrate complex (Fig. 2.1.5) (Fenegel and Wegener, 1984; Sjöström, 1993) which might be responsible for structural enforcement of lignocellulose structure. The covalent binding between lignin and hemicellulose can be either of an ester or ether type (also glycosidic bonds are possible).
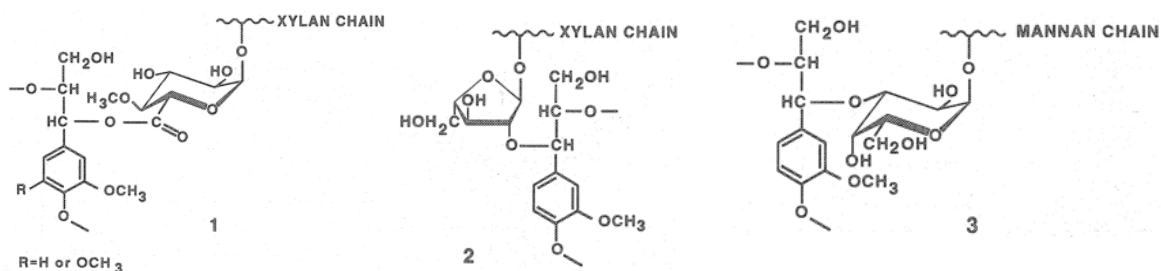
Figure 2.1.5 Examples of suggested lignin-carbohydrate bonds; 1) an ester linkage to xylan through 4-O-methyl glucuronic acid; 2) an ester linkage to xylan through arabnofuranose unit; 3) an ether linkage to galactoglucomannan through a galactopyranose unit.
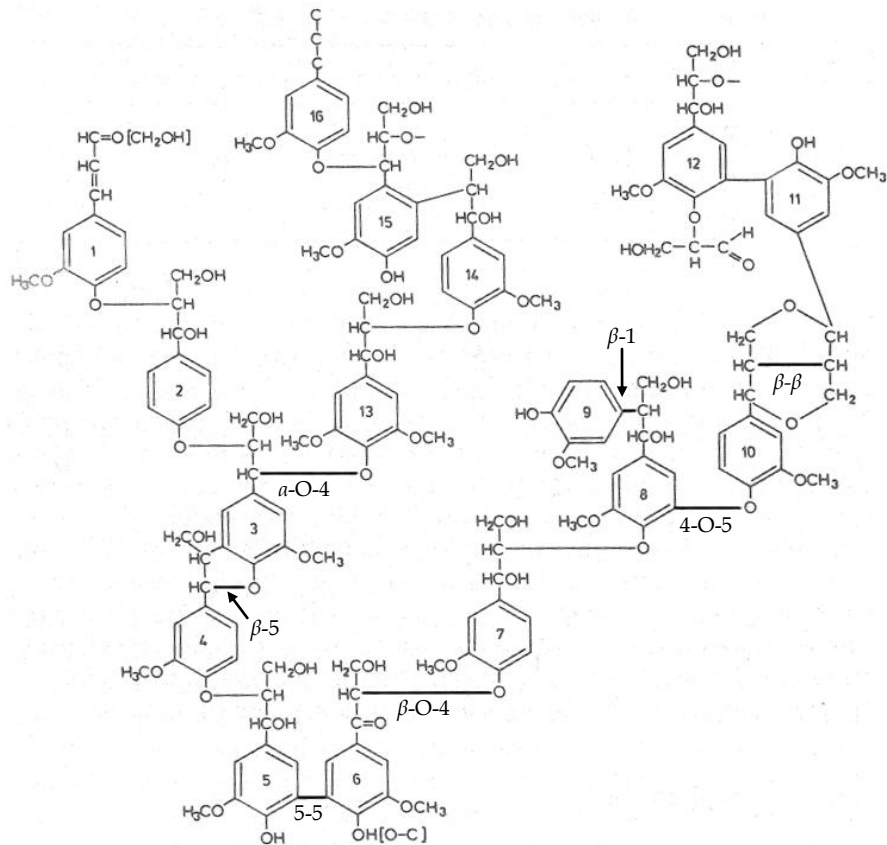
Figure 2.1.4 Adler's (1977) fragment of softwood model lignin. The most common linkages are bolded black.

Table 2.1.3 Percentage of different types of linkages connecting the phenylpropane units in lignin (Adler, 1977; Sjöström, 1993)

Linkage type[a]	Dimer structure	Percent of the total linkage	
		Softwood (spruce)	Hardwood (birch)
β-O-4	Arylglycerol-β-aryl ether	50	60[b]
α-O-4	Noncyclic benzyl aryl ether	2-8	7
β-5	Phenylcoumaran	9-12	6
5-5	Biphenyl	10-11	5
4-O-5	Diaryl ether	4	7
β-1	1,2-Diaryl propane	7	7
β-β	Linked through side chains	2	3
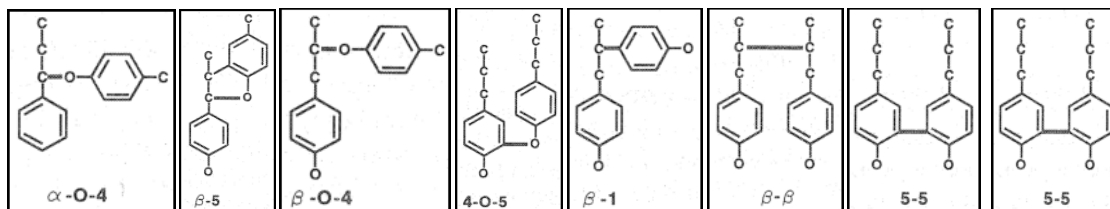
[a] The corresponding structures are presented below

[b] 40% of the arylglycerol-β-aryl ether structure is of guaiacyl type and 60% is of syringyl type

7

2.1.3.1   Fate of Lignin – Factors Limiting the Cellulolytic Hydrolysis of Biomass

The steam explosion is a pretreatment process where biomass is subjected to high-pressure steam followed by a rapid decompression, which forces the fibrous material to "explode" into component fiber and fiber boundless (Martin-Sampedro et al. 2011). The high pressure steam modifies the structure of the plant cell wall, resulting in a partial hydrolysis of hemicellulose, leaving cellulose, the rest of the hemicelluloses, and chemically modified lignin (Moilanen et al. 2011). Therefore, steam explosion is effective in opening up biomass structure and increasing accessibility of cellulosic fibers to cellulases (Zimbari et al. 2002). What exactly happens to the lignin structure is not known. Boussaid et al. (2000) suspects that lignin is not substantially removed in the pretreatment, but is rather momentarily solubilized and relocated. On the other hand Ximenes et al. (2010) claims that during the pretreatment process the lignin structure is undergoing significant changes, which mainly include its break down to small phenolic compounds. Additionally, from the work on aspen wood it has been concluded that during steam explosion processes almost simultaneous depolymerization and repolymerization reactions in lignin may take place by the formation of carbonium ions which is caused by the acidity created during the pretreatment (Li et al., 2007). Both reactions types are thought to originate from benzyl alcohol structures in the lignin.

Pretreatment of lignocellulosic materials is essential in order to enhance the cellulose surface area to the cellulases. However, the pretreatment step tends to generate the production of the inhibitory compounds that originate from the breakdown of cellulose, hemicellulose and lignin (Figure 2.1.3.1).

- 5-hydroxymethyl furfural (HMF)

- Phenolics (vanillin, 4-hydroxybenzaldehyde, ferulic acid, *p*-coumaric acid, syringaldehyde)

Cellulose

Lignin

Hemicellulose

- Furfurals
- 5-hydroxymethyl furfural (HMF)
- weak acids (acetic acid, formic acid, levulinic acid)
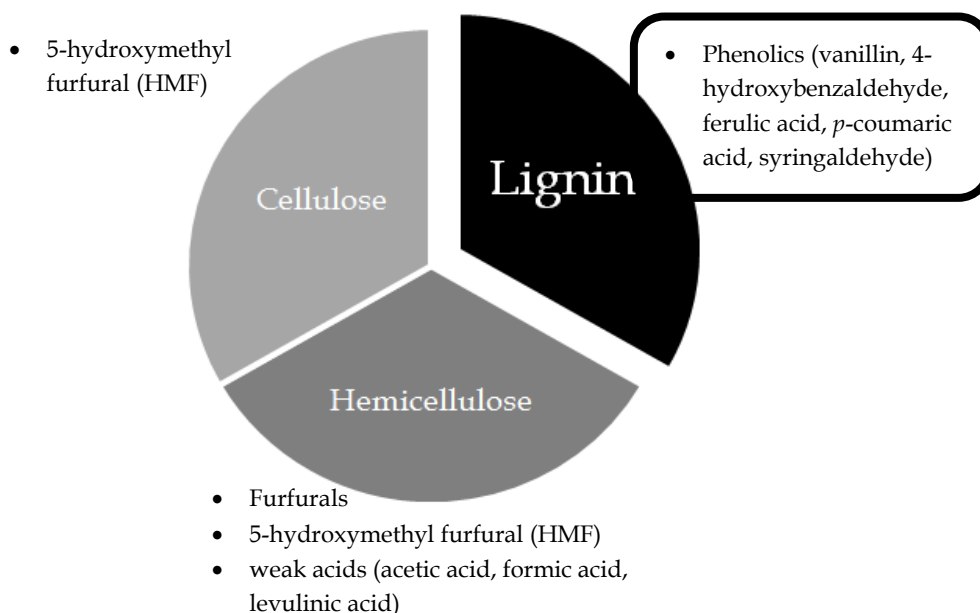
Figure 2.1.3.1. Plant cell wall derived inhibitors after pretreatment process. The amount and type of the inhibitor may vary depending on the pretreatment method applied. Figure adapted from Chandel (2011).

The phenolic substrates, which originate from the lignin degradation and have been discovered to significantly inhibit cellulase activity, at µM to mM concentrations causing the inactivation of cellulases by as much as 20-80% are; 4-hydroxybenzoic, *p*-coumaric, ferulic, tannic, gallic and hydroxybenzoic acid followed by guaiacol and vanillin (Klinke et al. 2004; Ximenes et al. 2011). Out of all inhibitory compounds mentioned, the one that deactivates cellulases in the larger extent is tannic acid (Ximenes et al. 2011). The same substances can additionally inhibit the fermentative organisms by affecting their cellular physiology (disturbing the function of biological membranes, leading to poor microbial growth) (Chandel et al. 2011). Furthermore, the use of cellulases in the hydrolysis of pretreated lignocellulosic biomass is limited by the presence of unmodified during pretreatment step lignin, which is also known to reduce the action of cellulases on the carbohydrate substrate by binding them nonspecifically (Moilanen et al. 2011).

It seems as if the cellulolytic hydrolysis of biomass was directly dependent on the fate of lignin and its modifications during the pretreatment process. Therefore, it is essentially important to optimize the enzymatic hydrolysis of the pretreated biomass. In the study described in **Paper I and Paper II,** an effect of laccase addition to the cellulase catalyzed hydrolysis of pretreated (by steam explosion) sugarcane bagasse was evaluated. The laccase from *G. lucidum* was found to increase the yields of cellulose-to-glucose conversion, overcoming the presence of cellulase-limiting factors by modification and/or removal of lignin. A hypothetical mechanism of such detoxification was also proposed (**Paper II**).

10

FUNGAL DEGRADATION OF LIGNOCELLULOSIC PLANT MATRIALS

3.1   White-rot Fungi – Superior Lignin Degraders

The only organisms known to extensively degrade lignin are fungi (Kirk and Farrell, 1987; Reid, 1995). They are divided into three different groups based on the type of the rot they produce during they proliferation on wood.

The most effective lignin degraders are basidiomyceteous *white–rot fungi* (Gilbertson, 1980). They generally degrade hardwood better than softwood, which is suspected to be due to higher presence of guaiacylpropyl (G) vs. syringylpropyl (S) units in softwood (Blanchette et al., 1988). In general (G) units are more resistant to degradation. White-rot fungi are able to completely mineralize both the lignin and carbohydrate components of wood. Some species (simultaneous rots) remove lignin and carbohydrate (in the form of hemicellulose) at the same proportional rate; others (selective white rots) remove lignin faster than cellulose (Blanchette 1995; Reid 1995). Although, some white-rot fungi always appear to either selectively or simultaneously degrade wood, there are many examples of white-rot fungi where one fungus produces both types of attack in the same substrate (see section; *Ganoderma lucidum* – a medicinal fungus with lignin degrading ability). It is suspected that lignin degradation is a result of phenol oxidase activity produced by fungi (Reid, 1995) in the culture broth and therefore extracellular peroxidases as well as laccase may contribute to the wood degradation phenomenon (see section; *Ligninolytic enzymes – why focus on laccases?*). In spite of the fact that white-rots are the most effective lignin degraders, their action on wood is limited by too severe environment conditions (i.e., excessively wet or dry sites) or substrates that do not favor their growth and development. These conditions can be however overcome by the soft-rot fungi (Blanchette, 1995).

Soft-rot fungi are classified in Ascomycota and Deuteromycota and the type of rot they produce has generally been associated with wood in wet environments. Two forms of soft rot have generally been recognized; type I which consists of distinct biconical or cylindrical cavities that are formed within the secondary cell walls, and type II that refers to en erosion form of degradation where the entire secondary wall is gradually degraded (Blanchette, 1995). The fact that the middle lamellae is not eroded and persists even in advanced stages of decay distinguishes the type II form of soft rot from a simultaneous white rot (Blanchette 1995). Therefore, the soft-rot fungi described by Nielson and Daniel (1989) failed to erode pine wood traheids and middle lamellae in birch.

Brown-rot fungi are basidiomycetes, and are classified in the same familiy as white-rot genera. In contrast to the white-rot fungi, however, the brown-rot fungi utilize the hemicellulose and cellulose of the cell wall, leaving the lignin essentially undigested, albeit modified by demethylation and oxidation (Green III and Highley, 1997). During the changes that occur in lignin, the polysachcharides are completely removed causing substantial losses in wood strength

properties. Without cellulose, the lignified residue lacks its integrity and may fracture to dust like particles (Blanchette, 1995; Ride, 1995). Brown-rot is most common for softwood, and the fungi are suspected to evolve from white-rot (Gilbertson, 1980).

It is clear from the available literature, which dates back to 1980, that the white-rot fungi are the superior lignin degraders. The exact mechanism of their performance is not known albeit the enzymes that can potentially take part in the wood degradation are mentioned. Lignin peroxidase, manganese peroxidase, and laccase are among the most serious candidates (see section; *Ligninolytic enzymes – why focus on laccases?*)

In this work, in order to test the hypothesis that the white-rot fungi may harbor abilities to produce new enzymes for lignocellulose degradation, a total of 44 strains were selected and tested for their growth performance on lignin in liquid media (**Paper I**). Of these strains, 40 were selected from a very large and well-known filamentous fungi culture collection tended by Professor Jens C. Frisvald (IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark, Lyngby, Denmark) and additional four fungi were purchased from CBS Fungal Biodiversity Collection (Utrecht, The Netherlands).

As explained in **Paper I**, none of the 40 strains from IBT Collection could grow on the medium supplied with a lignocellulosic material – sugarcane bagasse, which is why **Paper I** mainly focuses on the four strains obtained from the CBS Fungal Collection. These four fungi; *Trametes versicolor, Polyporus brumalis, Polyporus ciliatus*, and *Ganoderma lucidum* exhibited growth in liquid media supplemented not only with sugarcane bagasse but also with more recalcitrant, lignin alkaline. Out of these four mentioned fungi, *G. lucidum* quickly proved to exhibit superior growth on both lignin-containing supplemented to the MEA medium substrates (**Paper I**).

3.1.1   *G. lucidum* – Medicinal Fungus with Lignin Degrading Ability

> *"… In ancient times, it was said that if you discovered Reishi growing in the forest, you must keep the secret even from your closest relative – it was that highly regarded…"*

King and Jordan, 2008

The *Ganoderma lucidum* has been classified into Kingdom- Fungi, Phylum- Basidiomycota, Class- Basidiomycetes, Sub-clas- Homobasidiomycetes, Order-Polyporales, Family- Ganodermataceae, Genus- *Ganoderma* and Species- *lucidum* (Adaskaveg and Gilbertson, 1986).

In China and Korea *Ganoderma lucidum* is known as Ling Zhi, which is translated into "herb of spiritual potency" and in Japan it is called Reishi or mannetake, which means "ten thousand year mushroom" (Sanodiya et al., 2009). The name *Ganoderma* is derived from Greek *ganos* "brightness, sheen", hence shining and aptly describes this mushroom fruiting body, and *derma* means "skin" (Liddell and Scott, 1980).

The history of the healing properties of *G. lucidum* reaches back to the Eastern Han dynasty of China (AD 25-220). In those times Ling Zhi was worshipped as a kind of herbal medicine, the emperors of the great Chinese and Japanese Dynasties drank with their tees to achieve greater vitality and longer life (Sanodyia et al. 2009). In medicine *G. lucidum* was considered so promising that its medicinal value had been attested in a 2000-year-old Chinese medical text (*Shen Nong's Herbal Classic*) known as an authentic textbook of Oriental medical science (Zhu and Woerdenbag, 1995; Sanodyia et al. 2009).

Nowadays, there are a number of publications describing the medicinal and healing properties of *G. lucidum*. Some of the most compact and well-written are reviews of Zhu and Woerdenbag (1995), and especially the one of Sanodiya (2009) that described thoroughly the therapeutic effect as well as some of the bioactive compound produced by *G. lucidum*. It is difficult to list all of them since the basidiocarp, mycelia and spores of this fungus contain approximately 400 different bioactive compounds. Some of them, which mainly include triterpenoids, polysaccharides (mainly β-glucan), nucleotides, sterols, steroids, fatty acids, proteins/peptides, and trace elements have been reported to have a number of pharmacological effects including immunomodulation (Tasaka et al, 1988; Kim et al. 1997), anti-sclerotic, antioxidative, anti-inflammatory (Joseph et al., 2009), chemo preventive, antitumor (Sone et al. 1985; Wang et al., 1997), chemo and radio protective, sleep promoting, antibacterial, antiviral (including anti-HIV) (Gao et al. 2003), anti-diabetic (Gao et al., 2004), anti-aging (King and Jordan, 2008) properties and many and more.

Except for its healing properties, *G. lucidum* (Aphyllophorales) is also acknowledged as a white-rot fungus producing lignin oxidizing enzymes that efficiently degrade hardwood (Adaskaveg et al., 1990; Blanchette, 1995; D'Scouza et al., 1999). Common hardwood hosts in North America and Europe include oak, maple, sycamore, and in the southwestern United States and Mexico include mesquite, olive and grape (Adaskaveg and Gilbersten 1986). Additionally, Blanchette (1991) includes *G. lucidum* in a list of the most serious wood decayers. The study of Luna et al. (2004) on fungal decay in Poplar (*Populus deltoids*), reveals that *G. lucidum* performs a combination between i) selective and ii) simultaneous delignification. This result is in agreement with Blanchette (1984), who discovered that some white-rot fungi are able to produce both types of decay in the same wood. Delignification of poplar by *G. lucidum* resulted in a 60% wood weight loss in 2-5 months (Adaskaveg and Gilbertson, 1986; Luna et al. 2004). The structures of wood that were the most resistant to *G. lucidum*'s attack were vessels (Luna et al., 2004). The reasons why vessels might be more robust towards degradation by oxidative enzymes produced by white-rot fungi might be due to a larger S1 and S3 vs. the smaller S2 layer in the cell walls of the wood, high lignin content and high relative concentration of guaiacyl vs. syringyl lignin (Blanchette, 1988). Regardless of the degree of delignification, lignin is not degraded without the loss of some polysaccharides, however, it is suspected as if the hydrolysis of cellulose was due to the prolonged incubation of fungus on woody substrate since during delignification stage cellulolytic enzymes are repressed or nonfunctioning (Adaskaveg et al. 1990). Furthermore, Kirk and Moore (1972) found that the rate of lignin/carbohydrates removal by fungi varied depending on the wood used as substrate.

From the light and scanning microscopy, as described by Luna et al. (2004), it seemed as if the selective delignification, a step where mostly cell walls of wood are separated, was a first stage of

lignin delignification by *G. lucidum* (Fig. 3.1.1.1). The second stage (Fig. 3.1.1.2-8) was a simultaneous delignification during which the presence of erosion troughs (Fig. 3.1.1.1, 2), cell wall thinning (Fig. 3.1.1.2), bore holes (Fig. 3.1.1.3-4), rounded pit erosion (Fig. 3.1.1.5), and erosion channels were observed. Additionally, polysaccharides depletion was accompanied by chlamydosphores and hyphal accumulation in the difficult to degrade vessel remnants (Fig. 3.1.1.7).
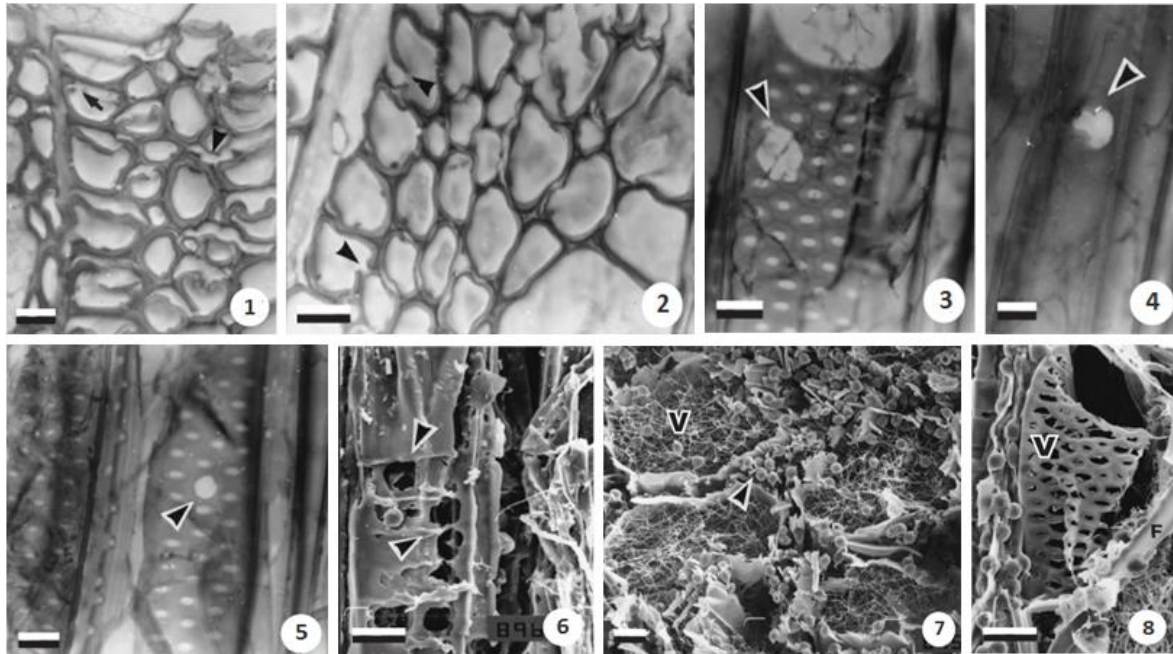
Figure 3.1.1 Anatomical features of decay by *Ganoderma lucidum* in *Populus deltoids* wood after 75 (1), and 150 (2-8) days of fungal inoculation. Figures and description of the figures adapted from Luna et al. (2004); 1 and 2-8 represent selective and simultaneous delignification of poplar, respectively; 1: centrifugal direction of delignification with remnants of lignin in cell corners and portions of middle lamellae (arrowhead) and erosion through in fiber wall (arrow); 2: advanced cell wall thinning and cell wall interruptions in fibers (arrowheads); 3: large hole in vessel wall (arrowhead); 4: large hole in fiber wall; 5: rounded pit erosion in vessel wall (arrowhead); 6: large holes in ray cells (arrowhead);7: vessel remnants (v) with abundant hyphae and chlamydoshores; 8: portions of vessels (v) and fiber wall (f).

In this study, the presence of the oxidative enzymes was measured on the ABTS and syringaldazine (**Paper I**) in the crude protein extracts of the four white-rot fungi that were able to grow on sugarcane bagasse and lignin alkaline supplemented media. It was evident that *G. lucidum* expressed by far the highest activity of laccase under these cultivation conditions. The activity data corresponded well with its vivid growth.

Therefore, the further experiments (SDS-PAGE, Native PAGE, and MALDI-TOF analysis) showed that the oxidative activity was mainly due to the presence of laccase (see also *Substrates for Detection of Laccase Activity* and *IEF of G. lucidum Crude Protein Extract* section in the experimental considerations section).

Hence, this work confirmed the hypothesis that it was possible to identify the oxidative enzymes (or at least one enzyme) by such an exploratory approach. As explained in **Paper I**, the further

analysis of the laccase-rich protein extract confirmed that its oxidative activity was able to enhance the cellulose degradation when added simultaneously with cellulase cocktail to the pretreated lignocellulosic biomass (sugarcane bagasse).

In the work, the laccase was subjected to further examination and it turned out that the laccase from *G. lucidum* was a unique enzyme with a molecular weight of 62.5 kDa (**Paper I**).

LIGNIN MODIFYING ENZYMES – WHY FOCUS ON LACCASES?

The degradation of lignin in the wood is seen upon as a complex process involving different classes of enzymes. The purpose of this chapter is to introduce the known enzymes whose specificity of action may contribute to the depolymerization of wood, rather than their detailed description which can be found in a number of review papers (Bourbonnais and Paice; Martinez et al. 2005; Kersten and Cullen, 2007; Wang, 2009).

Lignin digestion is considered as an "enzymatic combustion" process, involving several oxidoreductases such as laccases, ligninolytic peroxidases and peroxide generating oxidases. The enzymes involved in lignin digestion are furthermore classified in four major groups, based on their EC number; lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), versatile peroxidases (VP; EC 1.11.1.16), and laccases (EC 1.10.3.2). Based on their sequence and catalytic properties, the extracellular fungal LiP, MnP, and VP belong to class-II peroxidases (Wong, 2009). Moreover, the "enzymatic combustion" process is further enhanced by synergistic action of several accessory enzymes, which might include glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18) (Martinez et al. 2005; Kersten and Cullen, 2007; Wang, 2009).

For a long time laccase was not considered as an enzyme that could contribute to the ligninolysis due to the two aspects; its redox potential was too low, meaning that a laccase could only oxidize phenolic lignin structures, which contain less than 10% of the woody materials, and *P. chrysosporuim* that could delignify wood was suspected not to express laccase. Only research by Bourbonnais and Paice (1990) revealed that redox potential of laccase can be modulated by addition of an artificial laccase substrate, ABTS, which enabled a laccase catalyzed oxidation of non-phenolic lignin compounds (Eggert et al. 1997). Additionally Eggert (1996) demonstrated that this small redox tuning molecule can be also of natural origin, such as *Pycnoporus cinnabarinus* metabolite, 3-HAA (3-hydroxyanthranilate) (Eggert et al. 1996). The role of laccase in the wood decay was therefore contradictory and it was rather difficult to understand its exact contribution to the lignin-in-wood degradation. However in the course of study and during the literature search I came across a number of research papers that claim that laccase is essential for lignin degradation. The arguments for that hypothesis and additional features of laccase that make it an important enzyme during wood decay are presented below:

❖ Laccase catalyzes oxidative reactions of lignin with atmospheric oxygen as an electron acceptor (in contrast to lignin peroxidase and manganese peroxidase that are dependent on a continuous supply of H_2O_2) (Martinez et al. 2005)

❖ The combination of laccase with either LiP and/or MnP, in white-rot fungi, is a much more common combination of phenyloxidases than the LiP/MnP pattern found in *Phanerochaete chrysosporium* (Peláez et al. 1995; Tour et al. 1995; Eggert et al. 1997)

❖ Laccase has been visualized cytochemically in degraded wood cell walls (Youn et al. 1995)

❖ In some fungi, e.g. *Pycnoporus cinnabarinus* – a very efficient lignin degrader expressing only laccase and no LiP and/or MnP, the knock-out of the laccase gene unables the fungus to metabolize ^{14}C ring-labeled DHP (dehydrogenative polymer). However, an addition of a purified laccase to the laccase-less mutant from *P. cinnabarinus* restores its lignin degradation ability (Eggert et al. 1997; Bermek et al. 1998)

❖ The most recently discovered genome of *Ganoderma lucidum* (Chen et al. 2012) revealed an existence of 14 different laccase genes vs. 8 genes belonging to either lignin peroxidase, manganese peroxidase or versatile peroxidase. This finding might indicate laccase superior role in allowing *G. lucidum* to efficiently grow on media supplemented with lignin alkaline (**Paper I**)

❖ Laccase is capable of oxidizing high molecular weight lignin compounds even though they are much too large to directly penetrate and donate electrons to the T1 copper site (Piontek et al. 2002; **Review paper**). The process is suspected to happen directly through a long-range electron transfer process, since the T1 copper site is at least 7 Å away from the protein surface (Shleev et al. 2006)

❖ Laccase in the presence (NHA; Palonen and Viikari 2004; HBT; Gutierrez et al. 2012) and absence (Jurado et al. 2009; Moilanen et al. 2011; **Paper II**) of the mediator is able to significantly detoxify the pretreated lignocellulosic materials (by up to 75%) and improve its glucose release yields (by up to 33%). The possible mechanism during laccase treatment of pretreated lignocellulosic materials is condensation and/or polymerization of low molecular weight compounds, which is seen upon as a possible role of laccase protection of the fungus mycelium from the toxic substances (Youn et al. 1995; Rochefort et al. 2002; Shleev et al. 2006)

❖ Laccase is used for enzymatic detoxification of pretreated lignocellulosic hydrolysates from phenolic lignin degradation compounds, prior to fermentation processes and in turn yields higher ethanol yields than lignin peroxidase (Jönnson et al. 1998)

❖ Laccase is a focus of much attention due to its wide range of applications in food industry (crosslinking of polysaccharides (Zaidel and Meyer, 2012), beverages, fruit juice processing, baking), paper industry (biobleaching and biopulping) bioremediation applications (waste water detoxification and decontamination), textile industry (dye decolorization, denim processing), personal and medical care applications, and biosensor and analytical applications (Madhavi and Lele, 2009)

❖ Laccase is likely to contribute to the incipient decay of wood by brown-rot fungi *Postia placenta*, enabling it the access to polysaccharides (Wei et al. 2010).

4.1   Laccase from *G. lucidum* CBS229.93, LacGL1

Rather to attempt to purify the native laccase from *G. lucidum* crude protein extract, it was decided to study the enzyme further by subcloning of its gene to the expression vector and its heterologous expression in *P. pastoris* (see *Cloning of Laccase from G. lucidum* section in experimental considerations and **Paper II**).

As outlined in **Paper II**, the 520-bp LacGL1 laccase, expressed in *P. pastoris*, appeared to be a novel enzyme with a highly conserved sequence in the copper binding regions and a 91% identity on the amino acid level, to the *G. lucidum* described by Joo et al (2007). It also turned out that the LacGL1 was extensively glycosylated. This however, did not influence either its oxidation ability towards the artificial phenolic substrates tested or its synergistic effect on the total glucose yields released during ongoing cellulase catalyzed hydrolysis of pretreated sugarcane bagasse. On the contrary, the addition of the LacGL1 to the Cellic®CTec1 cellulase preparation, under experimental conditions that were close to the optimal for laccase (pH 4.7, and 40°C), increased the glucose yields from 17 to 33% (**Paper I** and **Paper II**). This result confirmed the hypothesis that a monocomponent enzyme, with an oxidative activity, can help to enhance the glucose yields during cellulase catalyzed hydrolysis of cellulose. Additionally, this finding proved that the LacGL1 has some unique properties that enable it to significantly enhance the cellulase catalyzed cellulose-to-glucose conversion. The exact mechanism of the LacGL1 laccase in the glucose yield improvement is not known, however, it is speculated (based on MD simulations) that the LacGL1 laccase acts towards small phenolic compounds released during the pretreatment of lignocellulose biomass (see section; *Fate of Lignin – Factors Limiting the Cellulolytic Hydrolysis of Biomass*) and that particularly amino acids such as 164Leu and 265Phe may play a significant role in that mechanism (**Paper II**). The importance of these two amino acids on the oxidation of phenolic compounds was also reported by Koschorreck et al. (2008).

This PhD study also provides an insight into the function of the catalytic pocket of the LacGL1 laccase, which made it possible to confirm that the LacGL1 laccase electron transfer proceeds via a well described T1-Cys-His-T3β pathway (Solomon et al. 2008; Augustine et al. 2010; **Review paper**). The four, one-by-one electron transfer in the catalytic pocket of laccases is driven by the presence of two acids in the vicinity of the four copper ions. D94 and E487 have been confirmed, to be also placed close to the copper ions in case of the LacGL1 (**Review paper**). An additional feature that may influence the oxidation rate of the phenolic substances is a presence of a non-coordinating phenylalanine in the distance of ~3.5 Å from the T1Cu site, which based on the available knowledge influences the ability of the enzyme to abstract the electrons (widely known as redox potential) from the lignin phenoxy groups and classifies the laccases to the enzymes having a high redox potential. Laccases are also able to oxidize non-phenolic compounds, for these reactions, they need a small, diffusible molecule – a mediator (**Review paper**).

Additionally, the results from the 3D modeling of the LacGL1 structure revealed that the laccase from *G. lucidum* has three domains and two disulphide bridges that keep the functional structure together (**Review paper**). These features are common for the fungal laccases (Hakulinen et al. 2002). However, one needs to be cautious about any structural mutations, especially to the C-terminal end which may lead to an inactive protein (see section; *Transformation and Expression of the LacGL1_NC*).

20

EXPERIMENTAL CONSIDERATIONS

Lignin and lignin derived phenolic substrates pose a barrier for an efficient cellulase action. Therefore, in an ideal biofuel production scenario, there is no lignin and lignin related products but instead there are high yields of fermentable sugars. So far cellulolytic enzymes are the main components of the commercial preparation cocktails however, new enzyme activities are being considered. This chapter focuses on the process and aspects that were challenging and those that led to the finding that a laccase activity is able to boost cellulase catalyzed hydrolysis of pretreated biomass and yield higher overall glucose yields, so desired in the biofuel industry.

5.1   Substrates for Detection of Laccase Activity

A special care needs to be undertaken when detecting laccase activity in the fungal crude protein extracts due to the reasons mentioned below:
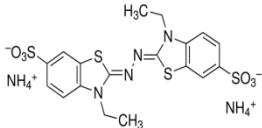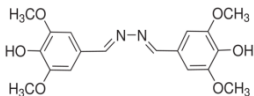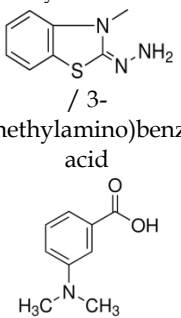
- ❖ nature of laccase substrates, most of them are phenol homologs that are or might be carcinogenic

- ❖ presence of oxidases that produce H_2O_2 which may auto-oxidize laccase substrates

- ❖ presence of peroxidases (lignin and manganese peroxidase), that catalyze H_2O_2 – dependent oxidation of laccase substrates

- ❖ ability of laccase substrates to be oxidized by catalyze, that instead of removing H_2O_2, acts as a peroxidase and uses H_2O_2 to oxidize laccase substrates

Therefore, it is crucially important to consider the above mentioned obstacles that can interfere with the true laccase activity (**Research paper I**), since the detection of laccase activity is a balance between the amounts of peroxidases but also the amount of endogenous H_2O_2 produced in the fungal crude protein extracts.

Table 5.1 gives an overview of the most used substrates for detection of laccase activity and simultaneously focuses on their advantages and disadvantages.

The best laccase substrate seems to be syringaldazine, which is said not to be influenced or oxidized by peroxidases and H_2O_2 present in the crude protein extracts. Harkin et al. (1974) overrules as if the concentration of endogenous H_2O_2 present in the crude protein extracts was high enough to catalyze oxidation of syringaldazine by peroxidases. Additionally, syringaldazine do not auto-oxidize and form organic hydroperoxides. Therefore syringaldazine is said to be a true laccase substrate.

Table 5.1 Substrates used for detection of laccase activity in the crude protein extracts

Substrate	Advantages	Disadvantages	Ref.
ABTS 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	• non toxic • H_2O soluble (at a concentration of 10 – 50 μM) • immediate oxidation reaction after addition of laccase • suited for laccase detection in the crude protein extract in the *absence* of catalyses, peroxidases, and H_2O_2 • not reactive with tyrosinases	• oxidized by catalyse, during removal of H_2O_2 • not suited for laccase detection in the crude protein extracts in the *presence* of catalyses and H_2O_2 or peroxidases, and high levels of H_2O_2 • auto-oxidation with high H_2O_2 levels (0.3% w/w)	Wolfanden et al. 1982; Lonergan and Baker, 1995
Syringaldazine (3,5-dimethoxy-4-hydroxybenzylidene) hydrazine	• soluble in methanol or 95% ethanol • rapid color formation at all pH levels • suited for laccase detection in the crude protein extracts in the *presence* of catalyses and H_2O_2 or peroxidases, and low levels of H_2O_2 • not reactive with tyrosinases • not reactive with H_2O_2 alone	• toxic • color tends to fade rapidly outside the pH 3 to 7 • not suited for laccase detection in the crude protein extracts in the presence of lignin peroxidase and H_2O_2 (high level) and Mn^{2+}, manganese peroxidase and H_2O_2	Harkin and Obst, 1973; Harkin et al. 1974
MBTH/DMAB 3-methyl-2-benzothiazolinone hydrazone / 3-(dimethylamino)benzoic acid	• H_2O soluble • high rate of reaction (only slightly lower than in case of ABTS • color stable, fades slowly over several hours	• toxic • requires treatment with catalyse prior to laccase detection (oxidation by lignin and manganese peroxidase at low H_2O_2 concentrations) • in presence of Mn^{2+} and 0.05 mM H_2O_2 is used for manganese peroxidase activity	del Pilar Castillo et al. 1994; Lonegan and Baker, 1995

5.2   IEF of *G. lucidum* Crude Protein Extract

Two MALDI-TOF MS analyses were performed (referred in Table 5.2 as 1st and 2nd round, respectively). The first analysis focused solely on the single protein band that showed activity towards phenolic substrate (1,8-DAN, described in detail in **Paper I**). After discovering that this protein band was a laccase, the short amino acid stretches served as the regions where the primers for the laccase gene amplification (**Paper II**) were designed. The second MALDI-TOF analysis was supposed to reveal what exact enzymes are being released during *G. lucidum* growth and whether or not the laccase expression pattern differs depending on the lignocellulosic substrates present in the cultivation medium. The analysis of all the enzymes in the crude protein extract were crucially important because the addition of laccase-rich broth boosted total yields of released glucose and that no cellulase activity was detected during AZCL cellulase assay. Therefore to exclude that the higher yields of glucose were due to the presence of cellulases and the reason behind it was laccase activity, the evaluation of the presence of cellulolytic enzymes, and especially GH61 (glycoside hydrolase), called a cellulase-enhancing factor (Quilan et al. 2011), was at stake.

The outcome of the 1st round MALDI-TOF analysis resulted in a successful amplification of the *LacGL1* gene, even though the short amino acid stretches differed slightly in the amino acid sequence with the expressed LacGL1 laccase. The 2nd round of MALDI-TOF analysis failed to identify all proteins expressed by *G. lucidum*. It revealed however two aspects; the expression pattern of proteins differed depending on the carbon source used (Fig. 5.2) – more proteins were expressed when Avicel was added than when SCB was present, and *G. lucidum* expressed at least four different laccase isoforms (spots 3, 4, 5, and 7; Fig. 5.3) – which differed between each other with the amount of homologous amino acid short peptides (A-E; Fig. 5.3). The last result corresponded well to the genome sequencing data from another *G. lucidum* strain (14 different candidate laccase genes (Chen et al. 2012)). Surprisingly, spot number 6 did not show any homology with the LacGL1 sequence. However, it would be very unlikely that another type of protein would be situated between laccase isoforms.

Table 5.2 MALDI TOF analysis of proteins from the crude extract of *G. lucidum*

MALDI-TOF MS analysis of the proteome peptides		Amino acid mutations in the LacGL1 laccase
1st round	2nd round	
TTSIHWHGFFQK		S
FPLGSDSTLINGLGR		---
DDDSTVTTLADWYHVAAR		I, T
TTSNADIAPDGFTR		I, V, Y
	FPLGSDSTLINGLGR	---
	SAGSTVYNYDNPVWR	---
	ANPNFGNVGFTDGINSAILR	---
	GIGPKDLTISNADVAPDGFTR	Y
	NLLLETDLH	N, V

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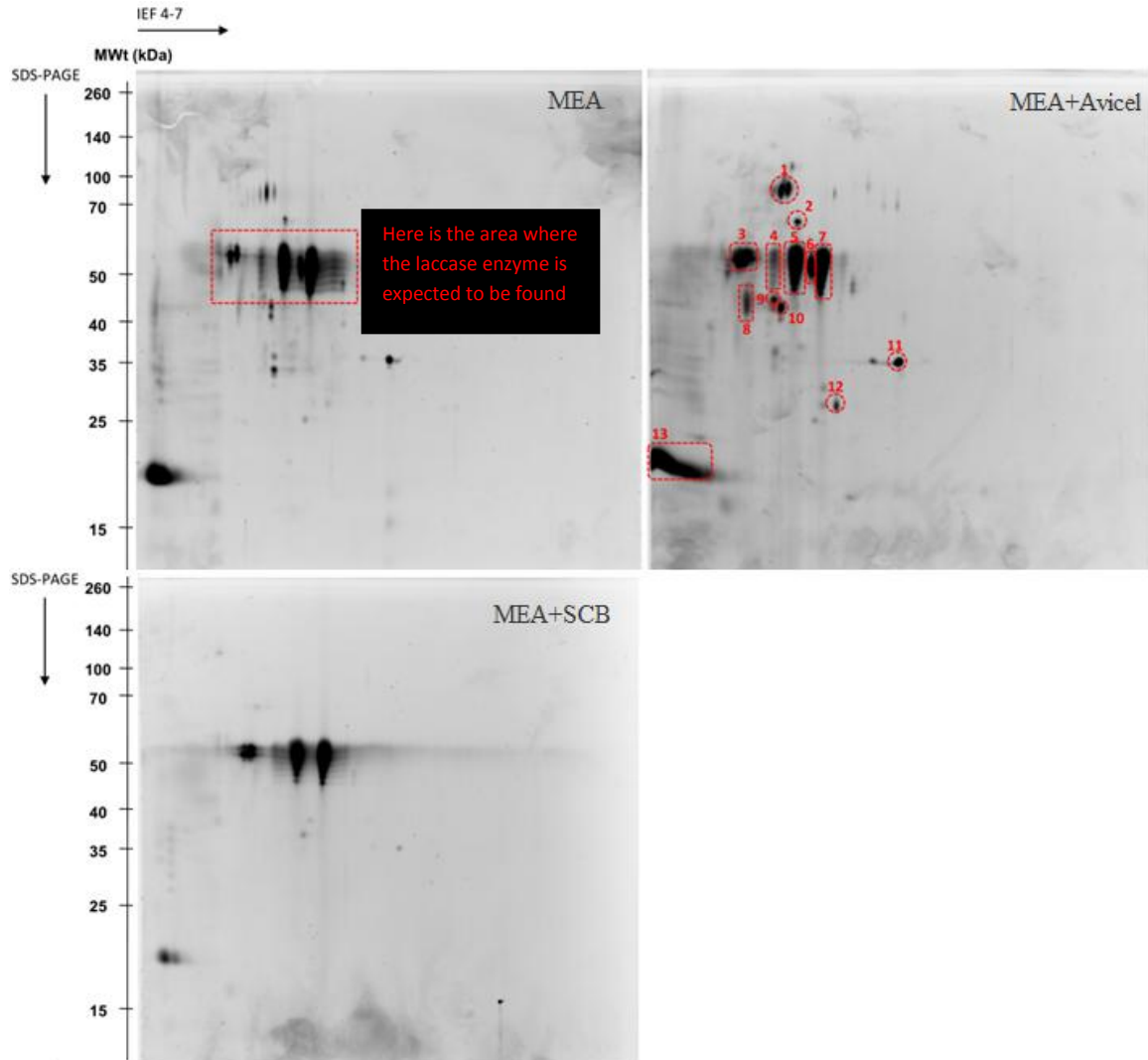
Figure 5.3 Two-dimentional proteomic profiles of *G. lucidum* CBS229.93. Aliquots containing 50 μg of proteins, from the fungal crude protein extracts, were subjected to IEF and separated by molecular weight by SDS-PAGE electrophoresis. Following electrophoresis, resolved proteins were stained with Sypro Ruby stain. The crude protein extracts were collected from fungal cultivation media on malt extract medium (MEA), malt extract medium supplemented with Avicel (MEA+Avicel), and malt extract medium supplemented with sugarcane bagasse (MEA+SCB). The resolved protein spots were in-gel digested with trypsin and analized using MALDI TOF MS/MS. The protein bands were analyzed by MALDI-TOF MS/MS. Four out of thirteen spots (3, 4, 5, and 7) belong to laccases. The figure has been prepared in collaboration with Adelina Rogowska (Southern University of Denmark (SDU), Odense M, Denmark).
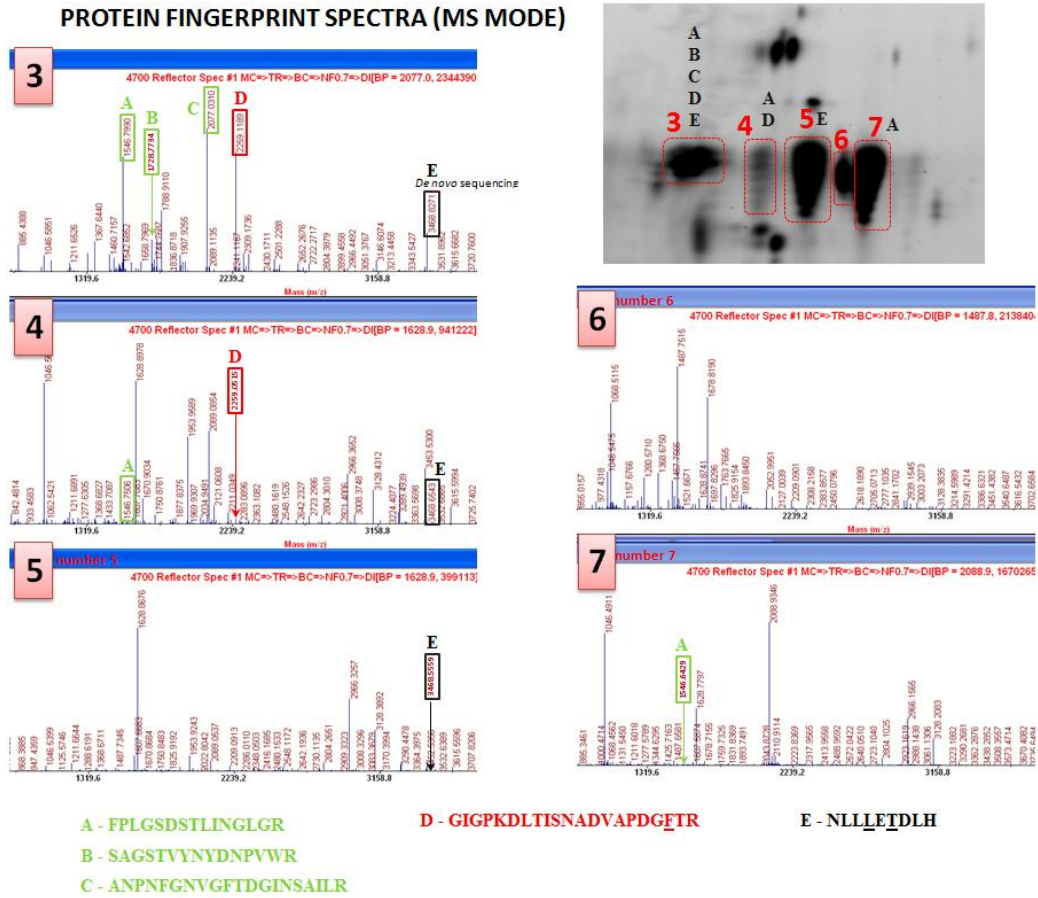
Figure 5.2 Peptide mass fingerprints of protein from the crude extract of *G. lucidum* CBS229.93. Fingerprint mass spectra were generated via MALDI-TOF MS analysis. The predicted peptide fragments of laccase corresponding to their m/z values are shown at the bottom of the figure. Short peptide sequences colored green are 100% homologous to the LacGL1 laccase sequence from G. lucidum CBS229.93. Short peptide sequence colored red has one single mutation (F is Y in LacGL1 laccase expressed in *Pichia pastoris*) and Short peptide sequence colored black has two single mutations (L is N and T is V in LacGL1 laccase). The two-dimentional proteomic profile of G. lucidum (in the right upper corner of the figure) shows how many and what type of short peptide sequences each of the protein spots contains. It seems as if a protein spot number 3 corresponded to the *P. pastoris* expressed LacGL1 laccase (**Paper II).** This figure has been prepared in collaboration with Adelina Rogowska (SDU, Odense M, Denmark).

5.3   Cloning of Laccase from *G. lucidum*

A MALDI-TOF analysis of trypsin digested protein that showed activity towards 1,8-diaminonaphthalene (**Paper I**), resulted in four short amino acid sequences (Table 5.2, 1st round) that belonged to laccase. Among them, two amino acid stretches: TTSIHWHGFFQK and FPLGSDSTLINGLGR showed to be highly conserved within laccases from different *G. lucidum* strains. Therefore, the primers for amplification of the laccase gene, *LacGL1*, were designed based on those short peptides (Table 1, **Paper I**). The primer set, WADGP_fwd3 and LINGLP_rev1, gave a 500-bp-streatch of nucleotides that when blasted in the UniProt database, revealed high sequence homology to laccase from *G. lucidum* 7071-9 (UniProt identifier; Q9GH17). Please note that due to the nature of the DNA amplification method (described in detail in **Paper II**), the

described WADGP_fwd3 primer is situated in a close proximity to the TTSIHWHGFFQK, however is not a part of it. Following the overlapping primer method for amplification of the entire *LacGL1* gene, I obtained a genomic DNA fragment containing 1963 bp. In the search for introns and exons a manual, knowledge-based approach (Padget et al. 1984) was used. It was in turn verified with the obtained intron-depleted sequence, by a software-intron-prediction analysis (Stanke and Morgenstern 2005). The manual and software based prediction of positioning of the introns in the obtained genomic DNA sequence revealed a 100% match. The intron-free DNA sequence contained 1563 bp. Because the obtained *LacGL1* gene sequence was not a full laccase sequence it was decided to fill the missing 8 amino acids in the N- and 8 amino acids in the C-terminal ends, based on the highest score for these sequences among laccases from different *G. lucidum* strains. The signal peptide was not taken under consideration since a leader sequence was already incorporated into a pPICZαA vector. Such constructed laccase gene, *LacGL1_NC*, containing 1563 bp nucleotide fragment from *G. lucidum* CBS229.93 and having first 8 N-terminal and last 8 C-terminal amino acids from *G .lucidum* 7071-9 (UniProt identifier; Q9GH17), was send off to DNA2.0 (DNA2.0, Menlo Park, CA, USA) for subcloning into the pPICZαA vector, (containing a STREP® purification tag, Fig. 5.3.1) and codon optimization for expression in *P. pastoris*.

Please note that the two amplified laccase genes were designated as *LacGL1* and *LacGL1_NC*. *LacGL1* is a 1563 bp nucleotide fragment coding for a laccase gene from *G. lucidum* CBS229.93, while *LacGL1_NC* is a hybrid between *LacGL1* fragment with 8 N-(GVGPKADLT) and C-(DALSADDH) terminal sequences from *G. lucidum* 7071-9, the strain that showed the highest nucleotide as well as amino acid homology to the 1563 bp fragment from *G. lucidum* CBS229.93. The underlined amino acids will later show (after RACE technique), that they differed from the real sequence of *G. lucidum* laccase (**Paper II**).

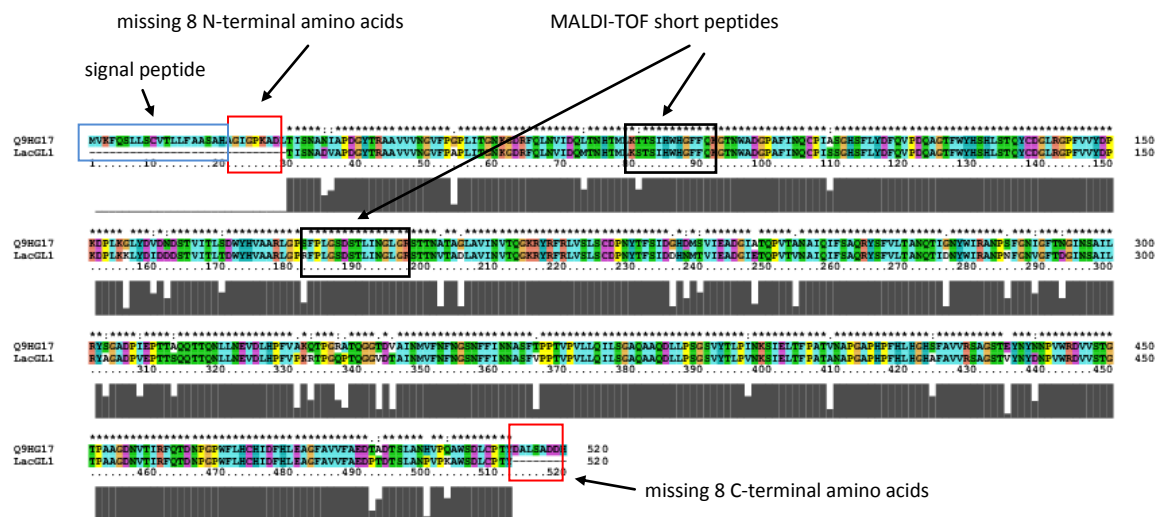**Figure 5.3** Alignment of an amplified, intron-free laccase gene (*LacGL1*) with its closest laccase homolog from *G. lucidum* 7071-9. The blue, red, and black boxes represent signal peptide sequence, missing N- and C-terminal amino acids, and short peptide stretches obtained by the *de novo* MALDI-TOF MS analysis, where the primers for 500-bp gene amplification fragment were designed, respectively.

5.3.1 Transformation and Expression of LacGL1_NC

The transformation and expression of LacGL1_NC laccase gene followed the steps:

❖ Assessing the plasmid DNA (DNA2.0_LacGL1_NC) from the GFC filters, supplied by DNA2.0 (DNA2.0, Menlo Park, CA, USA) – the constructed vector contained *AOX* promotor, *LacGL1_NC* gene, and a purification tag (STREP®tag) in the multicloning site.
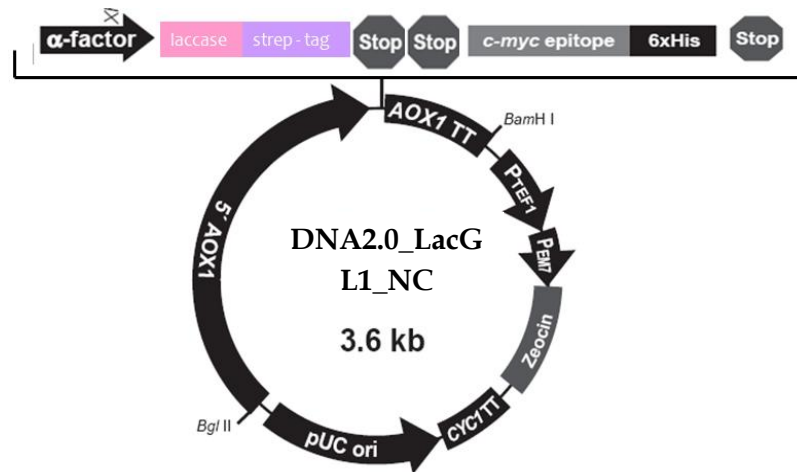
Figure 5.3.1 The map of the recombinant plasmid DNA2.0_LacGL1_NC used for expression of *LacGL1_NC* laccase gene in *Pichia pastoris* X-33. The DNA2.0_LacGL1_NC vector contains the sequence coding for the signal peptide from *Saccharomyces cerevisiae* α-mating factor pre pro peptide and gives a fusion of STREP®tag to the C-terminal end of the subcloned *LacGL1_NC* laccase gene.

❖ Transformation of DNA2.O_LacGL1_NC plasmid into *E. coli* (TOP10 and Match1-T1 (faster growing strain)) – *E. coli* was grown at 30°C due to a lower death rate of *E. coli*, (company guidelines). As a result, very low transformation ratio (50-100 colonies), especially for TOP10 *E. coli* was obtained (plating on Low Salt Medium plus 25 μg/mL Zeocin)

❖ Isolation of the DNA2.0_LacGL1_NC plasmid and its sequence conformation – *AOX* promotor sequence plus the sequence of the multicloning site in the DNA2.0_LacGL1_NC vector construct were as designed and optimized by DNA2.0. No single mutations were observed

❖ DNA2.0_LacGL1_NC plasmid linearization and transformation to *P. pastoris* by electroporation – different concentration of Zeocin on plates; 100, 500, 1000, and 2000 μg/mL Zeocin

❖ Conformation of existing insert and no mutations in the *AOX* promoter in *P. pastoris* transformants – randomly chosen 3 transformants for sequencing out of 10 that were "positive" in the digestions – existence of the LacGL1_NC gene, *AOX* promoter, α-factor

- ❖ Expression of the protein from the recombinant DNA2.0_LacGL1_NC plasmid – BMMY medium (recipe detailed in **Paper II**), 0.5% MetOH, 1mL antifoam, 0.1 mM $CuSO_4 \cdot 5H_2O$, 20°C, expression of 50 colonies on the plate, none positive

- ❖ Evaluation of the intracellular and secreted proteins by SDS PAGE – many bands, hardly noticeable whether the expressed protein was there. Additionally, the wild type *P. pastoris* strain had a protein at approximately 60-65 kDa (Joo et al. 2007).

- ❖ Western Blot analysis – antibodies against the Strep-tag – no visible results

Following all eight steps, it was found out that no active laccase was expressed. Therefore, I speculated the following:

- ❖ α-factor lider sequence from *S. cerevisiae* – higher expression with a native lider sequence from *G. lucidum* – maybe expression was so low that it could not be monitored. This hypothesis was however, excluded by the Western Blot analysis – no detectable response of antibodies to the STREP®tag

- ❖ expression at lower temperature – to ensure the proper protein folding and decrease the protease activity – literature even down to 10°C, and then no protease activity as compared to 28-30°C

- ❖ methanol toxicity to the host – I used 1 and 0.5 %

- ❖ laccase toxicity to the host – low transformation efficiency ca. 5 - 20 colonies

- ❖ X-33 is a protease non deficient strain – can laccase be degraded by a protease since the laccase sequence was optimized so that it will not be recognized by protease

- ❖ shaking and oxygen access to the growing culture? – too powerful shaking, or too slow, oxygen access through stoppers

- ❖ pH of the fermentation broth – kept at 5, but maybe acidic proteases act on laccase

- ❖ SDS-PAGE gel electrophoresis show a large amount of bands – stress??, dead cells protein extract??

After a careful validation of each of the presented problems, I found out, by 3D structural prediction of the LacGL1_NC laccase using Phyre2 software (Kelley and Sternberg 2009), that the protein is missing one of the disulphide bridges (Fig. 4.3.2), so important for the functionality of an enzyme.
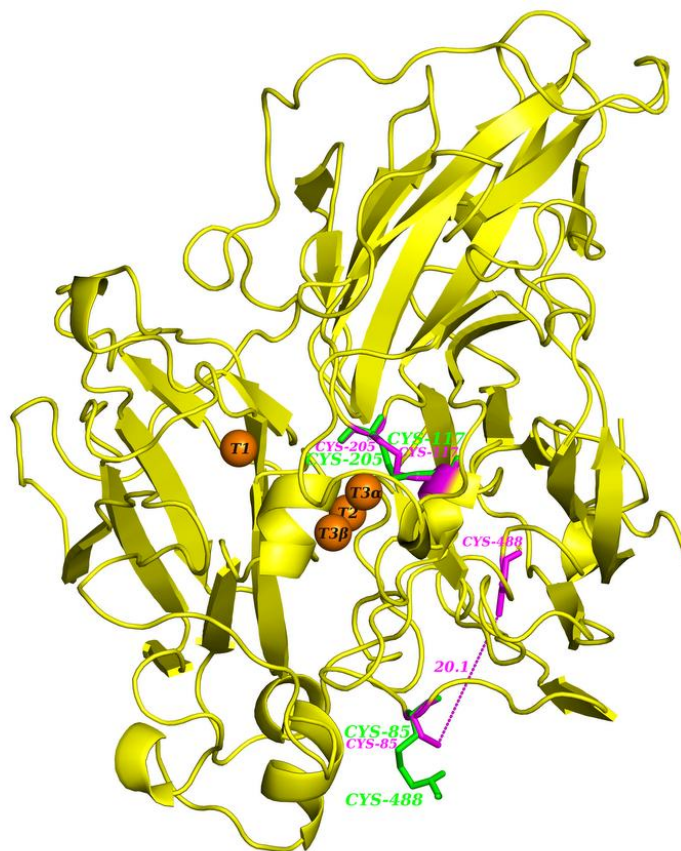
Figure 4.3.2 A graphical representation of the LacGL1_NC laccase gene expressed in *Pichia pastoris* illustrated using the PyMOL Molecular Graphical System version 1.5 (Schrödinger, LCC). The yellow colored 3D structure of the laccase was predicted by submitting its full amino acid sequence to the 3D comparative protein modeler – Phyre2 (Kelley and Sternberg 2009). The pink and green sticks (belonging to *Trametes versicolor* (PDB ID: 1GYC) and *G. lucidum* CBS229.93, respectively) represent cysteine residues responsible for formation of the disulphide bridges. The brown spheres represent copper ions. *T. versicolor* crystallographic structure of laccase was used as a benchmark for the 3D model structure of LacGL1_NC laccase.

Therefore, in order to determine the exact amino acid sequences on the N- and C-terminal end a RACE technique was applied (the exact methodology is described in **Paper II**). The obtained mRNA sequence of *G. lucidum* laccase, translated into amino acid sequence differed from the complimentary LacGL1_NC laccase sequence (Fig. 4.3.3). The single mutations occurred exclusively in the N- and C-terminal ends of the laccase sequence. Based on the fact that the RACE technique revealed the entire laccase sequence and that it was identical to the laccase sequence obtained from the genomic DNA amplification, I chose to name it LacGL1.
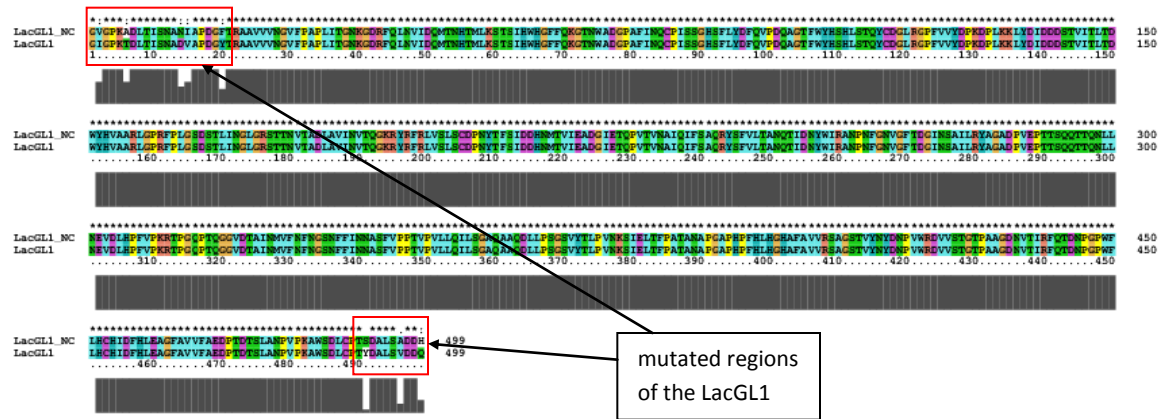
Figure 4.3.3 Alignment of mRNA from the LacGL1_NC, and LacGL1. The LacGL1_NC was obtained by and artificial insertion of the N- and C-terminal end sequences belonging to *G. lucidum* 7071-9, while the LacGL1 N- and C-terminal ends were obtained by a RACE technique (**Paper II**). The red boxes represent the regions in LacGL1 that were altered in respect to the LacGL1_NC, and which in turn could cause improper folding of the LacGL1_NC (Fig. 4.3.2).

CONCLUSIONS

In the process of the PhD study two research papers, a review, and a patent have been produced. The performed work concludes the following aspects:

❖ Out of forty strains tested, belonging to Ascomycota and Basidiomycota, only four white-rot fungi were chosen for further experiments carried on lignin alkaline (LA) – an oxidative enzyme inducer source. The choice of the white-rot fungal strains for further experimental analysis was made based on low growth performance of Ascomycota when sugarcane bagassewas added to the cultivation medium

❖ Out of four white-rot fungi tested, only one, namely *G. lucidum,* could perform a vivid growth on medium supplemented with LA. Therefore, **Paper I** solely focuses on the laccase enzyme that we think was responsible for its outstanding growth performance, as compared to the growth of other fungi tested

❖ Separation of the fungal crude protein extract of *G. lucidum* under denaturing conditions revealed presence of an enzyme that had a similar (slightly lower) molecular weight than that of a commercial laccase from *Trametes versicolor*

❖ Activity staining of the fungal crude protein extract of *G. lucidum* under native conditions (Native PAGE) revealed a presence of an enzyme having activity towards a phenolic substrate (1,8-diamononaphthalene) and corresponded well with the MW of the control laccase from *Trametes versicolor*

❖ *G. lucidum's* laccase activity (measured with syringaldazine as a substrate) corresponded well with the growth pattern. The more vivid was the growth of *G. lucidum*, the more laccase was produced

❖ An in-gel trypsin digestion of the potential laccase protein and a *de novo* MALDI-TOF analysis resulted in obtaining four short peptides whose sequence were highly homologous (but not identical) to the laccases deposited in the UniProt database

❖ Addition of the laccase-rich fungal protein extract to the cellulase catalyzed hydrolysis of the pre-treated SCB enhanced the yields of the released glucose by 17% (**Paper I**)

❖ A cloned and successfully expressed in *P. pastoris,* 520-amino-acid long laccase from *G. lucidum* (LacGL1) enhanced the total glucose yields, during cellulase catalyzed cellulose hydrolysis, by as much as ~20 to up to 33% for Cellic®CTec2 and Cellic®CTec1, respectively (**Paper II**). Therefore, the LacGL1 laccase can find application in e.g. biofuel industry

❖ The K_m value of the expressed LacGL1 laccase was 0.122 mM and correlated well with the K_m of the laccase in the crude fungal protein extract

❖ The pH optimum for the LacGL1 laccase was 4.7, and the thermal stability studies revealed 85% remaining activity after 60 min at 50°C. This property of laccase might have been of a great help during the delignification/detoxification mechanism that enabled cellulases to perform a better action during cellulose-to-glucose conversion

❖ LacGL1 had 91% and 81% homology to another strain of *Ganoderma lucidum* and *Polyporus brumalis*, respectively

❖ The sequence alignment with other fungal laccases revealed a high homology in the copper binding regions, which are highly conserved within laccases

❖ The molecular weight of the expressed laccase was extensively glycosylated, however after 24-hour-inccubation at 37°C the MW of laccase was identical (~62.5 kDa) as that observed in the fungal crude protein extract. Additionally, a similar reactivity towards tested substrates and inhibitory substances (**Paper II**), for the native and heterologously expressed laccase, suggested that neither the HIS tag nor the glycosylation had significant functional implications on the LacGL1 activity

❖ This functional neutrality of the HIS-tag was further supported by the conservation of the structure excluding the HIS-tag and Phyre2 homology models of the native and the expressed laccase

❖ The molecular dynamics simulations revealed that docking of *p*-coumaric acid (the compound found in the pretreated hydrolysates of SCB and causing severe cellulase inhibition) in the LacGL1 binding pocket and is exposed to the solvent caused by the shift in 164Leu and 265Phe might favor the detoxification/modification reactions of small phenolic substrates that inhibit cellulases action and therefore cause lower yields of glucose when laccase activity is not present

❖ The four coppers in the laccase active site need to be in a reduced form in order to be able to reduce atmospheric oxygen to two molecules of water. Two acids in the vicinity of the catalytic copper site take part in the mechanism

❖ The four electrons that are abstracted from four substrate molecules are transferred one by one from T1 Cu site to T2/T3 Cu site via the T1-Cys-His-T3β (**Review paper**)

❖ The oxidation of the phenolic and non-phenolic substrates follows the same primary mechanisms (C_α-C_β cleavage, C_α-oxidation, or alkyl-aryl cleavage). However one needs to remember that oxidation of non-phenolic substrates is mediated by the presence of a small, diffusible compound – a mediator

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❖ It seems like laccases that have a high redox potential have a non-coordinating phenylalanine residue in the vicinity of the T1 Cu site, instead of a methionine that makes the T1Cu area more rigid

❖ Based on the literature review and own unpublished data, it has been observed that the mutations and the alterations to the C-termini can completely deactivate laccase function and activity

❖ The 3D modeling of the LacGL1 showed the existence of the common features for fungal laccases; the LacGL1 is a three domain protein with copper ions situated in domain 1 and 3 and two disulphide bridges that keep the structure together

FUTURE WORK

In the present study, we showed that the addition of the laccase-rich medium from *G. lucidum,* as well as the laccase-containing broth from *P. pastoris,* results in the enhancement of glucose yields, released during the cellulase catalyzed hydrolysis of cellulose. This result, followed by the patent application, is crucially important for industrial application in biofuels sector considering the fact that cellulase preparations, namely Cellic®CTec 1 and Cellic®CTec 2 are improved enzyme preparations in respect to different types of inhibitors. Unfortunately, the exact mechanism of laccase action in the reaction mixture is not known, and would require additional experiments. The most acute future research tasks are presented below:

❖ IEF data showed existence of at least four additional isoforms of laccase. It would be highly beneficial to evaluate the synergistic potential of all four laccases with the cellulase ocktail on the total yields of released sugars

❖ Since the mechanism of laccase action is not known, it would be interesting to evaluate the type and amount of the phenolic compounds present in the pretreated sugarcane bagasse substrate before and after the laccase treatment

❖ If the laccase follows the detoxification mechanism, as also suspected from the MD simulations (**Paper II**), and in this way helps cellulases to convert more cellulose to glucose, then a thorough identification of soluble, phenolic compounds, present in the pretreated sugarcane bagasse should be evaluated and their different concentration dosages could be added to the previously washed, soluble, phenolic-free pretreated biomass (or Avicel)

❖ If the laccase follows the lignin depolymerization mechanism, then different lignin concentrations could be added to Avicel and the yields of cellulose converted to glucose during ongoing cellulase catalyzed reaction could be tested

❖ The LacGL1 laccase was only tested on the pretreated sugarcane bagasse, however an evaluation of its impact on other pretreated lignocellulosic substrates could be compared

❖ The LacGL1 laccase has a 91% amino acid sequence identity to another laccase from *G. lucidum*, described by Joo et al. (2008). It would be interesting to evaluate this laccase potential in the enhancement of cellulose-to-glucose conversion during ongoing cellulase hydrolysis of pretreated sugarcane bagasse

❖ The LacGL1 laccase dosage has been evaluated based on the total concentration of proteins that followed expression of laccase; therefore a purification step would be necessary to evaluate the real laccase dosage during cellulase catalyzed hydrolysis of pretreated biomass

❖ It would be also interesting to subclone the laccase coding gene to a vector with a metalloprotein-neutral-purification tag (such as STREP®tag) and crystallize the laccase structure for more accurate molecular dynamics simulation with the appropriate substrate

❖ Different cellulase mixtures, available commercially, would also be evaluated for the synergistic laccase catalyzed improvement of the sugar yields on different types of lignocellulosic materials (beyond sugarcane bagasse)

❖ The positive effect of laccase on the total glucose yields during small scale, cellulase catalyzed hydrolysis of cellulose to glucose was evaluated. It would be interesting to find out if the overall output could be scalable to large volumes

❖ The effect on treating the pretreated sugarcane bagasse with laccase (and other oxidases; lignin peroxidase, and manganese peroxidase) would be visualized in the SEM microscope, to evaluate the changes to the lignin structure that undergo during that reaction

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Identification of a Highly Active Laccase from Ganoderma lucidum CBS229.93, able to Enhance Cellulase Catalyzed Lignocellulose Degradation

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Identification of a highly active laccase from *Ganoderma lucidum* CBS229.93, able to enhance cellulase catalyzed lignocellulose degradation.

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■ **Abstract** Based on a differential pre-screening of 44 white-rot fungi on a lignocellulose-supplemented minimal medium, four basidiomycetes were selected for further study: *Ganoderma lucidum, Polyporus brumalis, Polyporus ciliatus* and *Trametes versicolor*. In the next round, *G. lucidum* was found to be the only one being able to grow vividly on media supplemented with lignin alkaline. The crude protein extract of this *G. lucidum* exhibited high laccase activity of ~3 U/mL towards syringaldazine. This activity was 13 to 17 fold higher than the corresponding activity of the crude protein extracts of *P. brumalis, P. ciliatus* and *T. versicolor*. The presence of laccase in the crude *G. lucidum* extract was further evaluated by SDS and Native PAGE electrophoresis. The enzyme had a molecular weight of ~62.5 kDa, and a K_m value of 0.107 mM (determined on ABTS). A partial amino acid sequence analysis of this laccase, obtained by *de novo* sequencing using MALDI-TOF MS/MS analysis, revealed 64 to 100% homology to the closely related laccases found in the UniProt database, and also indicated that certain sequence stretches had a relatively low homology. When the laccase-rich broth was added to lignocellulosic biomass (pretreated sugarcane bagasse) together with a state-of-the-art cellulase enzyme preparation (Cellic®CTec1, Novozymes), the laccase activity boosted the cellulosic glucose yield by 17% after 24 h (pH 5.1 and 50°C). These results indicate that this particular laccase from *G. lucidum* has unique properties that may prove significant in industrial upgrading of lignocellulosic biomass.

INTRODUCTION

Lignocellulosic materials, such as agro-industrial residues (e.g. straw, bagasse, stover) and forestry materials are recalcitrant substrates that are currently studied over the world to allow their utilization for biofuel industry. They comprise of cellulose, hemicellulose, and lignin. The latter one is a complex, aromatic biopolymer consisting of phenolic units mainly *p*-hydroxyphenyl, guaiacyl, and syringyl-type phenylpropane, which are linked together by ether and carbon-carbon bonds [1]. In plants lignin has a function to act as a cementing agent between cellulose and hemicellulose fibers, protecting them from microbial and enzymatic attack, and enabling the water transport through vessels and tracheids from roots to upper trunks and branches [2,3]. The presence of lignin in the lignocellulose materials, used for biofuel production, has been suggested to retard the cellulases during their catalyzed hydrolysis of cellulose [4].

White-rot fungi are able to grow on wood materials, and are to a certain extent looked upon as undesirable because they cause decay of wood [5]. The decay is partly a result of the fungal production of oxidative extracellular enzymes that can both efficiently catalyze the degradation of lignin and catalyze detoxification of lignin-derived compounds [6,7]. In contrast, brown-rot fungi, can

only utilize the hemicellulose and cellulose of the cell wall, leaving the lignin essentially undigested, albeit modified by demethylation and oxidation [8]. *G. lucidum* (*lingzhi*) is one of the representatives of white-rot fungi (*Basidiomycota*) and is highly adaptable to grow on and degrade a wide variety of agro-industrial lignocellulosic waste and wood. This ability is presumed to be due to its ability to synthesize relevant hydrolytic (cellulases and hemicellulases) and various oxidative enzymes – some of these oxidative enzymes are presumed to be able to catalyze the oxidation of lignin [9]. In the study of Adaskaveg and Gilbertson [10] it was shown that *G. lucidum* generally caused greater weight loss of all woods tested, than a *Ganoderma tsugae* isolate. However, despite the documented ability of *G. lucidum* to efficiently degrade wood, little is known about its potential lignin modifying enzymes.

The expression system of oxidative enzymes of white-rot fungi is highly regulated by the nutrients that the fungi are exposed to [11]. The main hypothesis of this study was that if a fungus can grow on a medium supplemented with lignin, then it may also produce extracellular enzyme(s) that can promote degradation/modification of lignin or lignin-derived components. Secondly, if such enzyme activity is then added to a lignocellulosic substrate undergoing cellulolytic hydrolysis, then the added activity may boost the conversion of the cellulose. Accordingly, assessing the growth of a number (44) of white-rot fungi, known to grow on plant and decaying wood material, we hoped to identify i) a fungus with the ability to grow on a recalcitrant lignocellulosic substrate and/or directly on lignin supplemented medium, ii) the enzyme/s that had significant (relevant, oxidative) enzyme activity on lignin-mimicking substrates, and iii) assess whether this enzyme activity in turn could burst lignocellulose degradation.

Our focus was turned into a laccase, considering the fact that some fungi are able to degrade lignin even though they lack the ability to produce lignin peroxidase and manganese peroxidase [12].

Laccase (EC 1.10.3.2) is a blue copper containing enzyme, which catalyzes the removal of an electron and a proton from phenolic hydroxyl or aromatic amino groups to form free phenoxy radicals and amino radicals, respectively. During this reaction one molecule of atmospheric oxygen is reduced to two molecules of water, so that the catalysis is taking place *via* transfer of 4 electrons per round of catalysis [13]. Laccase is also able to catalyze the oxidation of non-phenolic lignin units (C_4-esterified) to radicals, and during this reaction laccase acts *via* a mediator [14].

In this paper, we present the discovery that *G. lucidum* can grow on lignin supplemented media and we annotate the presence of laccase activity in the broth derived from this *G. lucidum* growth. We also show that the addition of the *G. lucidum* derived laccase activity increased the yields of released glucose by 17% from pretreated sugarcane bagasse when added to a commercial cellulase preparation (Cellic®CTec1). We also report a partial amino acid sequence of the laccase together with a fast ABTS based screening methodology for evaluation of presence of the oxidative enzymes in the fermentation broth.

MATERIALS AND METHODS

Organisms

40 strains of the genus *Alternaria, Fusarium, Memnoniella, Stemphylium,* and *Ulocladium* were obtained from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark, (Lyngby, Denmark). The fungal isolates were listed by the IBT numbers (S1, Table 1). In addition, four white-rot fungal strains of *Ganoderma lucidum* (CBS 229.93)*, Trametes versicolor* (CBS 100.29)*, Polyporus brumalis* (CBS 470.72)*, Polyporus ciliatus* (CBS 366.74), were purchased from CBS Fungal Biodiversity Center (Utrecht, The Netherlands). All cultures were maintained on **M**alt **E**xtract **A**gar (MEA) slants at 4˚C.

Substrates

Sugar**c**ane **b**agasse (SCB) was obtained from the American Society of Sugar Cane Technologists, Florida Division (LaBelle, FL, USA). The raw biomass was washed in distilled water (5 times) to remove sand particles. The washed SCB was then dried at 50°C for 44 h to a **d**ry **m**atter (DM) content of 97% (w/w), milled using a coffee mill, and sieved to pass a sieve size of 500 μm (Endecotts, London, UK). The raw SCB contained: 51.1% (w/w) cellulose, 23.1% (w/w) hemicellulose, and 21.6% (w/w) acid insoluble lignin. For the enzymatic lignocellulose hydrolysis study, the SCB was pretreated by steam explosion: 15% dry matter (w/v) at temp. 175°C for 10 min., 11 bars pressure, and a double addition of oxygen (3 min. each session) as described previously [15]. After the steam explosion the filter cake and the hydrolysate were mixed together, dried at 55°C for 44 h, and milled using a coffee-mill and sieved to pass a sieve size 210 μm (Endecotts, London, UK). The pretreated SCB contained: 43.8% (w/w) cellulose, 13.8% (w/w) hemicellulose, and 19.3% (w/w) insoluble lignin.

The content of DM and the biomass composition was determined according to the NREL procedure [16]. The levels of glucose and xylose liberated after strong acid hydrolysis were determined by HPAEC using Dionex BioLC system equipped with Dionex CarboPac PA1 analytical column (Dionex, Sunnyvale, CA, USA) and an electrochemical detector used in the pulsed amperiometric detection mode principally as described previously [17] and as detailed by [18].

Lignin **a**lkaline (LA) was purchased from NacalaiTesque (Kyoto, Japan), Avicel was from Sigma-Aldrich (Steinheim, Germany).

Media

Malt **E**xtract medium (MEA), contained: malt extract 20 g/L; peptone 1 g/L; glucose 20 g/L, and agar 20 g/L for agar slants. MEA medium was supplemented with 1 mL of a stock trace metal solution (1 g/L $ZnSO_4*7H_2O$ and 0.5 g/L $CuSO_4 \cdot 5H_2O$).

Minimal **M**edium (MM), was prepared as described before [19] and contained NH_4NO_3 1 g/L; Na_2HPO_4 0.2 g/L; KH_2PO_4 0.8 g/L; $MgSO_4*7H_2O$ 0.5 g/L.

For the differential pre-screening of the 44 fungal strains, MM medium was supplemented with 10 g/L of SCB (the MM medium was supplemented with 2 g/L of glucose when 10 g/L SCB was added).

For the subsequent growth evaluation of the 4 selected white-rot fungi (*G. lucidum, T. versicolor, P. brumalis, P. ciliatus*), the following media were used: MEA medium supplemented with 10 g/L SCB; MM medium and MEA medium each supplemented with a combination of 5 g/L SCB and 5 g/L Avicel; MM medium and MEA medium each supplemented with 10 g/L LA (the MM medium was additionally supplemented with 2 g/L of glucose when 10 g/L LA was added).

The pH of all media was adjusted to 5.6 with 2 M NaOH prior to autoclavation. All fungal cultures were cultivated at 25˚C for a period of 16 and 30 days on the MEA and MM supplemented media, respectively. The growth of the fungi was always performed on a solid, floating support of Leca® beads (Johannes Fog, Lyngby, Denmark). The Leca® beads were added and autoclaved together with the medium principally as described in [20].

All the media used were inoculated with a 50-mm-diameter-disc cut out of the most peripheral side of the MEA agar plate, after a 7-day cultivation of the respective fungus.

Freeze-drying of crude fungal protein extracts

After growth in the respective liquid media, the crude fungal protein extracts were obtained after removal of mycelia and Leca® beads by centrifugation at 10.000 rpm for 20 min. The crude extract was frozen for 2 h prior to freeze-drying. Freeze-drying of the crude liquid extracts (15 mL) was performed on a Lyovac GT 2 (Germany) for 2 days until a visible dry pellet was formed. The dry pellet was then concentrated 10 times by solubilization in 1.5 mL of water and used for the ABTS plate assays, SDS-PAGE and Native-PAGE analysis.

Protein quantification was performed using the Pierce BCA (**Bi**cinchoninic **A**cid) protein assay kit microplate procedure according to manufacturer's instructions (Thermo Fisher Scientific, Rockford, US) as described before [21]. Bovine Serum Albumin (BSA) obtained from Sigma-Aldrich (Steinheim, Germany) was used as a protein standard.

ABTS plate assay for laccase activity visualization

The assay was performed as described before [22]. Development of a green halo (positive response for oxidative enzyme activity) was followed by placing 25 µL of 10x concentrated crude extract solution, resuspended in water (as described above) into a 4 mm well in the ABTS-agar plate. 25 µL of boiled crude extract solution was used as a negative control, while 25 µL of commercial preparation of laccase (Cat. no. 51639), lignin peroxidase (Cat. no. 42603) and manganese peroxidase (Cat. no. 93014) were used as enzyme activity controls. All enzymes were from Sigma-Aldrich, (Steinheim, Germany) and were diluted to reach the same concentration of U/mL, prior to ABTS-plate assay. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) was also from Sigma-Aldrich.

The false-positive effect of ABTS being oxidized by other than laccase factors/enzymes present in the crude protein extract, was evaluated by addition of H_2O_2 (final concentration of 0.003%, w/v) to all commercial protein preparations, and by addition of pure H_2O_2 (final concentrations of 30, 0.3, 0.03 and 0.003%, w/v) to the well of ABTS plate.

Laccase activity assay

Laccase activity was measured spectrophotometrically with syringaldazine (Sigma Aldrich, Steinheim, Germany) as a substrate, based on a method of Ryde [23]. Laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of syringaldazine per minute at 30°C and pH 6.5. Oxidation of syringaldazine was monitored at 530 nm ($\varepsilon_{530} = 65000$ $M^{-1}cm^{-1}$, [24]) for 10 min. The assay mixture contained: 100 mM phosphate buffer (2.2 mL, pH 6.5), syringaldazine (0.3 mL, 0.216 mM) and a pre-diluted fungal crude extract (0.5 mL) that ensured the linear range of Michaelis-Menten kinetics. The pre-diluted fungal crude extracts were filtered through 0.45 µm filter (MiniSart-plus, sterile, Sartorius, Germany) prior to activity measurements.

SDS-PAGE

The yield of expressed proteins into the fungal crude extract was evaluated by **S**odium **D**odecyl **S**ulphate polyacrylamid (SDS-PAGE) electrophoresis, using a Criterion XT gel system (Bio-Rad, CA, USA). The protein samples (65 µL) were diluted in XT sample buffer (25 µL, cat. no. 161-0791) and 500 mM dithiothreitol (10 µL, Sigma Aldrich, Germany). The samples were boiled at 95˚C for 5 min

before being loaded into a 10% separation gel (cat. no. 345-0118). Electrophoresis was carried out at a constant voltage of 125 V for 2 h using 5 times diluted XT MOPS as the running buffer (cat. no. 161-0788). The separated proteins were visualized by staining with Coomassie Blue G-250 (cat. no. 161-0786). Estimation of **M**olecular **W**eights (MW) of the proteins was made against molecular (stained) standards (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa) (cat. no. 161-0374). All chemicals used during SDS-PAGE were purchased from Bio-Rad, CA, USA.

Native PAGE

In order to assign the activity of protein at ~62.5 kDa to laccase, a native PAGE electrophoresis was performed and proteins in the gel were stained with laccase specific substrate (1.8-diaminonaphthalene, DAN). Freeze-dried supernatant samples were mixed in a ratio 1:1 with XT sample buffer, containing no reducing agents, and loaded onto a Zymogram gel (cat. no. 345-0080) without thermal denaturation. Running buffer and protein standards were the same as for the SDS-PAGE electrophoresis. The separated proteins were visualized by incubation of the gel in a 50 mM sodium acetate buffer (pH 5) containing: dimethyl sulfoxide (1%) and DAN solution (2 mM), as detailed by [25].

Kinetic studies of laccase isolated from *G. lucidum's* cultivation broth.

Michaelis constant (K_m) was calculated from a Hanes-Wolf plot. The substrate used in this study was ABTS at a final concentration of: 0.0113, 0.0085, 0.006, 0.0025, 0.001, 0.0005, and 0.0001 M [26]. The conditions for the enzymatic reaction were as detailed in [27].

MALDI Q TOF MS/MS analysis and conformation of laccase sequence.

The band of interest (~62.5 kDa) was excised from SDS-PAGE gel and in-gel digested with trypsin prior to MALDI-TOF analysis as previously described in [28] and as detailed in [29]. The trypsin digestion and sample analysis were carried out at the Department of Biochemistry and Molecular Biology at the University of Southern Denmark (Odense, Denmark). The short amino acid peptide sequences were obtained by the *de novo* sequencing and were analyzed using 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in MS/MS mode, followed by manual interpretation of the obtained MS/MS spectra by the AminoCalc program (Protana, Odense, Denmark).

Glucose release from pretreated SCB during laccase and cellulase catalysis

The effect of glucose release during a simultaneous laccase and cellulase catalysis of 5% (w/v) dry matter of pretreated SCB was evaluated in 0.1 M citrate-phosphate buffer pH 5.1 and 50°C (optimal for the cellulase preparation). The commercially available cellulase cocktail preparation Cellic®CTec1 (0.064% **E**nzyme/**S**ubstrate ratio (E/S), w/w; Novozyme, Denmark) was used in conjunction with a laccase-rich broth from *G. lucidum* (0.4% E/S, w/w). The hydrolysis reactions were collected after 0, 1, 3, 5, 16, and 24 hours and stopped by incubation at 99°C for 15 min. Afterwards, the samples were centrifuged at 10.000 rpm for 2 min, the supernatants were taken out and filtered through 0.2 μm filter

and the yields of released glucose were quantified using D-glucose-HK kit (Megazyme, Denmark) at 340 nm in an Infinite 200 microtiter plate reader (Tecan, Salzburg, Austria). Glucose yields released over time were corrected by the glucose amount present in the hydrolysis sample at time 0. E/S dosage was based on the total protein concentration used. Cellic®CTec1 is based on the *Trichoderma reesei* cellulase complex (exo-glucanase, endo-glucanase, and β-glucosidase activities) with additional β-glucosidase and glycoside hydrolase family 61 hydrolyse boosting proteins [30]. All determinations of the enzymatic hydrolysis samples were performed in duplicates.

RESULTS

44 white-rot fungal isolates belonging to the phyla Ascomycota (strains of the genus *Alternaria, Fusarium, Memnoniella, Stemphylim,* and *Ulocladium* (S1, Table 1)) and Basidiomycota (strains of the genus *Ganoderma, Trametes*, and *Polyporus*), were pre-screened for growth on minimal medium (MM) supplemented with sugarcane bagasse. The sugarcane bagasse was the natural model lignocellulosic substrate employed in this study. The 44 strains were selected based on *a priori* knowledge of their lignocellulosic/plant material habitat. The result of this differential pre-screening showed that only four of the basidiomycete isolates, namely *Ganoderma lucidum, Trametes versicolor, Polyporus brumalis,* and *Polyporus ciliatus* were able to grow on the sugarcane bagasse medium (within a 2 week growth period). In order to further evaluate the possible ability of any of these four strains to exhibit growth on lignin – and then implicitly express lignin-modifying or degrading enzymes – these four strains were inoculated on MM and MEA medium each supplemented with lignin alkaline (LA) and with sugarcane bagasse + Avicel, respectively.

Out of the four basidiomycetes fungi tested, only *G. lucidum* exhibited significant growth on both media supplemented with LA (Table 1). *P. brumalis, P. ciliatus* and *T. versicolor* obtained a faint mycelial growth on the MM medium supplemented with LA in comparison to a good growth of *G. lucidum* under the same conditions, but they were unable to grow on the MEA medium when LA was added (Table 1). The *G. lucidum* strain also grew well on the MM medium with SCB+Avicel and exeptionally better on the MEA with SCB+Avicel. Its growth was vivid and the colony size was much bigger (in diameter) and thicker when compared to that of *P. brumalis, P. ciliatus* and even the well-studied oxidative enzyme producer – *T. versicolor* (Table 1).

Table 1. Evaluation of the fungal growth[1] on the recalcitrant substrate supplementing both MM and MEA medium

Cultivation media used[2]		*Ganoderma lucidum*	*Polyporus brumalis*	*Polyporus ciliatus*	*Trametes versicolor*
LA [3]	MM	+	±	±	±
	MEA	++	-	-	-
SCB + Av. [4]	MM	++	+	+	+
	MEA	+++	+	+	+
SCB [5]	MM	++	+	+	+
	MEA	+++	+	+	+

[1]The grading of the fungal growth on recalcitrant substrate supplementing MM and MEA medium on a (+), (-) scale. (±) faint growth (single mycelium colonies), (+) good growth (mycelium mat covering the whole diameter of the experimental tube), (++) better growth (as in case of good growth but mycelium mat thicker), (+++) exceptional growth (thick mycelium mat plus spreading fungal growth on the walls of the experimental tube), and (-) no growth. The grading of the fungal growth was based on the area of mycelium (colony size) floating on the Leca® beads support (see Material and Methods section) added to the cultivation medium.

[2]Cultivation media used in this study; MEA (malt extract medium) and MM (minimal medium)

[3, 4, 5]Cultivation media supplementation: Lignin Alkaline (LA), sugarcane bagasse and Avicel (SCB + Av.), and sugarcane bagasse (SCB), respectively.

Oxidative enzyme activity assessment of the fungal crude protein extracts on ABTS

ABTS oxidation was used to evaluate the possible presence of oxidative enzyme activity in the crude fungal extract: The oxidation of ABTS in the agar plate assay followed the growth ability pattern on the different media tested, meaning that if a fungus could grow on a medium supplemented with SCB (with or without Avicel) or LA, then it also expressed enzymes that enabled activity on ABTS. The strongest response in the ABTS plate assay (appearance of a green halo, data not shown) was observed for the crude protein extract of *G. lucidum* for all media tested. No response for oxidation of ABTS was observed for *P. brumalis, P. ciliatus* and *T. versicolor* for the MEA medium supplemented with lignin alkaline (LA), which was in accordance with their lack of growth on this medium (Table 1). Oxidation level of ABTS for the LA supplemented MM medium was very low for *P. brumalis, P. ciliatus* and *T. versicolor* and in contrast significant for *G. lucidum*, also in accordance with the growth ability data (Table 1).

To assure that the ABTS oxidation was not a "false-positive" auto-oxidative response of ABTS, the addition of hydrogen peroxide in concentrations of 30, 0.3, 0.03 and 0.003% (w/v) were tested. Only the H_2O_2 at a level as high as 30% (w/v) was able to auto-oxidize ABTS (data not shown). That result excluded the possibility that the oxidation of ABTS was caused by H_2O_2 produced by fungal oxidases. Moreover, neither lignin peroxidase nor manganese peroxidase added, were able to oxidize ABTS unless 0.003% (w/v) H_2O_2 was added to the extract mixture (data not shown).

Kinetic studies of laccase present in the crude extract of *G. lucidum*

The kinetic studies for the *G. lucidum* laccase present in the crude protein extract were performed using ABTS as a substrate. ABTS was used in order to compare the obtained K_m value with those described in the literature. From the Hanes-Wolf plot, which gave a good distribution of the data points, the K_m value was calculated to be 0.107 mM (Table 2). This value of K_m places laccase from *G. lucidum* in the middle range of laccases presented in the Table 2, however is significantly higher than a K_m value belonging to another *G. lucidum* laccase, namely GaLc3 [31]. V_{max} value was not calculated due to lack of purity of the analyzed laccase containing sample.

Table 2. Kinetic constants of laccases and the pH value at which K_m was measured

Substrate	Laccase	K_m (mM)	pH	Reference
ABTS	*Pleurotus sajor-caju* Lac4	2.50	3.3	46
	Myceliophthora thermophila Lcc1	0.29	6	47
	Pleurotus ostreatus POXC	0.28	3	48
	Pleurotus ostreatus POXA2	0.12	3	48
	Ganoderma lucidum CBS229.93	0.107	5	this article
	Pleurotus ostreatus POXA1	0.09	3	48
	Rhizoctonia solani Lcc4	0.052	5.3	49
	Ganoderma lucidum GaLc3	0.037	5	31
	Trametes trogii POXL3	0.03	3.4	50

Laccase activity assessment for fungal crude protein extracts on syringaldazine

The highest activity of laccase, present in the fungal crude protein extract using syringaldazine as substrate, was obtained for *G. lucidum* cultivated on MEA medium supplemented with SCB or SCB+Avicel, and was 3.1 and 2.9 U/mL, respectively (Fig. 1). This level of laccase activity was

approximately 3.5 times higher than when *G. lucidum* was grown on MM medium under the same supplement conditions. These activity levels of laccase were consistent with the vivid growth of *G. lucidum* on the media supplemented with SCB. The laccase activity in the crude extracts from *P. brumalis*, *P. ciliatus*, and *T. versicolor*, respectively, was 13 to 17 times lower than that obtained for *G. lucidum* cultivated on the same media (i.e. MM or MEA supplemented with SCB or SCB+Avicel) (Fig. 1). Unfortunately, it was not possible to measure laccase activity on media supplemented with LA, due to the interference from the black coloring of lignin and inability to separate it from the cultivation media.
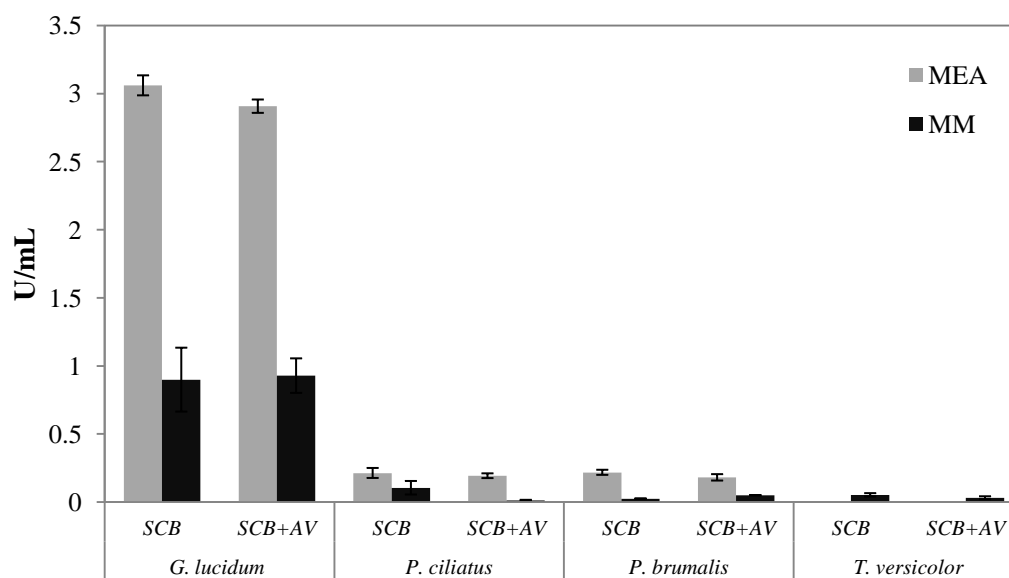

Figure 1. A comparison of the laccase activity in the crude protein extracts from *G. lucidum, P. ciliatus, P. brumalis,* and *T. versicolor*, cultivated on MEA and MM media, supplemented with SCB or SCB+Av, where SCB – sugarcane bagasse, and Av – Avicel. Laccase activity was measured using syringaldazine in accordance with suggestions from Lonergan and Baker [51]. The highest activity of laccase was observed for the crude protein extracts from *G. lucidum*. In general, laccase activity was higher when fungal strains were cultivated on MEA (SCB and SCB+Av) media. The activity of laccase for *T. versicolor* could not be determined. The gray and black bars indicate the amount of the laccase activity present in the fungal crude protein extracts from MEA and MM media, respectively.

Separation of crude protein extract from *G. lucidum* using denaturing (SDS-PAGE) and native (Native PAGE) conditions.

Ganoderma lucidum crude protein extract was analyzed by sodium dodecyl sulphate (SDS-PAGE) electrophoresis, due to the fungal ability to grow on media supplemented with LA and high laccase activity in media supplemented with SCB+Avicel, and SCB. Comparison of bands and MW of a control laccase from *T. versicolor* with the proteins from the crude extract indicated presence of laccase (Fig. 2). The band in the separated crude protein extract, corresponding to the laccase from *T. versicolor*, was approximately 62.5 kDa. It was slightly lower than a MW of the commercial laccase. Additionally, a sharp, distinct band of ~62.5 kDa was clearly visible in case of MEA medium supplemented with LA, regardless of the extensive, black background smearing.

In order to assign that the MW of the protein from *G. lucidum* belongs to laccase an in-gel activity staining was performed with the substrate specific for laccase (1.8-**di**amino**n**aphthalene, DAN). Only a single band at 62.5 kDa in each lane (Fig. 3) was stained with DAN. A specific dark brown band

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was observed which confirmed presence of an active laccase in the *G. lucidum* crude extract preparations derived from the different MEA supplemented media (MEA + SCB; MEA + SCB+Avicel; MEA + LA) (Fig. 3). The extracts from the MEA supplemented media were selected due to their higher laccase activity (Fig. 1). Additionally, no color development was observed neither for the commercial controls of lignin peroxidase and manganese peroxidase (data not shown), nor for the lower molecular weight proteins from the crude protein extract. The protein that showed activity towards DAN was examined for its amino acid sequence by MALDI-TOF.

Figure 2. An electrophoretic separation (SDS-PAGE under denaturating conditions) of the proteins present in the *G lucidum*'s crude extract, obtained under different cultivation conditions. All protein bands were stained with Coomassie Blue G-250. Wells in the gel represent; protein marker (M), laccase protein standard (4), and protein crude extract from MEA medium supplemented with SCB (1), SCB+AV (2), and LA (3). The laccase from *G. lucidum* (estimated to 62.5 kDa) had a slightly lower molecular weight than that from a control (*Trametes versicolor*, Cat. no. 51639, Sigma Aldrich, Germany).
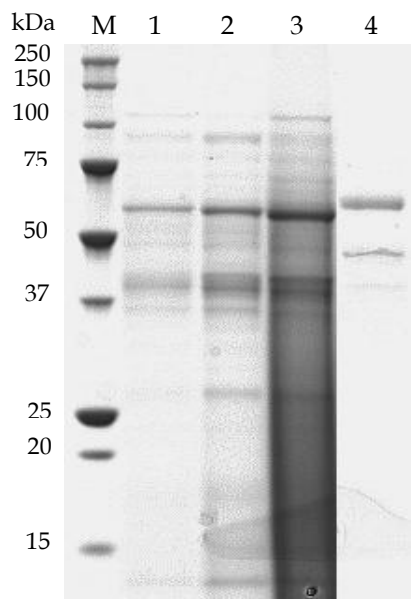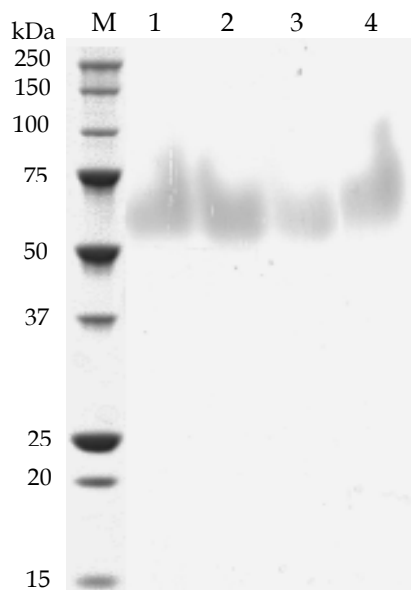
Figure 3. An electrophoretic separation (Native-PAGE under non-denaturing conditions) of the proteins present in the *G lucidum*'s crude extract, obtained under different cultivation conditions. All protein bands were stained with Coomassie Blue G-250. Wells in the gel represent; protein marker (M), laccase protein standard (4), and protein crude extract from MEA medium supplemented with SCB (1), SCB+AV (2), and LA (3). Native PAGE electrophoresis confirmed presence of active laccase at 62.5 kDa.

Partial amino acid sequence of laccase by MALDI-TOF/MS analysis

The protein band at 62.5 kDa, putatively identified as the laccase enzyme, was further analyzed by MS after an in-gel digestion with trypsin. The short peptide amino acid sequences discovered *de novo* by MALDI-TOF analysis had a high homology (64 to 100%) to laccase sequences from other basidiomycetes (Table 3). The blasting results in UniProt database [32] showed that the amino acid sequences between position 88 to 100 and position 185 to 197 are highly conserved within the family of *Ganodermataceae's*. The blasting results corroborated that the *G. lucidum* enzyme was a laccase. However, the fact that different peptide fragments, especially in the amino acid sequence regions of 245-263 and 452-466, exhibited less than 90% sequence identity to existing functional laccases put focus on the fact that the *G. lucidum* might harbor special sequential features that could indicate its uniqueness.

Table 3. Overview of the identity of four glycopeptides analyzed by MALDI-TOF from *G. lucidum* CBS229.93 to other laccases deposited in UniProt database

UniProt identyfier	*Identified organism*	*AA seq.*	*sequence identity of discovered glycopeptides*, (%)			
			[88]TTSIHWH GFFQK[100]	[185]GSDSTLI NGLGR[197]	[245]DDDSTVL TLADWYHV AAR[263]	[452]TLSNADI APDGFTR[466]
Q9GH17	*Ganoderma lucidum 7071-9*	520	100	100	80	81
Q9HDS8	*Polyporus ciliatus*	524	90	100	80	81
C5HL41	*Ganoderma lucidum TR6*	520	100	100	80	81
B5G552	*Ganoderma lucidum RZ*	520	100	100	75	88
Q308Q9	*Trametes versicolor*	522	100	93	85	64
A3F8Z8	*Polyporus brumalis lac1*	520	100	93	90	70
A3F8Z8	*Polyporus brumalis lac2*	524	90	93	n.a.	n.a.
Q9UVQ2	*Pycnoporus cinnabarinus lac1*	518	90	93	75	68

n.a. – not annotated.

Glucose release from pretreated SCB during laccase-cellulase catalyzed hydrolysis of SCB

Addition of the laccase-rich *G. lucidum* broth to lignocellulosic biomass (pretreated sugarcane bagasse) together with cellulase enzyme preparation (Cellic®CTec1, Novozymes A/S, Denmark) resulted in a 17% increase in the overall glucose yields, at the applied experimental conditions; 24h, pH 5.1, and 50°C (Fig. 4). The glucose yields were evaluated during cellulase catalyzed hydrolysis of pretreated sugarcane bagasse. That the effect was a result of the added laccase activity was corroborated by the finding, that the *G. lucidum* protein extract exhibited no cellulase activity on AZCL-cellulose.
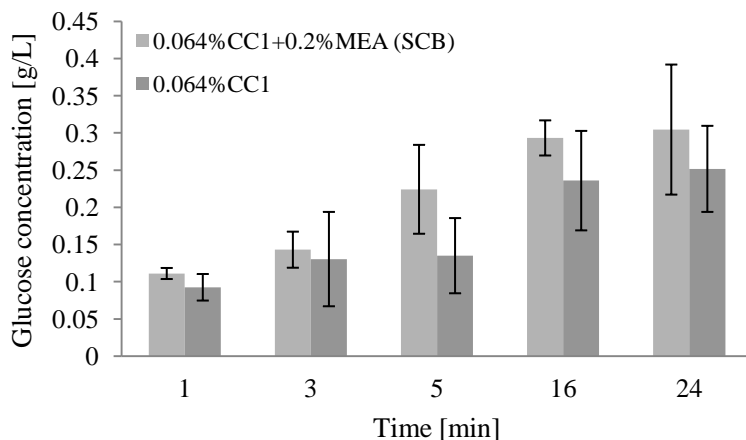
Figure 4. A graphical comparison of glucose yields released during a 24 h laccase-Cellic®CTec1 catalyzed hydrolysis of 5% (w/w) steam exploded SCB at pH 5.1 and 50°C. Cellic®CTec1 (CC1) and the laccase-rich crude protein extract from *Ganoderma lucidum* (MEA(SCB)) were added in an enzyme/substrate (E/S) ratio of 0.064%, and 0.2% (w/w), respectively. The light and dark grey bars indcate the amount of released glucose (g/L) for laccase-Cellic®CTec1 or Cellic®CTec1 catalyzed hydrolysis of pretreated SCB, respectively. The glucose yields were 17% higher when laccase-rich fungal protein extract was added to the cellulase catalyzed hydrolysis of the pretreated lignocellulosic material.

DISCUSSION AND CONCLUSIONS

The initial differential screening showed that only four out of 44 white rot fungi could grow significantly on media supplemented with lignocellulose (lignocellulose in the form of raw sugar cane bagasse). It was speculated that fungi that are capable of growing on SCB and lignin alkaline (LA), potentially express new interesting enzymes that may be able to catalyze modification of lignin in lignocellulosic biomass undergoing cellulosic hydrolysis. In turn, such lignin modification might yield a higher cellulose conversion during cellulase catalyzed hydrolysis of lignocellulosic substrates by increasing the available surface area and/or by diminishing the adsorption of cellulases to lignin [33]. The growth screening analysis showed that the growth of *P. brumalis, P. ciliatus*, and *T. versicolor*, which were able to proliferate on sugar cane bagasse, was apparently inhibited by addition of LA to a malt extract medium (regardless of the MEA being a sugar and nutrient rich medium). However, *G. lucidum* was found to grow significantly with LA added, i.e. *G. lucidum* grew faster and to a larger colony size than the other fungi tested. *T. versicolor* is a well studied oxidizing enzyme producer [34] and was chosen as a positive control for assessment of the growth ability on recalcitrant lignocellulose substrates and on LA. It was therefore surprising that the growth of *T. versicolor* was rather poor on LA since this fungus is known to produce laccase. Laccase from *T. versicolor* has been reported to detoxify lignocellulosic hydrolysates from wood via catalytic oxidation of monophenolic compounds and in turn improve the fermentability of these wood hydrolysates better than for example lignin peroxidase [35]. Based on the finding that *G. lucidum* could grow with LA added to the medium, it was conceived that the *G. lucidum* might produce one or more oxidative enzymes able to catalyze the modification of lignin constituents. Hence, the ability of *G. lucidum* – as the only fungus, to grow well on LA was indeed an indication that it had a unique, as compared to *P. ciliatus, P. brumalis*, and *T. versicolor*, enzyme system that allowed it to degrade/modify lignin or to overcome the toxic/inactivating effect of LA addition under carbon and nitrogen rich growth conditions (MEA medium). When coupling this interpretation to the growth data on MM or MEA supplemented with LA, it might be possible that the laccase from *G. lucidum* had a role in its ability to grow on the LA supplemented media.

This comprehension agrees with previously reported results that indicate that *G. lucidum* expresses lignin-modifying enzymes, notably laccase when cultivated on wood-derived lignocellulosic substrates (poplar, spruce, oak, and pine) [36]. Earlier studies indicate that white-rot fungi produce only laccase in defined media, and laccase and manganese peroxidase in cultures grown with poplar [37,38,39]. Paleaz et al. [9] reported that out of 68 fungal strains tested, laccase activity was found in 50% of them and that laccase had the highest activity among other oxidizing enzymes tested (namely, MnP, AAO, and LiP). MnP and AAO were present in 40 and 29% respectively, and LiP was not detected in any of the 68 fungi tested.

Tour et al. [40] classified different white-rot fungi into five groups, based on the oxidative type of enzyme(s) they produce, and laccase was frequently a dominant enzyme indicating its important role in lignin degradation. Laccase, being a phenol oxidase (EC 1.10.3.2), catalyzes the oxidation of phenols and phenolic lignin substructures by electron abstraction and during this reaction, the enzyme is presumed to simultaneously catalyze the formation of radicals that can re-polymerize or lead to depolymerization [41]. Laccase thus has polymerizing and depolymerizing activity while MnP and Lip only polymerize phenoxy radicals [40]. Due to their adaptable redox potential laccases may catalyze the oxidation of both phenolic and non-phenolic lignin derived substrates [42]. The specific laccase from *G. lucidum* was not induced by carbon depletion - as it might be in case of other white-rot fungi tested when low amounts of laccase were produced when minimal media were used. This finding indicates that the laccase expression might be induced by tryptophan or tyrosine being present in the malt extract [43]. It is worth noting that Eggert at el. [12] identified a tryptophan derivative (4-hydroxyindole) and tryptophane derived metabolite (3-hydroxy-2-aminobenzoate) acting as a mediator in laccase-catalyzed reactions performed by *P. cinnabarinus*. In general, a change in laccase activity corresponding to the type of media used (i.e. nitrogen and carbon source availability) is a controversial subject [11]. Some authors found that the "ligninolytic" enzyme activity production by certain white rot fungi increased under nutrient and carbon limiting conditions, and others reported the opposite dependence [19,39,44].

The ABTS plate assay was used to grade the oxidative enzyme activity based on the degree of green color development and the speed with which the oxidation of ABTS took place. Comparing these results with the initial growth screening (Table 1), it was found that the activity of fungal crude protein extracts on ABTS correlated well with the growth rate. Additionally, the laccase from *G. lucidum* had relatively high value of K_m (0.107 mM), indicating a low enzyme affinity to the substrate. The substrate selectivity, thermal robustness, and the response of the laccase activity to reaction conditions deserve a further study. The activity band staining of enzymes with a phenolic substrate revealed a presence of laccase with a MW of ~62.5 kDa which was further confirmed by MALDI-TOF analysis. The short amino acid sequences compared with the fungi tested for the growth ability (Table 3) revealed significant differences between the amino acids in the short stretches. Additionally, this laccase showed the highest short-peptide similarity to *Ganoderma lucidum* 7071-9 (Q9GH17, UniProt identifier). The laccase-rich crude protein extract from *G. lucidum* was able to increase the released glucose yields by as much as 17%. The laccase from *G. lucidum* might act on lignocellulose by opening of its very rigid structure and in this way allowing cellulases a more efficient action on cellulose fibers. Alternatively the presumed laccase promoted mechanism may involve enzymatic oxidation of the lignin derived components to lesser inhibitory compounds for the cellulase hydrolysis. This ability of laccase could be useful for the bioethanol industry, where high yields of fermentable sugars are desired. *G. lucidum* also happens to be a well known medicinal mushroom in traditional Chinese medicine and is commonly used for pharmaceutical purposes and in health foods [19,45]. Hence, the results presented may provide a step for multiple uses of *G. lucidum* in future bio-industrial applications.

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SUPPLEMENTARY MATERIAL

Alternaria arborescens	IBT BA1342
Alternaria arborescens	IBT BA500
Alternaria arborescens	IBT BA961
Alternaria infectoris	IBT BA1289
Alternaria infectoris	IBT BA1759
Alternaria infectoris	IBT BA562
Alternaria tenuissima	IBT BA1767
Alternaria tenuissima	IBT BA577
Alternaria tenuissima	IBT BA617
Alternaria tenuissima	IBT BA894
Alternaria triticina	IBT BA1207
Fusarium avenaceum	IBT 40847
Fusarium coeruleum	IBT 2630
Fusarium equiseti	IBT 40221
Fusarium equiseti	IBT 8752
Fusarium flocciferum	IBT 1434
Fusarium flocciferum	IBT 8932
Fusarium langsethiae	IBT 8051
Fusarium langsethiae	IBT 9955
Fusarium pose	IBT 9928
Fusarium pose	IBT 9982
Fusarium pose	IBT 9999
Fusarium sambucinum	IBT 1731
Fusarium sambucinum	IBT 2364
Fusarium sambucinum	IBT 2524
Fusarium sporotrichioides	IBT 1926
Fusarium sporotrichioides	IBT 9967
Fusarium subglutinans	IBT 40638
Fusarium torulosum	IBT 8580
Fusarium torulosum	IBT 8922
Fusarium tricinctum	IBT 1945
Fusarium tricinctum	IBT 2660
Fusarium tricinctum	IBT 8838
Fusarium venenatum	IBT 1338
Memnoniella echinata	IBT 9789
Memnoniella echinata	IBT 9791
Stemphylium versicarium	IBT BA910
Ulocladium atrum	IBT BA1539
Ulocladium cucurbitae	IBT BA1540
Ulocladium cucurbitae	IBT BA520

A Novel Laccase from Ganoderma lucidum, Recombinant Enzyme Expression, and the Enzymatic Boosting of Lignocellulosic Biomass Degradation

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A novel laccase from *Ganoderma lucidum*, recombinant enzyme expression, and enzymatic boosting of lignocellulolytic biomass degradation.

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Key words *Ganoderma lucidum*, laccase, RACE PCR, laccase-cellulase catalyzed hydrolysis, sugarcane bagasse, molecular dynamics simulations.

■ **Abstract** Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze the oxidation of aromatic compounds with concomitant reduction of dioxygen to water. A novel laccase, LacGL1, was identified from the crude enzyme extract of the fungal strain *Ganoderma lucidum* CBS229.93, belonging to the phylum Basidiomycota. The laccase gene sequence, *LacGL1*, was cloned by PCR involving an overlapping set of up- and downstream primers. The cloned gene consisted of 1963 bp, with its coding regions interrupted by 9 introns, each containing from 50 to 76 nucleotides, which all followed the GT-AG rule at the intron/exon junctions.   A full-length cDNA of *LacGL1* which contained an uninterrupted open reading frame (ORF) of 1563 bp, was also cloned. The cloned laccase gene coded for 520 amino acids including a putative 21-residue signal sequence, and the amino acid sequence had 91% and 81% identity with known laccases from *Ganoderma lucidum* and *Polyporus brumalis*, respectively. The LacGL1 laccase was also heterologously expressed in *Pichia pastoris*. The value of K_m on ABTS substrate of the expressed LacGL1 was 0.122 mM and the laccase activity on ABTS was 17.5 U/mL. The pH optimum was 4.7 and the enzyme was relatively stable at 50°C for 60 min. with a remaining activity of 85%. Generally, the kinetics observed for the expressed LacGL1 laccase were similar to those from a crude extract of *G. lucidum* CBS229.93. The LacGL1 laccase was also able to catalyze the oxidation of hydroquinone, guaiacol, and 2,6-dimethoxyphenol and was strongly inhibited by sodium azide. Most interestingly, the expressed enzyme was able to promote higher yields of glucose during cellulase catalyzed hydrolysis (no mediators used) of pretreated sugarcane bagasse by up to 33% and 19% for Cellic®CTec1 (pH 4.7 and 40°C) and Cellic®CTec2 (pH 5.1 and 50°C), respectively ("Cellic®CTec" series are the state-of-the-art *Trichoderma reesei* cellulase preparations). Through the molecular dynamics simulations of *G. lucidum* laccase and subsequent docking of the *p*-coumaric acid into the MD-averaged structure, we suggest a mechanism of the LacGL1 action that may contribute to obtaining higher glucose yields during cellulase catalyzed hydrolysis of lignocellulose.

INTRODUCTION

SugarCane Bagasse (SCB) is a fibrous residue of cane stalks that is left over after the crushing and extraction of the sugar rich juice from sugarcane (*Saccharum officinarium*). SCB mainly consists of cellulose 51% (w/w), hemicellulose 23% (w/w), and insoluble lignin 22% (w/w), and may be used as a biomass feedstock for cellulosic ethanol production.

One of the first prerequisites in such ethanol production is the efficient generation of a fermentable hydrolysate, rich in glucose, from the biomass feedstock. However, the efficient decomposition of the (ligno)cellulose to fermentable monosaccharides is currently a major bottleneck in lignocellulose-to-ethanol processes, notably because of the significant robustness of the lignocellulosic biomass to an enzymatic deconstruction (Selig et al. 2007). In order to increase the accessibility of the cellulose to the enzymatic attack, the lignocellulosic substrates are currently subjected to a hydrothermal pretreatment step prior to the enzymatic treatment (Pedersen et al. 2010). Although significant progress has recently been made with respect to improving the cellulolytic enzyme blends and minimizing the enzyme addition levels for the conversion of cellulose to glucose, the presence of lignin and lignin-derived phenolics after the pretreatment retards the enzymatic conversion (Palonen et al. 2004; Selig et al. 2007).Various attempts have been made to design integrated pretreatment systems for removing the lignin from the pretreated biomass (Koo et al. 2012; Yang et al. 2012) but there is a surprising scarcity of work directed towards enzymatic pacification of the lignin and the lignin-derived components to enhance cellulolytic lignocellulose conversion.

Laccases (benzenediol: dioxygen oxidoreductases, EC 1.10.3.2) are copper-containing blue oxidases that catalyze the oxidation of phenolic units of lignin and a number of phenolic compounds and aromatic amines (with co-presence of a mediator) to radicals, with molecular oxygen as the electron acceptor that is reduced to water (Solomon et al. 2008). Laccase has been used to decrease the toxicity of steam exploded wheat straw (Jurado et al. 2009) and sugarcane bagasse (Martin et al. 2007) for second generation bioethanol. The characteristic properties that define a good laccase include its redox potential (directly related to k_{cat}) but also the laccase affinity (K_m) to the substrate to be oxidized. In general, the higher the redox potential, the easier the electrons are abstracted from the phenolic phenoxy groups. In nature, laccase produces Mn(III) chelates which allow wood decaying enzymes to penetrate wood cell walls (Youn et al. 1995). Therefore, laccases are considered to be capable of degrading lignin.

In nature the best lignin degraders are white-rot fungi (Kawase 1962). *Ganoderma lucidum* (Ling Zhi) is a non-pathogenic, white-rot, basidiomycete macrofungus known as "the mushroom of immortality" which extract and bioactive compounds has been used extensively in China, Japan, Korea, and other Asian countries for 2000 years (Sanodiya et al. 2009). Moreover, *G. lucidum* is important due to its role as a decomposer of dead wood, and it can both grow on coniferous and hardwood species (Adaskaveg et al. 1990), having enzymes that allow it to break down wood components such as lignin and cellulose (Kirk and Moore 1972).

Based on our discovery that *G. lucidum* CBS 229.93 can grow on lignin and express high levels of laccase activity, the objective of the present study was to clone and express laccase from *G. lucidum* in *P. pastoris* to obtain the moncomponent enzyme and in turn evaluate the potential boosting effect of adding this laccase during cellulase catalyzed hydrolysis of pretreated lignocellulose. The motivation for this study was the documented detrimental effect of lignin on cellulase activity, for which one cause may be the decreased enzyme concentration due to the cellulase adsorption to lignin (Converse et al. 1990; Ooshima et al. 1990).

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MATERIALS AND METHODS

Strains and plasmids used in this study

Ganoderma lucidum (strain CBS229.93), a white-rot, basidiomycete fungus, was purchased from CBS-KNAW Fungal Biodiversity Center (Utrecht, The Netherlands) and maintained on Malt Extract Agar (MEA) slants (2% (w/w) malt extract, 0.1% (w/w) peptone, 2% (w/w) glucose, and 1.5% (w/w) agar). The medium was adjusted to a pH of 6.0 with 2 M NaOH, prior to sterilization (121°C, 20 min.).

Escherichia coli, strain Mach1™-T1^R (F- Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(rk-, mk+) Δ*rec*A1398 *end*A1 *ton*A; Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) was grown on a modified LB (Luria-Bertani) medium (1% (w/w) tryptone, 0.5% (w/w) yeast extract, and 0.1% (w/w) sodium chloride, instead of 1% (w/w) sodium chloride, pH 7), supplemented with 35 μg/mL kanamycin and transformed with a sequencing vector pCR®-BluntII-TOPO®, carrying genomic DNA and cDNA sequences of the *LacGL1* gene. *E. coli* was transformed using guidelines from the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA).

Pichia pastoris strain X-33 (wild type) was grown on Yeast extract Peptone Dextrose Sorbitol (YPDS) plates containing 100 μg/mL zeocin for 3 days at 30°C after being transformed with a *MssI* (*PmeI*) linearized plasmid pMLα_LacGL1, carrying a laccase gene with a C-terminal c-myc epitope and HIS tag and an N-terminal signal peptide from *Saccharomyces cerevisiae* α-mating factor pre pro peptide (MRFPSIFTAVLFAASSALA) (Fig. 1). *P. pastoris* X-33 negative control was transformed with *MssI* (*PmeI*) linearized pPICZαA plasmid. All plasmids were purified with QiAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany).

Genomic DNA and mRNA isolation

0.1 to 0.3 g of *G. lucidum*'s mycelium was collected from MEA plates using a sterile scalpel, transferred to a previously cooled mortar container, and grinded in liquid nitrogen with a pestle. The genomic DNA was isolated using the chloroform: phenol: isoamyl alcohol (25:24:1, v:v:v) method previously described by Lee and Taylor (1990). Extracted DNA pellets, from 10 isolation tubes, resuspended in 10 μL of TE buffer each (10 mM Tris-HCl, 100 mM EDTA, pH 7.2) were collected and precipitated together using 2 to 3 volumes of ice-cold 96% ethanol (v/v) and 1/10 volume of 3 M sodium acetate, pH 5.2. Such mixture was incubated at -20°C overnight and centrifuged at 14000 rpm at 4°C for 30 min. the next day. The supernatant was discarded, whereas the pellet was washed in 70% ethanol (v/v), dried at room temperature (but not over dried), resuspended in a desired amount of TE buffer, and used for further experiments.

In order to isolate mRNA, *G. lucidum* was grown in Minimal Medium (MM) medium (0.1% (w/w) NH$_4$NO$_3$, 0.02% (w/w) Na$_2$HPO$_4$, 0.08% (w/w) KH$_2$PO$_4$, 0.05% (w/w) MgSO$_4$*7H$_2$O) supplemented with 0.5% (w/w) raw SCB for 7 days at 25°C. The mycelial samples were harvested and treated with liquid nitrogen as described for DNA isolation. The total RNA was extracted using the Qiagen RNeasy Mini kit (QIAGEN, Hilden, Germany). The contaminating DNA was removed by carrying out an in-column DnaseI digestion, according to the manufacturer's recommendations. The DnaseI treated RNA samples were stored in water at -20°C.

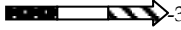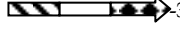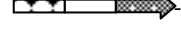## A two step partial amplification of *LacGL1* gene based on genomic DNA

Prior to the amplification and the *LacGL1* gene primer design, an in-gel digestion of electrophoretically separated by SDS-PAGE proteins from *G. lucidum* growth medium, was performed. Then, the short peptides, obtained by MALDI-TOF analysis (as described by Sitarz et al. 2012), were blasted in the UniProtKB database (http://www.uniprot.org/) to find similar protein sequences. The conserved stretches of amino acid sequences that corresponded to the sequences of the short peptides found within the organisms deposited in the database, served as the regions where primers for a 500 bp nucleotide fragment were designed (Table 1). The sequences of designed primers were complementary to the mRNA sequence of laccase from *G. lucidum* (UniProt database protein identifier; Q9HG17) and overlapped each other so that the 3' end of a first primer was repeated at the 5' end of the second primer. In total, two sets of four upstream and downstream primers were designed, which gave 16 pairs of primers to be tested. The *LacGL1* gene amplification of 500 bp was carried out by using 8U of RUN polymerase (A&A Biotechnology, Poland) per volume of 25 µL. The PCR temperature program was initiated at 94°C for 5 min., followed by 30 cycles of 94°C for 30 s., 58°C for 30 s., 72°C for 2 min., and a final extension at 72°C for 7 min. The amplified 500 bp fragment was purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), subcloned and sequenced in the pCR®-BluntII-TOPO® vector. The obtained nucleotide sequence was confirmed by a blast search in the NCBI database to belong to laccases and served as a template for designing 100% homologous outward primers (QYCDGLR_fwd1 and YHSHLST_rev1) that were used to amplify the 2000 bp *LacGL1* gene fragment. Due to a high nucleotide homology between LacGL1 and a corresponding 500 bp fragment from *G. lucidum* Q9HG17, it was assumed that there is also a high homology for the entire gene. Therefore the outermost primer sequences, close to N- and C-termini were designed as those for 500 bp fragment (Table 1). The PCR profile for obtaining a 2000 bp fragment of *LacGL1* gene was initiated by denaturation of the DNA strain at 96°C for 30 s., followed by 35 cycles at 96°C for 30 s., 63.4°C for 30 s., 72°C for 30 s., and a final extension at 72°C for 5 min., with the following aliquots of the PCR reaction mixture: 0.25 µL of 2U/µL Phusion polymerase (Finnzymes, Finland), 0.5 µL of 50 mM $MgCl_2$, 5 µL of 5xHF buffer, 0.2 µL 25 mM dNTPs (Fermentas, Denmark), 2.5 µL of 10 pmol/µL of corresponding P1 and P2 primer (see Table 1), and 1 µL of a properly diluted DNA. The amplified 2000 bp fragment was subcloned to a sequencing vector and sequentially verified.

Obtaining the full nucleotide sequence of *LacGL1* cDNA from total RNA of *G. lucidum* by 5' and 3' RACE

The first strand cDNA library construction and the Rapid Amplification of cDNA Ends (RACE) experiment were carried out using SMARTer™ RACE cDNA Amplification Kit (CloneTech, USA), in order to determine the missing 8% of the flanking regions of *LacGL1* gene sequence. 5' and 3' RACE PCR fragments were generated using the Universal Primer Mix, as supplied in the kit, and gene specific primer QKGTNWAD_fwd or SLANPVPK_rev (Table 1), respectively, designed based on the partial nucleotide sequence of the gene. The reaction cycle for the RACE technique was as follows: 94°C for 1 min.; five cycles of 94°C for 30 s, 72°C for 3 min.; five cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min.; and 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min. The generated RACE products were cloned into pCR®-BluntII-TOPO® vector and sequenced. The overlap of 5' and 3' sequence fragments enabled assembling of the full coding sequence of the *LacGL1* gene. The obtained full nucleotide sequence of the *LacGL1* gene was then amplified from the

cDNA library using MAKFQSL_fwd and LSVDDQ_rev primer (Table 1) and sequenced in a pCR®-BluntII-TOPO® vector.

Tabel 1. Oligonucleotide primers used for amplification of *LacGL1* gene

Primer name[a]	Primer sequence[b]
Primer 1	5′- ▭▭▭▶-3′
Primer 2	5′- ▭▭▭▷-3′
Primer 3	5′- ▭▭▭▶-3′
Primer 4	5′- ▭▭▭▷-3′

Primers used for amplification of 500bp fragment from LacGL1 gene from genomic DNA

WADGP_fwd3	5'-CTG GGC GGA TGG TCC CGC-3'
LINGLP_rev1[c]	5'-CGG CCA AGG CCA TTG ATG AG-3'

Primers for amplification of *LacGL1* gene fragment outwards from 500 bp fragment

QYCDGLR_fwd1	5'-CAG TAC TGC GAC GGT CTA AGA GG-3'
YHSHLST_rev1	5'-GGT GGA GAG ATG ACT GTG GTA CC-3'

Primers used for amplification of 2 kbp fragment of *LacGL1* gene from genomic DNA

IAPDGFT_fwd1[c]	5'-CAT CGC TCC CGA TGG CTT CAC TC-3'
DLCTS_rev1[c]	5'-CTG ACG TCG GGC AAA GAT CCG-3'

Primers used for amplification of a full nucleotide sequence of *LacGL1* gene from cDNA

SLANPVPK_rev	5'-CTT CGG CAC AGG GTT TGC TAG GGA G-3'
QKGTNWAD_fwd	5'-CAG AAG GGC ACG AAC TGG GCT GAC-3'
LSVDDQ_rev	5'-TAG CGC GGC CGC CTA TTA TCA TTG ATC ATC GAC CGA GAG CG-3'
MAKFQSL_fwd	5'-ATG CGA ATT CAT GGC GAA GTT CCA ATC GTT GC-3'
α-FACTOR_fwd	5'-ATG CGA ATT CGG CAT CGG TCC CAA GAC C-3'

[a] The name of the primer was based on the corresponding amino acid region in the laccase of *Ganoderma lucidum* Q9GH17.
[b] Oligonucleotide sequence was designed as shown in the figure supplementing the table. Primers for amplification of *LacGL1* gene sequence overlapped each other as shown with the pattern code, in order to maximize the positive response of the primer annealing to the genomic DNA of unknown LacGL1 gene sequence. The chosen amino acid regions for primers design were based on mRNA of laccase from *G. lucidum* Q9HG17.
[c] Underlined amino acids in the primer name represent single positional mutations in LacGL1 laccase as compared to laccase from *G. lucidum* Q9GH17.

Construction of an expression vector pMLα_LacGL1

To construct the expression vector, the laccase cDNA was amplified using a proofreading polymerase: Phusion® Hot Start II High-Fidelity DNA Polymerase (Finnzymes, Finland). For subcloning the laccase cDNA with the α-mating factor pre pro peptide from *Saccharomyces cerevisiae* (Brake, at al. 1983), primers α-FACTOR_fwd and LSVDDQ_rev (Table 1) were used. The obtained product was digested with *EcoRI* and *NotI* and cloned to pPICZαA plasmid to generate pMLα_LacGL1 vector (Fig. 1). The expression of the LacGL1 laccase yielded a recombinant protein containing a c-myc epitope and a HIS tag at the C-terminal end which accounted for 27 additional amino acids. The fidelity of all DNA constructs was confirmed by PCR and DNA sequencing.
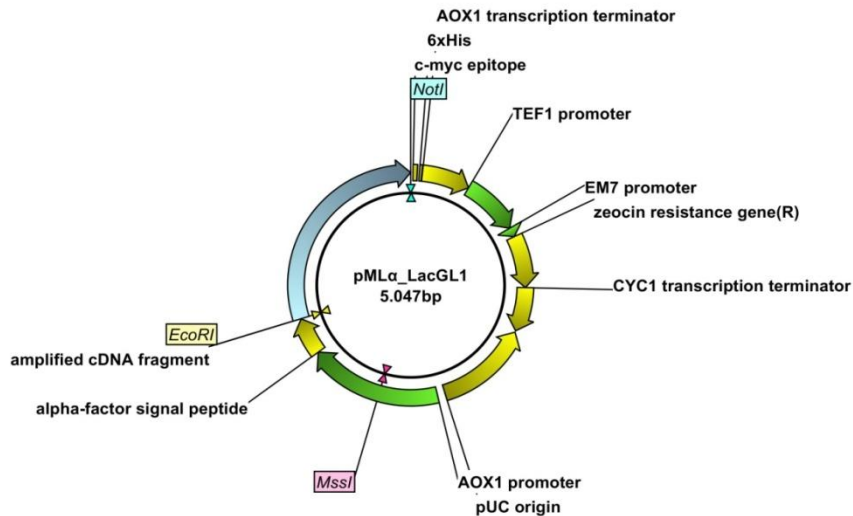
Figure 1. The map of the recombinant plasmid pMLα_LacGL1 used for expression of the LacGL1 laccase in *Pichia pastoris* X-33. The pMLα_LacGL1 vector contains the sequence coding for the signal peptide from *Saccharomyces cerevisiae* α-mating factor pre pro peptide and gives a fusion of HIS tag and c-myc epitope to the C- terminal end of subcloned *LacGL1* gene.

Expression of LacGL1 laccase in *P. pastoris* X-33 and visualization of its secretion

P. pastoris strain X-33 was transformed by electrophoresis with pMLα_LacGL1 vector. Plasmid pPICZαA was used as a negative control for laccase expression (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). All expression vectors were linearized with *PmeI* (*MssI*) prior to the transformation. The transformant cells were selected on Yeast Extract Peptone Dextrose (YPDS) agar plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 µg/mL zeocin. The in-agar expression of LacGL1 laccase from positive *P. pastoris* transformants was detected after 7 days at 30°C on Buffered Minimal Methanol (BMM) agar plates (100 mM potassium phosphate, pH 6, 1.34% Yeast Nitrogen Base (YNB), 4×10^{-5}% biotin, 1% methanol, and 1.5% agar) supplemented with 0.3 mM $CuSO_4$ and 0.04% guaiacol by appearance of brown-red halos.

Production of the recombinant LacGL1 laccase was also performed in liquid cultures prior to fermentation. Inoculation of the selected positive clones into Buffered Glycerol Medium (BGMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH6, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) was followed by incubation at 30°C overnight in a shaking incubator. The cells were harvested when the OD_{600} reached a value of 1, resuspended in Buffered Methanol Medium (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol) supplemented with 0.3 mM $CuSO_4$ and incubated for 5 days with an addition of methanol to a final concentration of 0.5%, every 24 h. The strain exhibiting the highest laccase specific activity for ABTS was chosen for fermentation.

Fermentation of LacGL1 laccase from *G. lucidum* in *P. pastoris*

P. pastoris strain X-33 carrying the pMLα-LacGL1 vector was inoculated overnight at 30°C, at 150 rpm in the shaking flasks in BMG (Buffered Minimal Glycerol) medium (100 mM potassium phosphate, pH 6, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) until the cell density reached a value of

1.7. This step was followed by inoculation of a 5 L Sartorius Biostat Aplus fermentor. The 5 L scale production of recombinant laccase in *P. pastoris* was performed essentially according to Stratton et al. (1999) and as detailed by Silva et al. (2011), except that the Methanol Fed-Batch phase was carried out at 20°C, in order to improve the enzyme's stability. Agitation was kept below 750 rpm to avoid excessive cell disruption of the *P. pastoris* cells and in turn to limit the downstream purification process. Additional oxygen was added automatically to accommodate optimal growth and enzyme expression. The total time for the fermentation process was 112 h.

In order to harvest the total protein, the fermentation broth was centrifuged at 5300 x *g* 5°C for 1 h. The supernatant was then subjected to sterile filtration, followed by concentration of the protein by ultrafiltration, using a cross-flow bioreactor system with a 30 kDa cutoff membrane (Millipore, Sartorius, Denmark), as described by Silva et al. (Silva et al. 2011). The enzyme aliquots containing 25% (w/v) glycerol were stored at -80°C.

Native PAGE and EndoH treatment

The native PAGE electrophoresis was performed according to the guidelines from Sitarz et al. (2012), however the substrate used for activity bands visualization was ABTS. The electrophoretically separated and fixed on the gel proteins from the crude extract of *G. lucidum* CBS229.93 and *P. pastoris* fermentation were submerged in 49 mL of 0.1 M citrate-phosphate and 1mL ABTS (4 mM) to visualize the activity staining of laccase.

The EndoH (endoglycosidase) treatment of the LacGL1 laccase was performed as described in New England Biolabs procedure (Ipswitch, MA, The USA). The EndoH treatment was performed for 24 h in order to be able to visualize activity staining.

Phylogenetics of LacGL1 laccase

From the query sequence of the LacGL1 laccase from *G. lucidum* CBS229.93, a dataset of putative homologous sequences was built by BLAST (Altschul et al. 1997) and run on the UniProtKB database (Apweiler et al. 2004). The raw dataset was filtered manually to eliminate potentially non-homologous sequences, disturbing alignments and duplicates. The sequences retrieved were only focused on a scope TaxeID = 4751 (fungal kingdom). An alignment was created using MUSCLE (Edgar 2004), and the phylogenetic tree (unrooted, squared) was generated using Seaview software (Gouy et al. 2010) using the maximum likelihood method (Felsenstein 1981). Bootstrapping was carried out with 100 replications.

Laccase activity assay

The laccase activity was measured based on a modified method of Ters et al. (2009). The laccase activity was defined as the amount of the enzyme required to oxidise 1 μmol of ABTS per minute at 25°C, and pH 4.7. The assay mixture contained: 0.1 M citrate-phosphate buffer (255 μL, pH 4.7), ABTS (5 μL, 4 mM (ABTS stock solution)), and pre-diluted laccase (5 μL) that ensured the linear range of Michaelis-Menten kinetics. The oxidation of ABTS was monitored at 420 nm (ε_{420} = 36800 $M^{-1}cm^{-1}$) for 2 min. in an Infinite 200 microtiter plate reader (Tecan, Salzburg, Austria). The activity evaluation on the other assay substrates, hydroquinone (248 nm), 2.6-dimethoxyphenol (470 nm), and guaiacol (436 nm) was done similarly with the substrate concentration as described in Table 2 using the following extinction coefficient values: 17.542 $M^{-1}cm^{-1}$, 35.645 $M^{-1}cm^{-1}$, and 6.400 $M^{-1}cm^{-1}$,

respectively (Minussi et al. 2007). The data collection was monitored by the program Tecan i-control version 1.5.14.0 (Tecan, Salzburg, Austria). The calculated laccase activity was corrected by the absorbance of a control sample which contained: 0.1 M citrate-phosphate buffer (255 µL, pH 4.7), ABTS (5 µL, 4 mM), and 5 µL of distilled water instead of the enzyme. All determinations of the laccase activity were performed in duplicates, with an average sample standard deviation less than 5%.

Evaluation of the influence of pH and temperature on laccase activity - MODDE

The effect of the pH and temperature on the LacGL1 laccase activity, expressed from *P. pastoris,* was modeled via randomized, full factorial, statistically designed experiment - MODDE program version 7.0.0.1 (Umetrics, Umeå, Sweden). The statistical design consisted of 12 experiments, including a triplicate repetition at the center point (pH 5 and 40°C), using ABTS as a substrate. The influence of the temperature and pH was monitored between 25-55°C and pH 4-6 (for 0.1 M citrate-phosphate buffer), respectively. The mixture with the ABTS and buffer at the defined pH value was incubated for 5 min. in a thermocycler set to a desired temperature and afterwards added to a previously pre-diluted enzyme in a microtiter plate which was incubated for 1 min. in a microtiter plate reader set to a desired temperature. After mixing the substrate and buffer with the enzyme, the initial rate of the product formation was measured as described above. The volumes and concentrations of enzyme, substrate, and buffer were identical as for the laccase activity assay.

Thermal stability studies and evaluation of substrate specificity, kinetic and inhibition parameters

The thermal stability studies of the LacGL1 laccase expressed in *P. pastoris* were performed by incubation of 5 µL of the enzyme and 255 µL of 0.1 M citrate-phosphate buffer, pH 4.7 at different temperatures (25, 40, 50, 60, and 70°C) for 2, 5, 15, 30, 45, and 60 min. in a thermocycler. Afterwards, 5 µL of 4 mM ABTS were added to the enzyme-buffer mixture and the initial rate of product formation was measured as described above.

The potential inhibitory effect of EDTA, NaN_3 and NaF on the rate of product formation was monitored with ABTS as a substrate, by addition of the inhibitor-substrate-buffer mixture to the 5 µL of LacGL1 laccase preparation. The final concentration of the potential inhibitor evaluated in this study was 0.0001, 0.0005, 0.001, 0.01, 0.1 and 50 mM for NaN_3, NaF and EDTA, respectively.

The Michaelis constant (K_m) was calculated from a Hanes-Wolf plot. The linear relationship of the data, where the substrate concentration over the reaction rate [S]/[v] is plotted against the substrate concentration [S], gave a slope of $1/V_{max}$, a y-intercept of K_m/V_{max} and an x-intercept of $-K_m$. The substrate used in this study was ABTS with a final concentration in the reaction mixture of: 0.015, 0.031, 0.041, 0.062, 0.077, and 0.092 mM. The rate of product formation was measured as described above.

Preparation of biomass sample for evaluation of laccase effect on released glucose yields

SCB (Sugarcane Bagasse) was obtained from the commercial American Society of Sugarcane Technologists, Florida Division (LaBelle, FL, USA). The raw biomass was washed in the distilled water to remove any sand particles and dried at 50°C, prior to the pretreatment. Afterwards, 15% dry matter (w/v) of SCB was pretreated by the steam explosion process at 175°C for 10 min., 11 bars

70

pressure, and a double addition of oxygen (3 min. each session) as described previously (Sørensen et al. 2007). After the steam explosion, the filter cake and the hydrolysate were mixed together, dried at 55°C for 44 h, and coffee-milled to pass a sieve size of 210 μm (Endecotts, London, UK). The pretreated SCB contained; 48.8% (w/w) cellulose, 13.8% (w/w) hemicellulose, and 19.3% (w/w) insoluble lignin.

The content of the dry matter and the biomass composition was determined according to the National Renewable Energy Laboratory (NREL) procedure (Sluiter et al. 2011). The levels of glucose and xylose liberated after strong acid hydrolysis were determined by HPAEC using Dionex BioLC system equipped with Dionex CarboPac PA1 analytical column (Dionex, Sunnyvale, CA, USA) and an electrochemical detector used in the pulsed amperiometric detection mode principally as described previously (Sørensen et al. 2003) and as detailed by (Pedersen et al. 2011).

Glucose release from pretreated SCB during laccase-cellulase catalyzed hydrolysis of SCB

The effect of the glucose released during a laccase-cellulase catalyzed hydrolysis of 5% (w/v) dry matter of pretreated SCB was evaluated in 0.1 M citrate-phosphate buffer at pH 4.7 and 40°C (optimal for the LacGL1 laccase) or pH 5.1 and 50°C (optimal for the cellulase preparations), respectively. The commercially available cellulase cocktail preparations: Cellic®CTec1 and Cellic®CTec2 (0.064% Enzyme/Substrate ratio (E/S), w/w; Novozymes, Bagsværd, Denmark) were used with the combination of the LacGL1 laccase (0.4% E/S, w/w). The hydrolysis reactions were collected after 0, 1, 3, 5, 16, and 24 hours and stopped by incubation at 99°C for 15 min. Afterwards the samples were centrifuged at 10.000 rpm for 2 min., the supernatants were taken out and filtered through 0.2 μm filter and the yields of released glucose were quantified using the D-glucose-HK kit (Megazyme, Denmark). The glucose yields released over time were corrected by the glucose amount present in the hydrolysis sample at time 0. The E/S dosage was based on the total protein concentration used. The Cellic®CTec1 and Cellic®CTec2, cellulase preparations, are based on the *Trichoderma reesei* cellulase complex (exo-glucanase, endo-glucanase, and β-glucosidase activities) with additional β-glucosidase and glycoside hydrolase family 61 hydrolyse boosting proteins (Harris 2010). All determinations of the enzymatic hydrolysis samples were performed in duplicates.

The protein quantification was performed using the Pierce BCA (BiCinchoninic Acid) protein assay kit microplate procedure according to manufacturer's instructions (Thermo Fisher Scientific, Rockford, US) as described before (Silva et al. 2011). Bovine serum albumin (BSA) was used as a standard.

Homology modeling

The crystal structure of the *Trametes versicolor* laccase (TvL) (PDB ID: 1GYC) (Piontek et al. 2002) was retrieved from the protein data bank. A homology model of the LacGL1 laccase was build using Phyre2 (Kelley and Sternberg 2009) in a threading mode using TvL as a template. Both structures were prepared for the molecular dynamics simulations using the protein preparation wizard in Maestro version 9.2 (Schrödinger LLC: New York, 2011).

Molecular dynamics simulations

The LacGL1 model and the TvL structure were protonated corresponding to the neutral pH and each of them was immersed in a cubic box of TIP4P water (Jorgensen et al. 1983) providing a

minimum layer of 13 Å of water on each side of the protein. A charge-neutral system was achieved by replacing randomly selected TIP4P molecules with Na$^+$. Each system was subjected to the steepest descent minimization to a gradient of 1 kcal mol^{-1} Å^{-1} followed by the default pre-simulation protocol employed in Desmond Molecular Dynamic System, version 3.0. (D.E. Shaw Research: New York). Following the relaxation protocol, a 20 ns NPT simulation was carried out with Desmond for each system at 300 K. The simulations were performed with the OPLS_2005 force field (Banks et al. 2005), periodic boundary conditions and employed the smooth particle mesh Ewald method (Essmann 1995) for treatment of long-range Coulomb interactions. MD trajectories were saved to disk at 20 ps intervals for subsequent analysis.

Docking

The structure of the substrate, *p*-coumaric acid, was generated and optimized in Maestro. The last snapshot of the 20 ns MD simulation of the LacGL1 laccase was chosen to represent the structure of LacGL1. After superimposition of the MD structure of the LacGL1 laccase and the crystal structure of TvL laccase, *p*-coumaric acid was substituted for 2,5-xylidine present in the crystal structure using the most plausible alignment of functional groups.

RESULTS

Characterization of laccase gene, *LacGL1*, and predicted protein, LacGL1

The MALDI-TOF analysis of the proteins in the crude extract of *G. lucidum* CBS229.93 revealed an existence of a laccase (Sitarz et al. 2012). Based on the homology between the five short peptides belonging to *G. lucidum* CBS229.93 laccase and the laccase from *G. lucidum* Q9HG17, two sets of four primers each were designed based on the mRNA sequence of *G. lucidum* Q9HG17. Out of the 16 pairs of primers (each of two sets of four primers, paired forward and reverse), one set (WADGP_fwd3 and LINGLP_rev1; Table 1) resulted in an amplification of a 500 bp fragment which when blasted in the UniProt database (Apweiler et al. 2004) confirmed a high homology to laccase from *G. lucidum* Q9HG17. The 500 bp fragment was positioned between the two short peptides (TTSIHWHGFFQ and FPLGSDSTLINGLG discovered *de novo* by MALDI-TOF analysis) and was 80% identical to the mRNA sequence of *G. lucidum* Q9HG17. This level of identity corroborated that the peptide fragment was most likely a laccase. Next, the identified sequence of 500 bp of *G. lucidum* CBS229.93 was used to design 100% identical outward primers, QYCDGLR_fwd1 and YHSHLST_rev1, respectively (Table 1). Therefore, an amplification of the genomic DNA with the outward primers and their respective fwd and rev partners, IAPDGFI_fwd1 and DLCTS_rev1, respectively, whose sequence was again based on *G. lucidum* Q9HG17, gave a product of 1963 bp belonging to the *LacGL1* laccase gene. The manual and software analysis (Stanke and Morgenstern 2005) of the introns and exons in this fragment revealed an existence of 9 introns, located at 183-250, 319-369, 490-547, 661-722, 790-866, 958-1009, 1166-1218, 1416-1476, and 1740-1796 bp which all followed a GT-AG rule at the exon/intron junctions (Padgett et al. 1984) and had a characteristic for *Basidiomycetes* motif within the intron – CTNA (Seitz et al. 1996). This fragment however, did not contain N- and C- terminal sequences of the LacGL1 laccase, which were found by applying the RACE technique (Ryu et al. 2008). In this way the missing 8% of the final sequence of the *LacGL1* gene was identified and amplified. A comparison between the cDNA and the 1963 bp fragment of the genomic DNA sequence of *LacGL1*, deprived of introns, confirmed that the sequences were identical. The final mRNA sequence of the *LacGL1* gene consisted of 1563 bp. The analysis of the predicted

cDNA gene sequence showed 87% identity with the laccase from *G. lucidum* strain 7071-9 (Joo et al. 2007) and 77% with *Lac*1 gene from *Polyporus brumalis* (Ryu et al. 2008).

The cDNA of the *LacGL1* laccase gene was translated into an amino acid sequence using an ExPASY translate tool (Gasteiger et al. 2005). The translation showed that the *LacGL1* laccase gene encoded for 520 amino acids including a 21 amino acid long signal peptide with its cleavage site predicted by SignalP software (Nielsen et al. 1997; Bendtsen et al. 2004). Afterwards, the amino acid sequence of the native laccase with the truncated signal peptide and the purification tag and the LacGL1 laccase with the truncated signal peptide but containing a 27-amino-acid purification tag were submitted to ExPASY proteomics tool, and the pI and the molecular weight of the proteins were calculated to be 5.07, 54.5 kDa and 5.21, 57.5 kDa, respectively. The mass of the native laccase corresponded well with SDS-PAGE results of 62.5 kDa (Sitarz et al. 2012). The slightly higher mass of the native enzyme revealed by the Native-PAGE (Fig. 2, lane 1) analysis was presumably due to the glycosylation since the sequence was found to contain seven potential N-glycosylation sites, computed by NetNGlyc server (Blom et al. 2004). Additionally, the analysis of the amino acid sequence of the LacGL1 laccase, resulted in 91% identity with a laccase from *G. lucidum* strain 7071-9 (Q9HG17 – UniProt identifier), and 81% with a laccase from *Polyporus brumalis* (A3F8Z8), respectively.
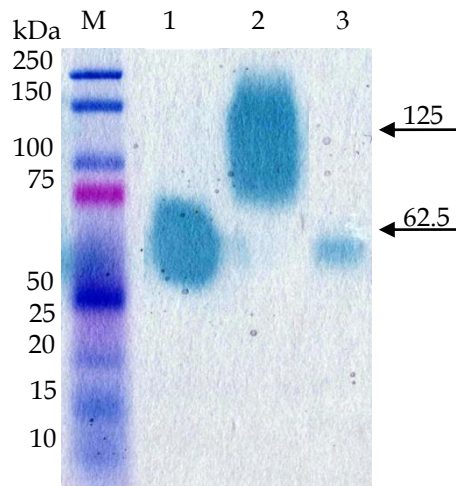

Figure 2. The activity staining of the laccase from *Ganoderma lucidum* CBS229.93 on Native PAGE, using an ABTS as a substrate. (M) molecular weight standards, (1) the laccase from a crude extract of *G. lucidum* CBS229.93, (2) the LacGL1 laccase expressed in *Pichia pastoris*, (3) the LacGL1 laccase expressed in *P. pastoris* after EndoH treatment.

The analysis of the amino acid sequence of the LacGL1 laccase from *G. lucidum* CBS229.93 revealed a high degree of homology in the highly conserved copper binding domains with their amino acids that take part in coordination to the four copper atoms (Fig. 3). These four regions, identified as R1-R4, consisted of the ungapped sequence regions that contain conserved residues of 1 cysteine and ten histidines that are involved in the binding of the four copper ions (Thurston 1994). The eight out of ten histidines appeared in a highly conserved pattern of HXH motifs (Fig. 2) in the protein. An X in this motif represents an undefined residue. Moreover, the HXH motifs were separated from each other by segments of 25 to 175 amino acids (Kumar et al. 2003). The most important residues that coordinated to copper ions were located in domain 1 and 3.

R1

R2

```
               βE          η2                        βG        η3        βH
c1gycA_
c1gycA_                     64 HWHGFFQAGTNWADGPAFVNQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Ganoderma_lucidum_CBS229.93 64 HWHGFFQKGTNWADGPAFINQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Ganoderma_lucidum_Q9HG17    64 HWHGFFQKGTNWADGPAFINQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Ganoderma_lucidum_B5G552    64 HWHGFFQKGTNWADGPAFVNQCPI   104 GTYWYHSHLSTQYCDGLRGPFV
Ganoderma_lucidum_B5G551    64 HWHGFFQKGTNWADGPAFVNQCPI   104 GTHWYHSHLSTQYCDGLRGPFV
Trametes_villosa_Q99044     64 HWHGFFQKGTNWADGPAFINQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Trametes_sanguinea_D7F485   64 HWHGFFQHGTNWADGVSFVNQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Trametes_versicolor_B8Y3J5  64 HWHGFFQAGTNWADGPAFVNQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Lenzites_gibbosa_G4XU44     64 HWHGFFQAGTNWADGPAFVNQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Polyporus_ciliatus_Q9HDS9   64 HWHGFFQKGTNWADGPAFVNQCPI   104 GTFWYHSHLSTQYCDGLRGPFV
Ganoderma_sp.En3_G0WM60     64 HWHGLFQRGTNWADGPAFVTQCPI   104 GTFWYHSHLSTQYCDGLRGPLV
```

R3

R4

```
               βY   TT              β^        α2          β_
c1gycA_
c1gycA_                    395 HPFHLHGH   447 GPWFLHCHIDFHLEAGFAIVFA
Ganoderma_lucidum_CBS229.93 395 HPFHLHGH  447 GPWFLHCHIDFHLEAGFAVVFA
Ganoderma_lucidum_Q9HG17   395 HPFHLHGH   447 GPWFLHCHIDFHLEAGFAVVFA
Ganoderma_lucidum_B5G552   395 HPFHLHGH   447 GPWFLHCHIDFHLDAGFAVVMA
Ganoderma_lucidum_B5G551   395 HPFHLHGH   447 GPWFLHCHIDFHLDAGFAVVFA
Trametes_villosa_Q99044    395 HPFHLHGH   447 GPWFLHCHIDFHLEAGFAVVFA
Trametes_sanguinea_D7F485  395 HPFHLHGH   445 GPWFLHCHIDFHLDAGFAVVMA
Trametes_versicolor_B8Y3J5 395 HPFHLHGH   447 GPWFLHCHIDFHLEAGFAIVFA
Lenzites_gibbosa_G4XU44    395 HPFHLHGH   447 GPWFLHCHIDFHLDAGFAVVFA
Polyporus_ciliatus_Q9HDS9  395 HPFHLHGH   445 GPWFLHCHIDFHLDAGFAVVFA
Ganoderma_sp.En3_G0WM60    396 HPFHMHGH   448 GPWFLHCHIDFHLQAGFAIALA
```
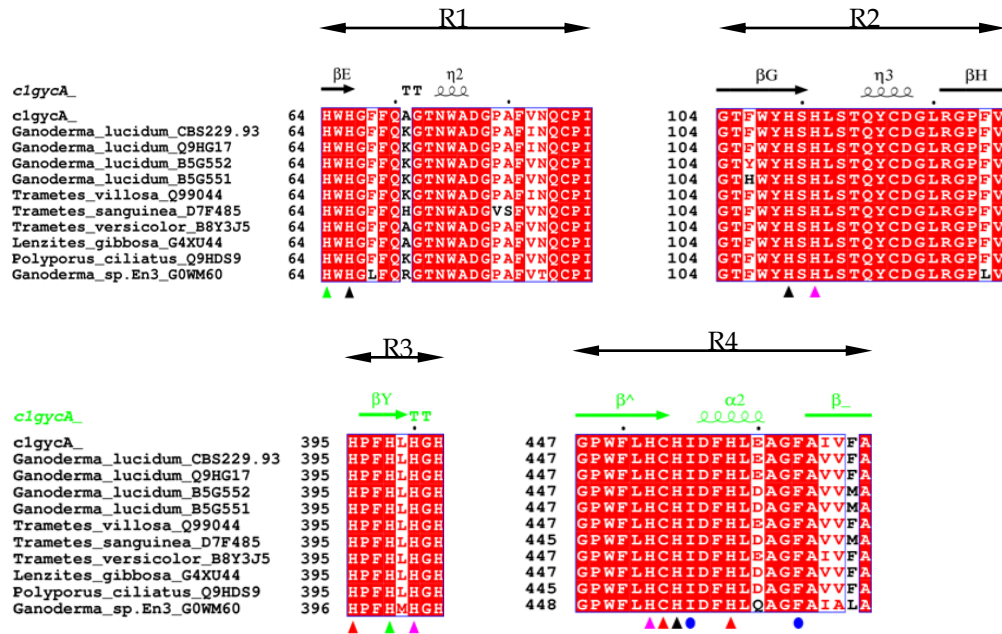
Figure 3. Selected alignment parts of randomly chosen enzymes, belonging to the multicopper oxidases family, which show the copper binding domains with their conserved amino acids that take part in coordination to the four copper atoms. The amino acids in the red boxes indicate fully conserved residues and the triangles below the red boxes point out to the 11 fully conserved amino acids coordinating to the four copper atoms. All 11 residues are positioned within the sequence segments (R1-R4), which are ungapped for fungal laccases and allow them to be distinguished within a broader class of multicopper oxidases. The red, green, pink, and black triangles indicate coordination to the T1Cu, T2Cu, T3αCu, and T3βCu copper ions, respectively. The blue circles indicate the residues positioned 4Å axial to the T1 copper ion. Note that the axial, non-coordinating isoleucine (455I) and phenylalanine (463F) are invariable among the selected fungal laccases. The secondary structures (α-helices and β-sheets) above the alignment are based on a crystallographic structure of *Trametes versicolor* (PDB ID: 1GYC) (Piontek et al. 2002). The α-helices and β-sheets colored black and green indicate domain 1 (residues 1-131, and 476-499), and domain 3 (residues 301-475), respectively. Domain 2 (residues 132-300) is not presented here due to the lack of residues serving a function in catalysis. The domain coloring in this figure corresponds to the coloration code of domains in Fig. 8B. The alignment of the sequences was made using the ClustalW 2.0 software (Goujon et al. 2010; Larkin et al. 2007) and ESPript for final output (Gouet et al. 1999). The signal peptides were cleaved off prior to the alignment using the SignalP software.

LacGL1 laccase phylogenetics

The finding of 91% homology between the laccase amino acid sequences of *G. lucidum* CBS229.93 and Q9HG17 (UniProt identifier) on the one hand confirmed that the LacGL1 enzyme was a laccase, but hinted that the 9% protein sequence dissimilarity can classify laccases to quite different phylogenetic groups (Fig. 4). Laccases belonging to *G. lucidum* B5G552 and B5G551 appear related to the LacGL1 laccase (but are not identical to LacGL1) while the laccases belonging to *Trametes* genus showed to originate from a different ancestor, grouping themselves in a separate family rather distant from *Ganoderma* family but still having regions R1 and R4 highly conserved (Fig.3 and 4).
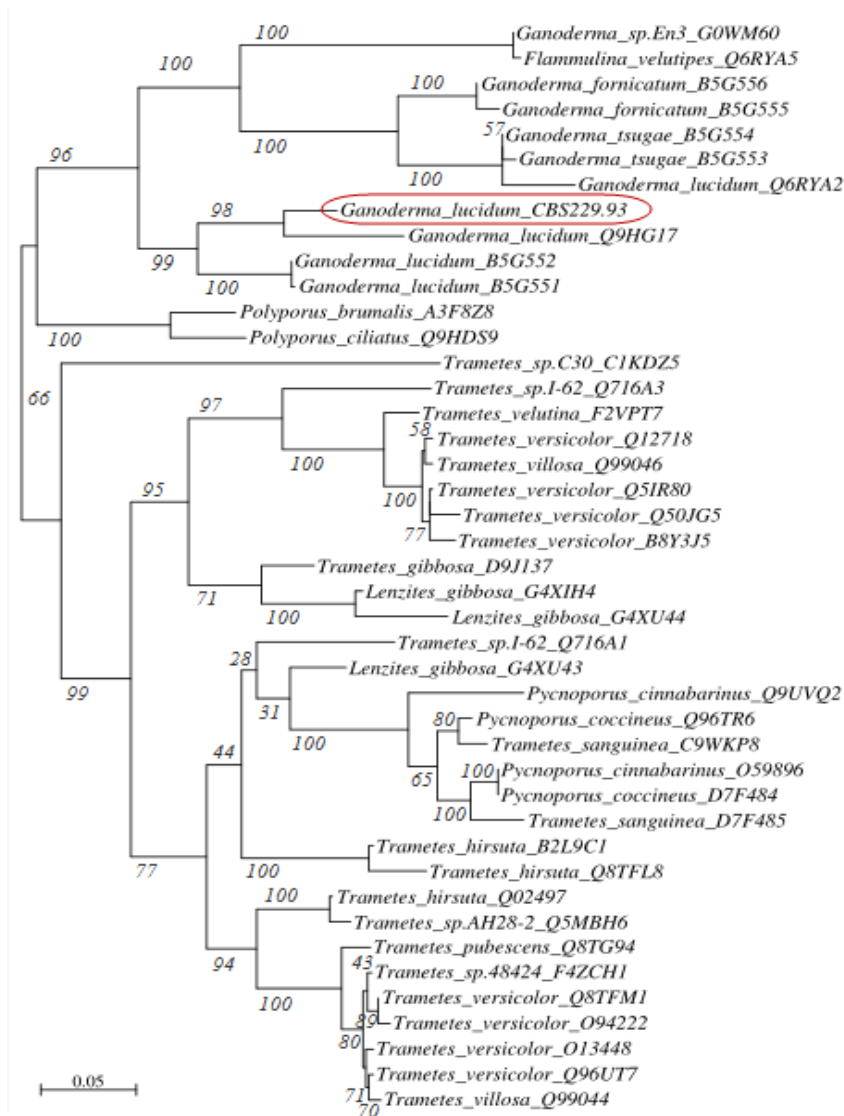
Figure 4. A maximum-likelihood phylogenetic reconstruction of the homologous sequences for the LacGL1 laccase. All proteins are named according to their Uniprot reference number. The LacGL1 from *G. lucidum* CBS229.93 (marked with the red loop) exhibits the shortest distance to laccase from *G. lucidum* Q9HG17. These proteins cluster together with two putative laccases from *G. lucidum* UniProt accession numbers B5G552 and B5G551, respectively.

Heterologous expression of the LacGL1 laccase in *P. pastoris*

Prior to the fermentation, the expression of the LacGL1 laccase was evaluated from two different vectors, one containing α-factor signal peptide from *Saccharomyces cerevisiae* (pMLα_LacGL1, Fig. 1) and the second one containing a native signal peptide from *G. lucidum* CBS229.93 (pML_LacGL1). Results showed a higher laccase activity when the α-factor signal peptide directed the transport of the LacGL1 protein (data not shown) and therefore pMLα_LacGL1 was used for laccase expression.

During the 5-L scale fermentation (112 h) of *Pichia pastoris* containing the recombinant *LacGL1* laccase gene, the methanolytic yeast growth was monitored by measurement of the OD_{600}, which increased from 1.7 to 630 during the cultivation period (data not shown). The level of the extracellular proteins, including the LacGL1, reached a concentration of 14.7 g/L. The protein expression was

followed by an in-gel activity measurement using Native PAGE electrophoresis and the LacGL1 laccase activity had response at 125 kDa (Fig. 4). That this relatively high Molecular Weight (MW) of the LacGL1 laccase was due to extensive glycosylation (Bohlin et al. 2006), was confirmed by Native PAGE electrophoresis which showed that the MW decreased to 62.5 kDa after EndoH treatment (Fig. 4). The LacGL1 laccase activity was 17.5 U/mL after the fermentation and sterile filtration.

Characterization of the LacGL1 laccase expressed in *P. pastoris*

The main purpose for expression of the LacGL1 laccase from *G. lucidum* was to obtain a sufficient amount of pure, monocomponent enzyme to evaluate its effect on the improvement of the total glucose yields released during ongoing cellulase hydrolysis of STEX SCB.

In order to determine the optimal pH and temperature conditions of laccase expressed in *P. pastoris*, a statistically designed, randomized, full factorial experiment was applied to evaluate the individual and interactive effects of two factors (pH and temperature) on the rate of the product formation. The highest relative activity of the LacGL1 laccase, predicted by the model was at pH 4.7 and 55°C and was almost independent of the temperature (Fig. 5)
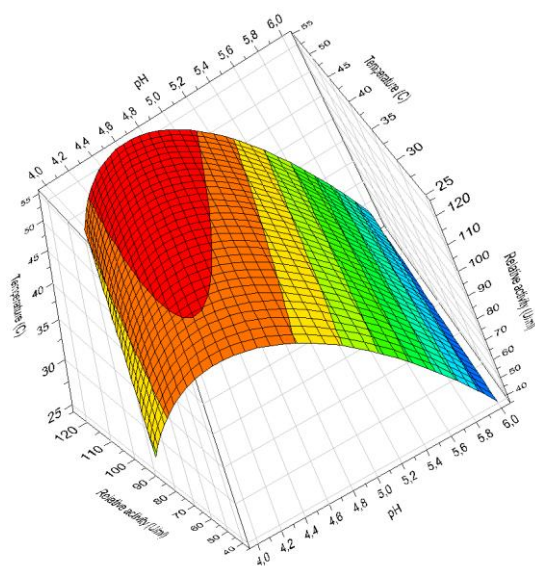

Figure 5. A surface response plot as a function of the pH and temperature on the relative activity (U/mL) of the LacGL1 laccase expressed in *Pichia pastoris*. The relative activity was calculated based on the LacGL1 activity at pH 4.7 and 25°C. The highest relative activity was calculated for pH 4.7 (red area of the plot) and was almost temperature independent. The lowest value of the relative activity was when the pH value dropped to 6 (blue area of the plot).

The model correlation coefficient R^2 (0.99) suggested that the fitted model could explain 99% of the total variation in the data. Together with the high values of predictivity Q^2 (0.97) and reproducibility (0.98) it could be concluded that the model was reliable. However, the model (Fig. 5) only predicted the potential and the most suited conditions for the LacGL1 laccase activity and therefore needed further validation. The relative activity measured at pH 4.7, of the LacGL1 laccase, did not change significantly for 25, 40, and even 50°C within an hour, however holding at temperatures of 60°C and 70°C lowered the laccase activity to 19 and 3%, respectively (Fig. 6). These results showed model correctness for temperature range from 25°C to 50°C. Additionally, the LacGL1

laccase showed a rather high stability at 50°C and 40°C for a prolonged time of incubation. It retained 33% activity after 24 h at 40°C in contrast to 23% activity after 5 h at 50°C (Fig. 7).
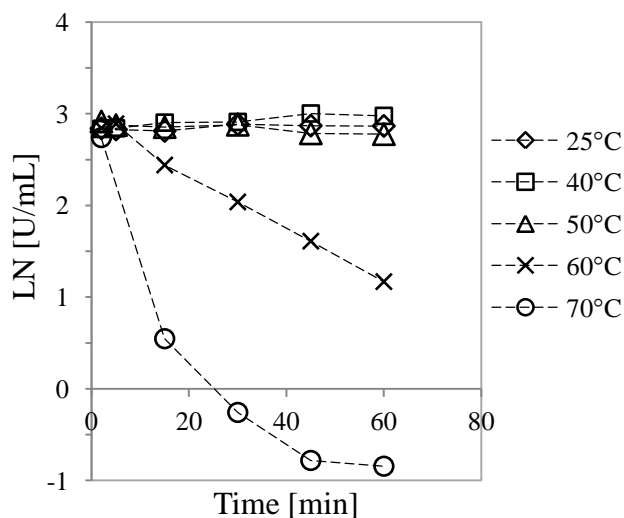

Figure 6. The temperature stability profile of LacGL1 laccase represented as a function of LN [U/mL] vs. incubation time at pH 4.7 and 0.1 M citrate-phosphate buffer. The highest impact on the rate of product formation under optimal pH conditions and incubation time at a specific temperature profile was observed for temp 60°C and 70°C, respectively. The relative difference between single measurements was <5%.
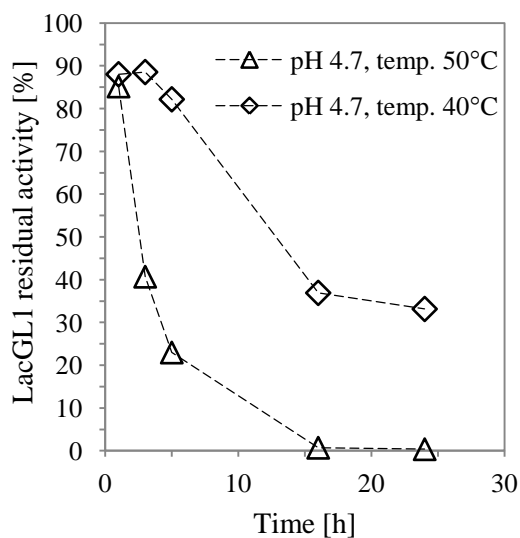

Figure. 7. LacGL1 laccase stability evolution plot at optimum pH of 4.7 and two different temperature values (40°C and 50°C). The plot is a continuation of temperature stability plot (Figure 6), therefore the first measurement is depicted after 1 h. Laccase residual activity [%] is calculated based on activity value of laccase measured at pH 4.7 and 25°C. The relative difference between single measurements was <5%.

Substrate specificity of LacGL1 laccase

Apart from ABTS, the LacGL1 laccase was able to oxidize other phenolic compounds (Table 2) such as; hydroquinone, guaiacol, and 2.6-dimethoxyphenol however in a slower rate than the one observed in the case of ABTS. The comparison of the rate of product formation of the laccase from the crude extract of *G. lucidum* (LacGLCE)*,* showed a similar pattern, however the expressed LacGL1 laccase was slightly faster.

From the Hanes-Wolf plot, which gave a good distribution of the data points, the K_m value for ABTS as substrate was calculated to be 0.122 mM and was identical with a value of K_m for the LacGLCE laccase (data not shown). V_{max} value was not calculated due to the lack of purity of the LacGL1 laccase.

Tabel 2. The evaluation of the rate of product formation for other laccase assay substrates.

Substrate [1 mM]	Laccase relative rate of product formation [%][a]	
	LacGL1[b]	Lac_GLCE[c]
ABTS	100	100
Hydroquinone	80	66
Guaiacol	66	57
2.6-Dimethoxyphnol	61	51

[a] All values represent the mean of duplicate measurements with a relative difference between single measurements of <5%.
[b] Laccase from *Ganoderma lucidum*, expressed in *P. pastoris*, and described throughout the paper
[c] Laccase from *Ganoderma lucidum* crude extract, used as a reference for LacGL1 laccase.

Additionally, the inactivation of the LacGL1 laccase and the laccase from the crude extract of *G. lucidum* by various concentrations of potential inhibitors is shown in Table 3. Both laccases were strongly inhibited by sodium azide (0.01 and 0.1 mM) and dithiothreitol (0.5 mM) and less but still significantly, by sodium fluoride. EDTA affected the laccases activity to a lesser extent.

Tabel 3. The effect of the inhibitory substances on the oxidation of ABTS by the LacGL1 laccase from *Ganoderma lucidum,* expressed in *Pichia pastoris*.

Compound	Concentration [mM]	Inhibition [%][a]	
		LacGL1	Lac_GLCE
Sodium azide	0.0001	16	18
	0.0005	50	47
	0.001	57	57
	0.01	100	89
	0.1	100	100
Sodium fluoride	0.0001	8	23
	0.0005	13	24
	0.001	19	28
	0.01	22	38
	0.1	46	56
EDTA	50	16	30
Dithiothreitol	0.5	100	100

[a] All values represent the mean of duplicate measurements with a relative difference between single measurements of <5%.
[b] Laccase from *G. lucidum*, expressed in *P. pastoris*, and described throughout the paper
[c] Laccase from *G. lucidum* crude extract, used as a reference for LacGL1 laccase.

Homology models created with Phyre2 (Kelley and Stenberg 2009) for LacGL1 with and without the 27-amino-acid purification tag (Fig. 8) showed almost identical structures for the common (non-HIS-tag) part of the sequence.
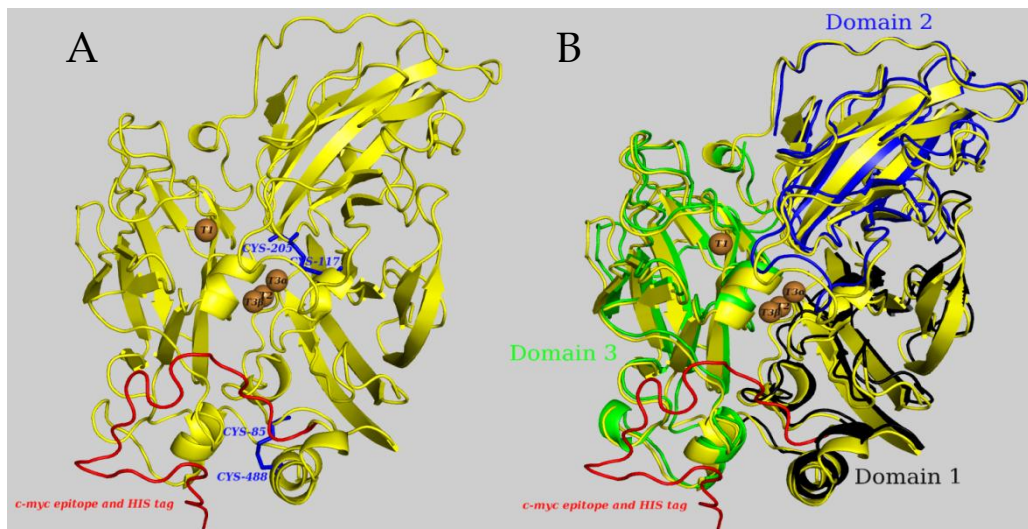

Figure 8. A graphical representation of the LacGL1 laccase expressed in *Pichia pastoris* (A) and its superimposition on a native laccase without a 27-amino-acid purification tag (B), illustrated using the PyMol Molecular Graphic System Version 1.5 Schrödinger, LLC. A) The yellow colored 3D structure of *Ganoderma lucidum* laccase was predicted by submitting its full amino acid sequence to the 3D comparative protein modeler – Phyre2 (Kelley and Sternberg 2009). The blue sticks represent cysteine residues responsible for formation of disulphide bridges, and the red tail is the 27-amino-acid purification tag; B) The three-colored, 3D structure of the native laccase from *G. lucidum* indicates positioning of the domains and compares the overall protein folding to the laccase containing a purification tag. Brown spheres represent four copper ions, which play an important role in catalysis. They are located in domain 3 (T1Cu) and between domain 3 and 1 (T2Cu, T3αCu and T3βCu). Domain 1 (residues 1-131 and 476-499) is colored black, domain 2 (residues 132-300) is colored blue, and domain 3 (residues 301-475) is colored green. The range of amino acid residues that are a part of each domain was predicted according to (Aleksandrov and Shinddyalov 2003) and found at www.pdb.org under *T. versicolor* (PDB ID: 1GYC) sequence.

Glucose release from pretreated SCB during laccase-cellulase catalyzed reaction

In order to evaluate any potential effect of laccase addition during the cellulase hydrolysis of cellulose, the LacGL1 laccase was added to the buffered solutions of the SCB containing cellulase protein preparations. The two protein preparations were investigated, namely: Cellic®CTec1 and Cellic®CTec2 (from now on referred to as CC1 and CC2, respectively). The expressed, but not purified laccase and the two cellulase preparations were dosed based on total protein concentration.

The experimental results showed that the laccase addition and the levels of the released sugars are dosage dependent. The more laccase was added the more glucose was released (data not shown). The combined laccase plus cellulase treatment of STEX SCB were performed at two different conditional set ups: pH 4.6, 40°C and pH 5.1, 50°C. The lower pH and temperature values were optimal for the LacGL1 laccase (as previously shown from MODDE pH-temp.-activity model, Fig. 5 and thermal stability plot, Fig. 6, 7). These conditions are however low for the optimal conditions of the golden cellulose standards CC1 and CC2 that perform optimally at 50°C and pH 5.1. The latter conditions were therefore also applied here. From the MODDE data, three different effects on glucose levels were observed and depended on the three factors: the type of the cellulase preparation, whether or not the LacGL1 was added, the temperature, and the pH of the reaction (Fig. 9, 10). Overall, the highest improvement of the cellulose conversion, considering also all of the aforementioned factors, was reported for LacGL1-Cellic®CTec2 catalyzed hydrolysis of STEX SCB at pH 5.1 and 50°C. The total

glucose yields were 19, and 27.5% higher, as compared with a reference CC2 hydrolysis at the same conditions, and pH 4.6 and 40°C, respectively. Moreover, the influence of the higher pH and temperature on LacGL1-CC2 catalyzed hydrolysis had a 12% improvement effect on the glucose yields released from SCB (Fig. 9).
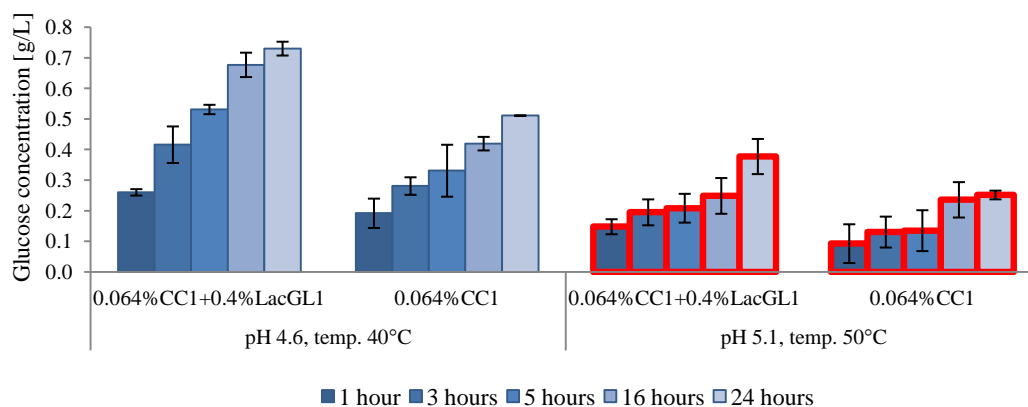

Figure 9. A graphical comparison of yields of released glucose over time in LacGL1-Cellic®CTec1 catalyzed hydrolysisof SCB at different pH and temperature values. The diagram bars without edge and those with a thick, red edge represent yields of released glucose at pH 4.6; temp. 40°C and pH 5.1; temp. 50°C, respectively. CC1 is an abreviation used for a cellulase preparation - Cellic®CTec1 and LacGL1 describes a laccase from *Ganoderma lucidum*, expressed in *Pichia pastoris* X-33. CC1 and LacGL1 were added in E/S ratio of 0.064%, and 0.4% (w/w), respectively.

On the other hand, the opposite effect of the temperature and pH on the LacGL1-Cellic®CTec1 catalyzed hydrolysis of the cellulose was observed. Here, the most optimal conditions were pH 4.6 and 40°C with improvement in glucose yields of 48, and 66% for LacGL1-CC1 and CC1 hydrolyzed samples at pH 5.1 and 50°C. Additionally, the LacGL1 addition to CC1 at pH 4.6 and 40°C resulted in 30% yield improvement (Fig. 10).
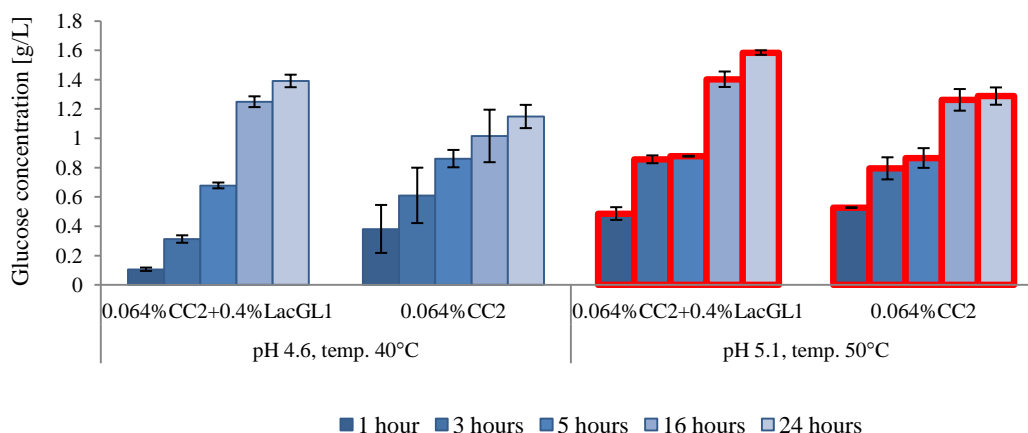

Figure 10. A graphical comparison of the yields of released glucose over time in the LacGL1-Cellic®CTec2 catalyzed hydrolysis of SCB at different pH and temperature values. The diagram bars without edge and those with a thick, red edge represent the yields of released glucose at pH 4.6; temp. 40°C and pH 5.1; temp. 50°C, respectively. The CC2 is an abreviation used for a cellulase preparation - Cellic®CTec2 and the LacGL1 describes a laccase from *Ganoderma lucidum*, expressed in *Pichia pastoris* X-33. The CC2 and the LacGL1 were added in the E/S ratio of 0.064%, and 0.4% (w/w), respectively.

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DISCUSSION

We have cloned a novel laccase (LacGL1) from a non-pathogenic basidiomycetes fungus *Ganoderma lucidum* CBS229.93, and expressed it in *P. pastoris* in order to evaluate its effect on the total glucose yields released during commercial cellulase catalyzed hydrolysis of the pretreated biomass. The role a laccase might play in lignin-like compound detoxification/degradation was indicated in our earlier studies (Sitarz et al. 2012) during fungal cultivation on lignin derivatives and evaluation of the proteomics profile of the expressed proteins.

Initially an evaluation of the dominating protein bands on the Native-PAGE gel was done by the MALDI-TOF analysis followed by a short peptide based amplification of the *LacGL1* gene. The *LacGL1* gene was translated into amino acid sequence and encoded for 520 amino acids, including a 21 amino acid signal peptide, which was predicted by SignalP software to be cleaved off at GIGPKT N-terminal sequence. This amino acid sequence was identical (GIGPKT) to the closest homolog of GaLC3 from *G. lucidum* strain 7071-9 (Ko et al. 2001), (Q9GH17 UniProt identifier), and was also similar but not identical (GIGPV) to the furthest positioned homologue of laccase isozyme I in the phylogenetic tree (Fig. 4), belonging to *Trametes villosa* (Yaver et al. 1996), (Q99O44 UniProt identifier). Additionally, the MW visualized by the Native-PAGE electrophoresis was 62.5 and 125 kDa for the native and the expressed laccase, respectively. The higher MW of the native laccase (as compared to software analysis prediction of 54.5 kDa) might be due to glycosylation, since there are seven potential glycosylation sites in the *G. lucidum* laccase sequence, which make a total of 13% of the sugar moieties attached to the laccase backbone. Such a phenomenon has also been reported for other laccases (Ko et al. 2001). As to the MW of the expressed in *P. pastoris* laccase, the almost 100% higher mass might be partially caused by implementing the purification tag (HIS tag) which is coupled with a c-myc epitope on the expression plasmid. The purification-epitope-tag complex accounted for 27 additional amino acids. The second reason for an observed higher MW is a probable extensive glycosylation that is common for a variety of proteins expressed in *P. pastoris* (Cereghino et al. 2002). However, the similar reactivity toward substrates (Table 2) and inhibitory substances (Table 3) for the native and heterologously expressed laccase (see below), suggested that neither HIS-tag nor glycosylation had significant functional implications on the LacGL1 activity. This functional neutrality of the HIS-tag was further supported by the conservation of structure excluding the HIS-tag and Phyre2 (Kelley and Sternberg 2009) homology models of the native and the expressed laccase.

The amino acid sequence analysis of LacGL1 laccase revealed a high degree of homology in the highly conserved copper binding regions, R1-R4 (Fig. 2), which together comprised the specific signature of laccases (Kumar, et al. 2003). Also a partial sequence, 452-HCHIDFHLEAGF-463, identical to that from *Trametes villosa* (Yaver et al. 1996) might indicate that the LacGL1 laccase also has a high redox potential (based on the presence of a T1 copper non-coordinating F residue).

The LacGL1 laccase showed maximum activity at an acidic pH of 4.7, which was quite similar to many fungal laccases whose pH optimum was in the range of 3.0 to 4.5 (Thurston 1994; Heinzkill et al. 1998). However, it had a relatively higher value of pH than GaLc3 (3.5). Additionally, the range of optimal temperature (50°C) was significantly higher than GaLc3 range (20-25°C). It was interesting that these two laccases differ so much in their pH and temperature regimes, considering the fact that their amino acid sequences were 91% identical. It is also worth noting that the K_m value of the LacGL1 laccase was rather high (0.122 mM) compared to the GaLc3 laccase (0.0037 mM), which might indicate a lower affinity to the substrate (in this case ABTS).

The native and *P. pastoris* expressed laccases were inhibited by sodium azide, an inhibitor of metaloenzymes (Thurston, 1994). This result was similar to the one observed for laccases, L1 and L2, from *Trametes versicolor* (Minussi et al. 2007) and *Poluporus pinsitus, Panaeolus sphincitrinus, Panaeouls papilionaceus* (Henzkill et al. 1998). Additionally, the two laccases LacGL1 and

LacGLCE, were also inhibited by sodium fluoride, dithiothreitol and EDTA, but in a lesser extent (Table 3).

The comparison of the pH and temperature range and also the effect of the inhibition and the laccase activity towards different substrates, between LacGL1 expressed in *P. pastoris* and laccase from the crude extract of *G. lucidum*, revealed a very similar dependence. Therefore, unpurified laccase LacGL1 was used for evaluation of its effect in cellulase catalyzed hydrolysis of pretreated biomass.

The LacGL1 laccase addition had a significant effect (19% increase) on the total glucose yields, released during cellulase catalyzed hydrolysis of the pretreated, unwashed biomass such as the sugarcane bagasse, when using a currently used methodology (pH 5.1, 50°C, CC2).We suspect that the LacGL1 laccase contributed to the higher cellulose-to-glucose conversion via detoxification of the steam-exploded sugarcane bagasse from the cellulase inhibitors. The putative mechanism is discussed in more detail below, but for now we note that the positive effect of laccase addition to cellulase catalyzed hydrolysis of the pretreated sugarcane bagasse probably occurs within the first 5 (at 40°C and 50°C) to 16 h (at 40° C) (Fig. 10A, B), due to the fact that after this time laccase loses its activity significantly. However, as the experimental data showed (Fig. 6, 7) addition of the LacGL1 laccase to cellulase catalyzed hydrolysis of biomass is a compromise between choosing the conditions that are close to the optimal for both enzymes. A similar improvement in the released sugar yields was observed during the hydrolysis of the steam-pretreated softwood (SPS) by Palonen and Viikari (2004), who observed a 11-13% and 21% improvement in total sugar yields during cellulase catalyzed hydrolysis of SPS, when no mediators and an artificial mediator such as N-hydroxy-N-phenylacetamide (NHA) was used, respectively. In the mentioned experiment however, a cellulase preparation of the first generation (Celluclast L1.5, and Novozym 188) was used, which is a much less robust enzyme preparation than the CC1 and CC2, which we have evaluated in our research. Additionally, the advantage of the LacGL1 laccase is significant when taking under consideration the fact that it does not need an artificial mediator for its action (that can both inactivate cellulase but also laccase itself (Pfaller et al. 1998)). However, some solubilized or colloidal lignin might act as a mediator in the oxidation process (Felby et al. 1997).What is more important is the fact that the LacGL1 laccase can further improve the sugar yields, for the already improved and robust to the inhibitors cellulase preparations, by 19%.

The enhanced glucose-yields upon the laccase treatment of the STEX-pretreated SCB observed in this work could be caused by a variety of molecular mechanisms. The identification of the mechanism would be useful in understanding the structure-function correlations to directly improve the performance of the laccases for enhancing sugar yields, e.g. by rationally directed mutagenesis and improved reaction conditions.

The STEX-pretreated SCB consists of a variety of degradation intermediates such as the large lignin-derived fragments, smaller aldehydes, and the phenolic compounds (Martin et al. 2007). As mentioned above, the effective cellulase concentration may be reduced due to encapsulation or other protein-surface interactions with lignin, which could possibly be prevented by direct oxidation of lignin fragments by the laccases. While direct degradation of the large lignin fragments would be challenging when compared to their proficient oxidation of smaller phenols, the oxidation via the long-range electron transfer has been observed on lignin fragments (Shleev et al. 2006).

A possible molecular mechanism of a direct lignin binding has been studied using the MD simulations for a *T. versicolor* laccase (Chen et al. 2011), providing a rationale for such a mechanism. However, other studies have found that among the lignin-degrading enzymes, only lignin peroxidase (LiP) specifically binds to the synthetic lignin, whereas laccases (from *Fomitella fraxinea*) did not (Johjima et al. 1999), consistent with a long-range electron transfer oxidation of lignin in the best case. It is known that laccases can remove phenols from the steam-exploded biomass for the detoxification purposes (Jönsson et al. 1998; Palonen and Viikari 2004; Chandel et al. 2007, Chandel

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et al. 2011) and that the small mono-phenolic compounds may act as direct inhibitors of cellulases (Ximenes et al. 2010; Ximenes et al. 2011). It is likely that the LacGL1 oxidizes some of these inhibitors to enhance enzymatic activity and glucose yield, although a combination of this mechanism and long-range oxidation may also occur.

Here we investigate a molecular mechanism where the LacGL1 directly oxidizes inhibitory phenolic compounds derived from the STEX-pretreatment of SCB. To substantiate such a mechanism, a previous study (Martin et al. 2007) identified three dominating phenols in the STEX-pretreated SCB, namely *p*-coumaric acid (0.17%), p-hydroxybenzaldehyde (0.06%), and ferulic acid (0.03%), (Fig. 11).
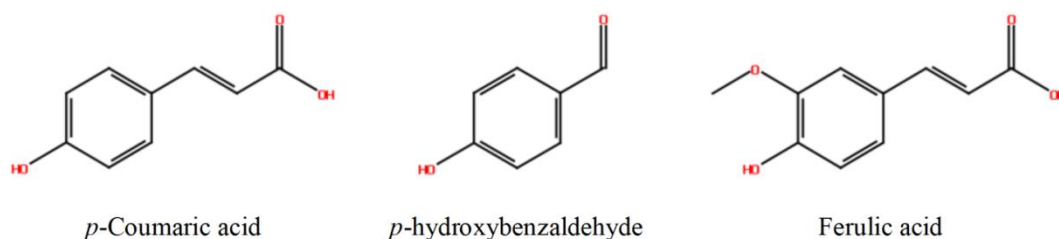

Figure 11. The three major phenols identified in the STEX pretreated SCB.

The most abundant of these, *p*-coumaric acid is a known β-glucosidase inhibitor (Ximenes et al. 2011), and was selected for proof-of-concept modeling of such a mechanism of the LacGL1 laccase. First, we made a homology model of the LacGL1 (see Methods and Supporting information), then performed the 20-ns MD simulations of the obtained LacGL1 structure to get a realistic, dynamically averaged structure of the protein including potential loop or turn movements.

Docking of *p*-coumaric acid into the MD-averaged (last snapshot) structure (Fig. 12) showed that many of the interactions (except F265 and L164) are similar to those in the crystal structure of the *T. versicolor* laccase complexed with 2,5-xylidine (Bertrand et al. 2002). This is evident from the alignment of the obtained MD structure of the LacGL1 laccase with this crystal structure (Fig. 13A).
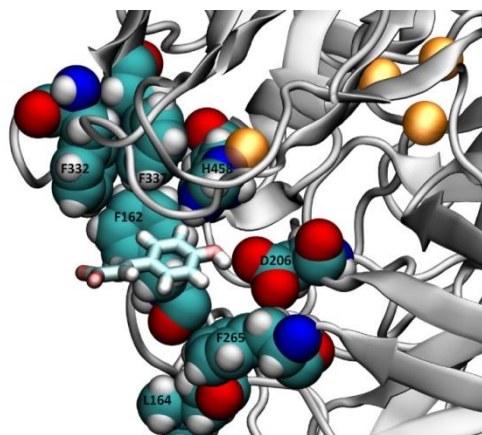

Figure 12. The *p*-coumaric acid (sticks) docked into the substrate binding site of the 20-ns MD-averaged structure of *Ganoderma lucidum* laccase (LacGL1). The residues of particular interest and their interactions with this phenol are shown in van der Waals representation. The copper ions are shown in yellow.

When taking into account the dynamics from MD in both systems, we obtained a comparison of the two proteins (Fig. 13B) without any bias from the effect of the solvent/MD dynamics vs. crystal packing. In both cases, the orientation of D206 allowed it to accept a hydrogen bond from the hydroxyl group on the phenolic inhibitor, which is commonly seen. Also the general arrangements of the hydrophobic F337 and F162 were commonly observed and similar in both proteins.

Interestingly, L164 and F265 (to the right in Fig. 13) have moved noticeably in the LacGL1, and the associated turn involving L164 has moved ~3 Å relative to both the crystal structure reference and the corresponding MD-structure of the TvL, implying that this effect was present regardless of the MD dynamics. The change for F265 was due to the rotation around the Cα-Cβ bond, positioning the phenyl ring closer to the substrate binding pocket, and due to a general change in turn position, possibly caused by the changed electrostatics in the LacGL1 (which has several more negatively charged residues on the outer part of the protein compared to TvL).
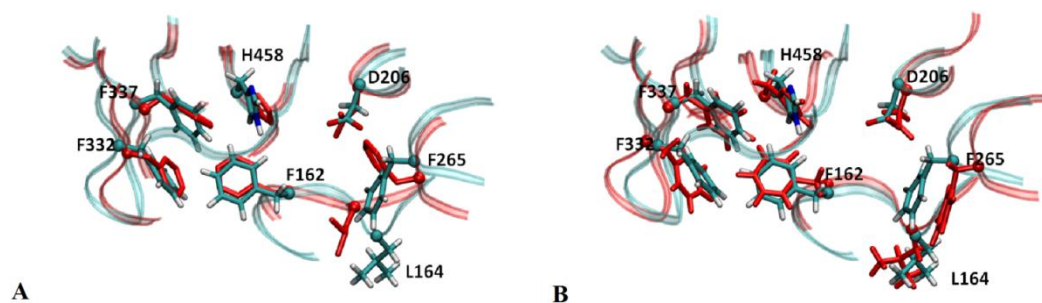

Figure 13. A) Superimposition of the substrate binding site in the LacGL1 MD structure (blue) and crystal structure of *T. versicolor* laccase 1KYA complexed with 2,5-xylidene (red). B) Superimposition of LacGL1 the MD structure (blue) and the corresponding MD-averaged TvL structure (red). Snapshots obtained at 20 ns. Superimposition was made of alpha carbons (shown as spheres) in the substrate binding site residues.

Also, the turn comprising F332 has moved approximately 3 Å, notably to the same effect of rendering the substrate binding site of the LacGL1 considerably (~5Å) more compact. This enhanced compactness most likely causes the small phenolic compounds to bind with stronger affinity to the laccase, probably improving the catalytic proficiency towards the smaller phenols such as *p*-coumaric acid, although this requires further experimental verification.

When docked into the MD structure of the LacGL1, the carboxylate of *p*-coumaric acid was favorably exposed to the solvent while preserving a stronger hydrophobic packing vs. TvL. Assuming that the phenols sharing the chemical similarity with *p*-coumaric acid (small, hydrophilic phenol acids or aldehydes without alkyl substituents) are the key inhibitors found in STEX-pretreated SCB, this provides a molecular model of the binding to laccase of these inhibitors and also suggest why the LacGL1 laccase may be particularly proficient in this degradation, by having a natural inclination towards smaller, more hydrophilic substrates commonly found in the STEX-pretreated SCB. Thus, the arrangement of *p*-coumaric acid in the substrate binding site suggests that e.g. the smaller *p*-hydroxybenzaldehyde may be docked in a similar manner.

The binding mode and enhanced compactness also immediately suggest rational improvements of the LacGL1 laccase for this purpose, although this would require a more careful docking and interaction study of several more phenolic compounds of high abundance and inhibitory effect derived from the process (and these fractions may be very sensitive to the reaction conditions (Jurado et al. 2009)).

CONCLUSIONS

A new laccase, having 91% sequence similarity to *G. lucidum* Q9GH17, was successfully cloned and expressed in *P. pastoris* using an α-factor signal peptide. The cloned laccase had maximum activity on ABTS at pH 4.7 and 55°C and showed ability to an extended incubation at 40°C for 24 h (however, with a drop in activity by 77%) and had a wide substrate specificity range with a calculated value of K_m of 0.122 mM. When added to the lignocellulosic biomass (pretreated SCB), the laccase contributed significantly to the release of glucose based on Cellic®CTec1 and Cellic®CTec2 hydrolysis (current, golden standard in commercial cellulase preparations) without the use of artificial mediators.

To our knowledge, we are the first to report a positive effect of laccase on cellulase catalyzed hydrolysis of SCB for an overall yield of released glucose on the currently used, new and improved commercial cellulase preparations Cellic®CTec1 and Cellic®CTec2. This finding has direct implications on decrease in the total enzyme dosage of Cellic®CTec2 on the pretreated biomass, which has a potential in decreasing of the overall operational costs of cellulose-to-glucose conversion and a simultaneous increase in the yields of the produced ethanol. The implementation of the LacGL1 laccase to the cellulase catalyzed hydrolysis reactions can boost their tolerance to inhibitory compounds produced during different forms of pretreatment, and can additionally save on using the process water on washing the biomass off of all inhibitors. The laccase addition has also a potential on influencing a faster turnover of the reaction in the hydrolysis tank, which has a direct interpretation in the lesser inventory space for lignocellulosic material and the lesser spoilage of it. However, more experiments are needed to evaluate if what happens in the reaction mixture is actually a SCB detoxification process.

We have also used 20-ns MD-averaged structures of the LacGL1 laccase and a reference laccase (TvL) with associated docking to demonstrate the molecular binding of likely small phenolic inhibitors derived from the process. Furthermore, we have suggested why the LacGL1 laccase may be particularly proficient for this purpose and have provided a rationale for a future optimization of the laccase for this purpose via site-directed mutagenesis.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION FOR MOLECULAR DYNAMICS SIMULATIONS

Methods

Laccase 3D Structures

The crystal structure of the *Trametes versicolor* laccase (TvL) (PDB ID: 1GYC[1]) was retrieved from the protein data bank[2].

A homology model of LacGL1 was build using Phyre2[3] in threading mode using TvL as a template.

Both structures were prepared for molecular dynamics simulations using the protein preparation wizard in Maestro[4]. Preparation included removal of small organic molecules, deletion of waters beyond 5 Å from hetero groups, addition of hydrogen atoms, and assignment of bond-orders. Due to the lack of force field parameters for copper, bonds to copper were automatically assigned zero bond order by Maestro, and all involved bond distances and angles were fixed to the crystal structure geometry. All copper ions were assigned a charge of +1. The oxygen species present at the T2 and T3 copper sites were modeled as single water molecules bound to copper. The hydrogen bonding network comprised by the protein and remaining crystal waters was refined by the Maestro protein preparation wizard. Due to lack of detailed knowledge about the relative glycosylation of LacGL1 and TvL, the carbohydrate (NAG) moieties found in the TvL crystal structure were removed prior to MD simulations.

Molecular Dynamics Simulations

Using Maestro[4] the LacGL1 model and the TvL structure were protonated corresponding to pH "neutral". The Desmond System Builder was used to immerse each prepared structure in a cubic box of TIP4P[5] water providing a minimum layer of 13 Å of water on each side of the protein. The automatic ionization procedure provided a charge-neutral system by replacing randomly selected waters with Na^+. System details are provided in **Table 1**.

Table 1. System details for MD simulations

Laccase	pH / protein charge	Atoms	#TIP4P waters
Ganoderma lucidum	Neutral / -11	140176	33156
Trametes versicolor (1GYC)	Neutral / -4	137755	32569

The OPLS_2005 force field parameters[6] were used for the systems, implying assignment the alkali parameters[8] for Na^+.

Each system was subjected to steepest descent minimization to a gradient of 1 kcal mol^{-1} Å^{-1} followed by the default pre-simulation protocol employed in Desmond consisting of (1) minimization with restraints on solute, (2) unrestrained minimization, (3) Berendsen[9] NVT simulation, T = 10 K, small time steps, restraints on heavy solute atoms, (4) Berendsen NPT simulation, T = 10 K, restraints on solute heavy atoms, (5) Berendsen NPT simulation with restraints on heavy solute atoms, and (6) unrestrained Berendsen NPT simulation.

Following the relaxation protocol, a 20 ns NPT simulation was carried out for each system at 300 K.

The temperature was regulated with the Nose-Hoover chain thermostat[10] with a relaxation time of 1.0 ps. The pressure was regulated with the Martyna-Tobias-Klein[11] barostat with isotropic coupling and a relaxation time of 2.0 ps. The RESPA[12] integrator was employed with bonded, near, and far time steps of 2.0 fs, 2.0 fs, and 6.0 fs, respectively. A 9 Å cutoff was used for non-bonded interactions. The smooth particle mesh Ewald method[13] with a tolerance of 10^{-9} was used for long-range Coulomb interactions. MD trajectories were saved to disk at 20 ps intervals for subsequent analysis. The root-mean-square backbone atomic positional deviation (RMSD) with respect to the initial structures (1GYC crystal structure for the 1GYC simulation, and 1GYC based homology model for the LacGL1 simulation) were calculated for each simulation (**Figure 1**).
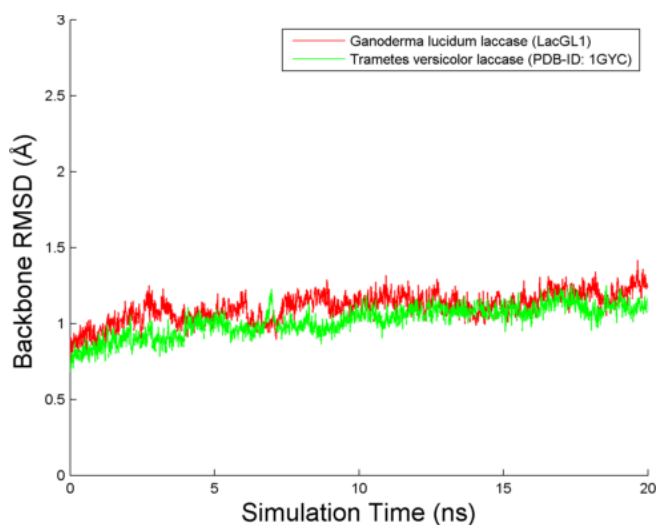

Figure 1. Backbone RMSD with respect to the initial structure for simulations of LacGL1 (red) and 1GYC (green).

RESULTS

RMSD for NPT simulations

It is evident from the RMSD plots for the simulations (**Figure 1**) that both LacGL1 and 1GYC have reached significant structural convergence at 10 ns. For both laccases, the low RMSD (~1.2 to 1.4 Å) for the simulated structures at 10 ns and beyond signifies that the overall structure in the initial structure (crystal structure for 1GYC, homology model based on 1GYC for LacGL1) is largely conserved in the simulations.

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Structure, Functionality, and Tuning Laccases for Industrial Applications

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Structure, functionality, and tuning laccases for industrial applications: A review.

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■ **Abstract** Laccases (EC 1.10.3.2) are copper-containing oxidoreductases that have a relatively high redox potential which enables them to catalyze the oxidation of phenolic compounds, including lignin-derived phenolics. The range of action of laccases can be expanded to non-phenolic substrates by means of supplementary mediators that essentially transfer the electron. The detailed mechanism for laccase-catalyzed oxidation of phenolics, followed by the concomitant reduction of dioxygen to water via copper catalysis, involves a complex series of electron transfer reactions balanced by a stepwise re-oxidation of copper ions in the active site of the enzyme. Based on a careful validation of the available models, a thorough discussion and in detail clarification of the catalytic four-copper mechanism of fungal laccases is presented. We also put focus on the features, regions, and modifications of laccases that make them desirable oxidoreductases for various industrial applications. The availability of 3D structure and of the regions of laccase that can be manipulated by either directed evolution, rational design or site-directed mutagenesis, gives a prerequisite for improved biorefinery processes.

CONTENTS

INTRODUCTION

Laccases (benzenediol: dioxygen oxidoreductases, EC 1.10.3.2) belong to the family of the blue copper oxidases along with ascorbate oxidase (Messerschmidt et al., 1992), bilirubin oxidase (Cracknell et al., 2011), ceruloplasmin (Zaitsev et al., 1996), cuprous oxidase from *Escherichia coli* (Singh et al., 2004), and ferroxidase from *Saccharomyces cerevisiae* (Taylor et al., 2005). The blue copper oxidases are characterized by the presence of at least four copper ions in the active site, which are distributed in cupredoxin domains (Hakulinen et al., 2002).

In nature, laccases play diverse roles, catalyzing reactions involved in lignification, delignification, oxidative in planta stress management, and fungal morphogenesis and virulence (Enguita et al., 2003).

Laccases are widely distributed among bacteria (Enguita et al. , 2003), bovine rumen flora (Beloqui et al., 2006), insects (Dittmer et al., 2004), plants (O'Malley et al., 1993), and fungi (Basidomycota and Ascomycota). Laccases produced by white-rot fungi (Basidiomycota) currently receive particular interest due to their apparent ability to catalyze the depolymerization (or modification) of lignin and in this way assist in the decomposition of plant material.

Laccases have a very broad substrate specificity with respect to the electron donor, and principally act on diphenols (and apparently also monophenols) and related compounds as electron donors, with oxygen as an electron acceptor according to the general overall reaction accrding to BRENDA - The Comprehensive Enzyme Information System (www. brenda-enzymes.org): 4 benzenediol + O_2 ↔ 4 benzosemiquinone + 2 H_2O (or as discussed later: 4 monophenols + O_2 ↔ 4 quinones + 2 H_2O). As biocatalysts, laccases can catalyze the oxidation of a large array of different substrates (phenols, polyphenols, benzenothiols, polyamines, hydroxyindols, aryldiamines), and their substrate range can be further expanded by the use of redox mediators (diffusible electron carriers from natural or synthetic sources) (Call, Mücke, 1997; Cañas, Camarero, 2010; Kunamneni et al., 2008; Maté et al., 2010). The laccases' wide substrate range is one of the reasons for them being intensively studied over other oxidoreductases. Moreover, their specificity for dioxygen as the electron acceptor, in contrast to requiring hydrogen peroxide as an acceptor, as it is in case of hydrogen peroxidases, is another advantage. Additionally, laccases can be classified as **L**ow (LRP) or **H**igh **R**edox **P**otential (HRP) laccases depending on their redox potential measured at the Type 1 (T1Cu) copper site (to be discussed later). The redox potentials for catalysis range from +0.43 V in bacterial and plant laccases to +0.79 V in some fungal laccases (Alcalde, 2007; Rodgers et al., 2009). The higher the redox potential of a laccase, the broader is the (donor) substrate versatility without the use of mediators.

Laccases have been widely explored to substitute chemical catalysis in various processes, notably in the paper and pulp industry. Currently, laccases are subject to intense investigation for pre-lignification of biomass to develop milder, more "green" processing of biomass e.g. for production of bioethanol from lignocellulosic plant resources (Lu et al., 2010).

The detailed enzymatic mechanism of the copper-catalyzed oxidoreduction process, notably the reduction of dioxygen to water, is complex. However, various details of the available models for the catalysis appear contradictory. In order to identify and develop suitable laccases for industrial use, it is crucially important to understand the factors that determine the catalytic conversion efficiency, notably the redox potential and the significance of the different amino acids coordinating to the T1Cu site. Recent developments in the structural elucidation of novel laccases now allow comparisons of structure - function relationships to be made, but the significance of specific structural traits of laccases for industrial functionality have not been systematically mapped. The purpose of the present review is to re-visit the detailed mechanism of the copper-catalyzed reaction mechanism of laccase, notably with respect to the reduction of dioxygen to water, and to provide an improved base for

defining the specific structural traits that are of significance for employing laccases to catalyze novel bioprocessing reactions. A specific purpose of the present review is thus to focus on important features for the catalytic reaction and enzyme robustness for industrial use and in turn to explore how laccases may be optimized for improved functional properties for extended use in industrial applications.

OXIDATIVE LIGNIN MODIFICATION BY MULTICOPPER OXIDASES.

Lignin is a tree-dimensional, heterogeneous, random, and water insoluble arrangement of phenylpropanoid units present in secondary plant cell walls. The highly-branched structure of lignin is made of three monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 1). Lignin can be efficiently depolymerized by enzymes produced by basidiomycetous white-rot fungi. By these extracellular enzymes the fungi can support their growth by gaining access to the carbon source in form of cellulose and hemicellulose, embedded in the lignin-cellulose complex. One of these enzymes which have gained special industrial attention is the laccase - a multicopper oxidase. This enzyme is able to catalyze the reduction of dioxygen to water by oxidation of lignin phenolic hydroxyl groups. This process leads to depolymerization of lignin and consists of two steps, where oxidation of phenolic substrates is the initial and essential step for the reduction of dioxygen to water, which is the final step.
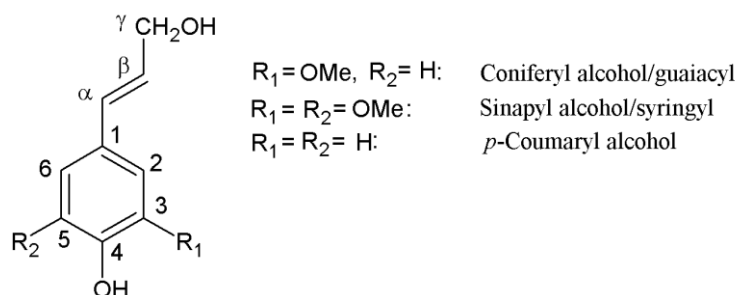

$R_1 = OMe$, $R_2 = H$:   Coniferyl alcohol/guaiacyl
$R_1 = R_2 = OMe$:   Sinapyl alcohol/syringyl
$R_1 = R_2 = H$:   *p*-Coumaryl alcohol

Figure 1. Schematic representation of three main monolignols forming a three-dimensional network of lignin.

Oxidation of phenolic substrates

Oxidation of phenolic substrates by laccases is the first step in the lignin depolymerization and takes place outside the enzyme, proximal to the T1Cu site, where the substrate interacts with amino acids of the binding loops of the laccase (Fig. 9 – to be discussed in section 3). In general terms, the oxidation of phenolic hydroxyl groups of lignin catalyzed by laccase, is a one-electron reaction, which generates resonance-stabilized phenoxy radicals (Fig. 2) (Kawai et al., 1988), which may undergo a second enzyme-catalyzed oxidation (fungal laccases) or a non-enzymatic reaction such as hydration, disproportionation or polymerization. However, the preference for either one of these mechanisms is not known. In terms of laccase catalyzed cleavage of bonds in lignin, they can follow one of the three primary mechanisms: a) C_α - C_β cleavage, b) C_α -oxidation, or c) alkyl-aryl cleavage (Fig. 3).They are presented on a dimeric β-1 lignin model compound.
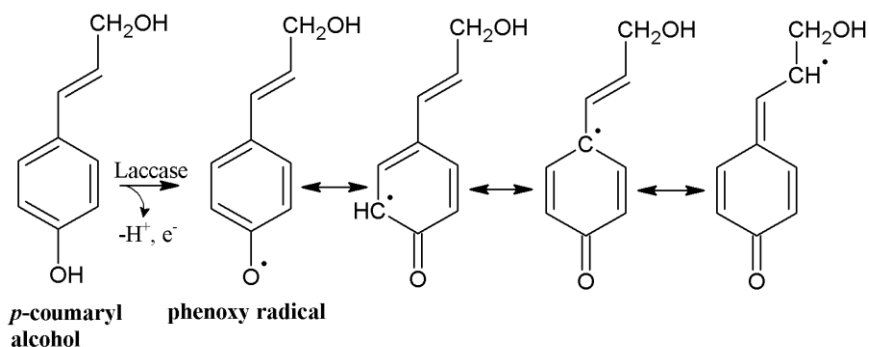
Figure 2. Formation of resonance-stabilized phenoxy radicals of one of the building blocks of lignin - *p*-coumaryl alcohol.

Furthermore, laccase-catalyzed oxidation of phenols, anilines, and benzenethiols correlates with the redox potential difference between the T1Cu site and the substrate. It becomes more difficult for the phenoxy group to be oxidized when electron withdrawing and bulky *o*- and *p*-substituents are present. It is due to their ability to reduce electron density at the phenoxy group making the phenol less reactive in surrendering the electron to the T1Cu of laccase. Moreover, bulky substituents may give rise to steric hindrance and thus proper docking of the substrate, resulting in decreased enzyme catalysis (Xu, 1996a).

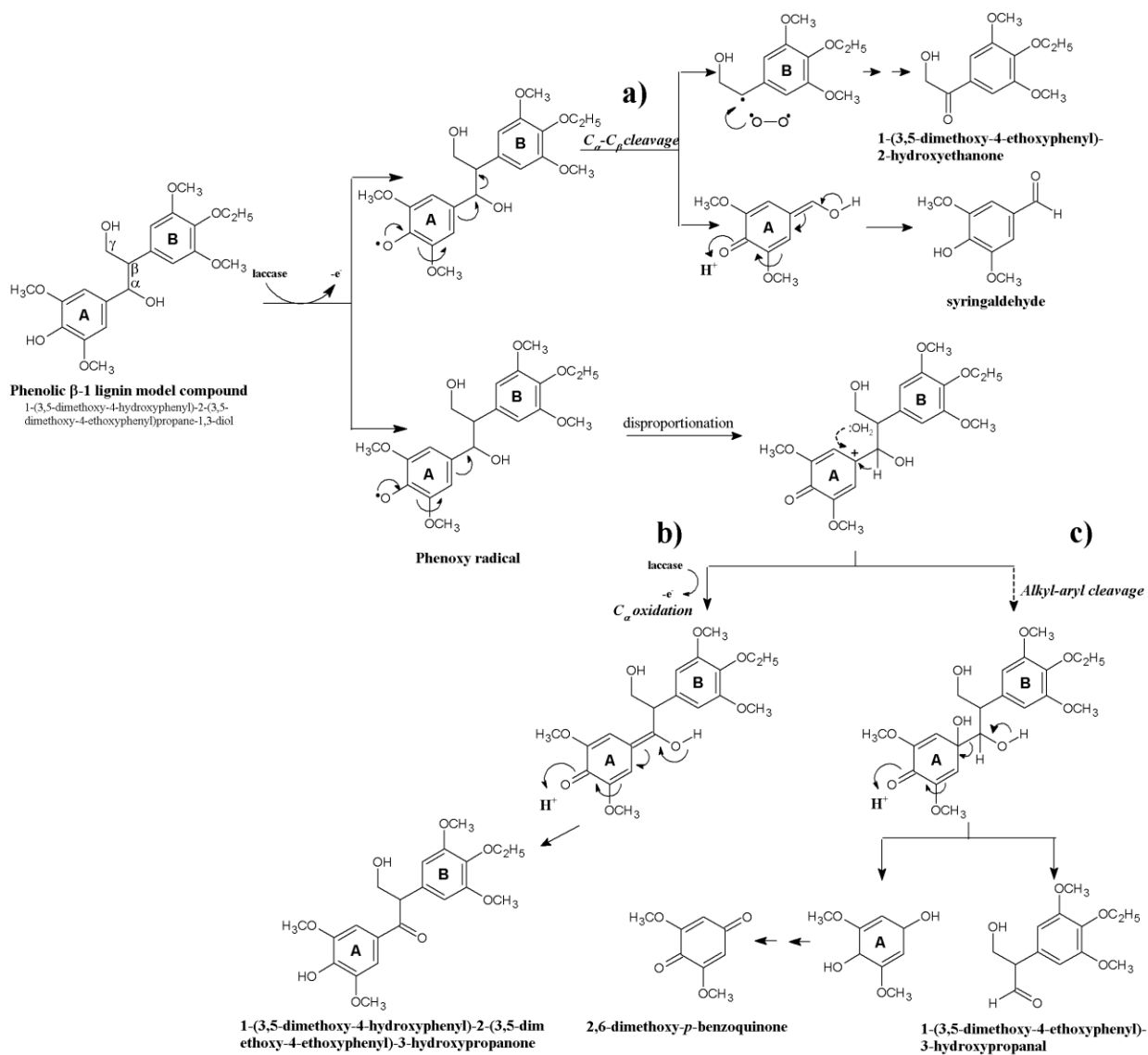Figure 3. Graphical visualization of possible degradation mechanisms of lignin, presented on a phenolic β-1 lignin model compound. The suspected primary degradation reactions include: (a) Cα-Cβ cleavage, (b) Cα oxidation and (c) aryl-alkyl cleavage. Model compound was adapted from (Kawai et al., 1988).

Reduction of dioxygen to water.

The second step that takes place simultaneously with lignin depolymerization is the laccase-catalyzed reduction of dioxygen to two molecules of water (Dwivedi et al., 2011; Quintanar et al., 2005; Rulíšek et al., 2005; Solomon et al., 2008). This reaction takes place *in vitro*, in the catalytic pocket of the enzyme, at the T2/T3 copper site, and requires four reduced copper ions. The oxidation of the copper ions can occur only when a total of four single electrons are abstracted from a phenolic hydroxyl group (referred as to a reducing substrate) (Fig. 4, a→b). The overall stoichiometry for the *in vitro* step is presented below:

$$4 \; Cu(++)^{a)}{}_{oxid.} \xrightarrow{4e^-} 4 \; Cu(+)^{b)}{}_{red.} + O_2 \rightarrow 2 \; [Cu(++)Cu(+)]^{c, d)}{}_{oxid./red.} \xrightarrow{4H^+, 4e^-} 4 \; [Cu(++)]^{e)}{}_{oxid.} \rightarrow 4 \; Cu(+)^{b)}{}_{red.} + 2 \; H_2O$$
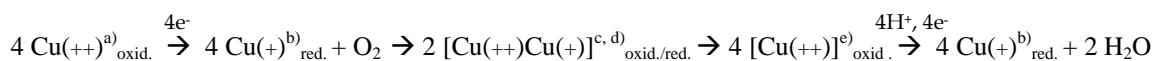
where small, superscript lettering represents singular steps in the catalytic cycle (Fig. 4), and stoichiometric forms in square brackets are the intermediate forms generated during the redox reaction.

The most prevalent and reliable proposal for the catalytic mechanism of multicopper oxidases (MCOs) including improvements of the intermediate state structure has been proposed by Solomon's group. This model is presented in Fig. 4.
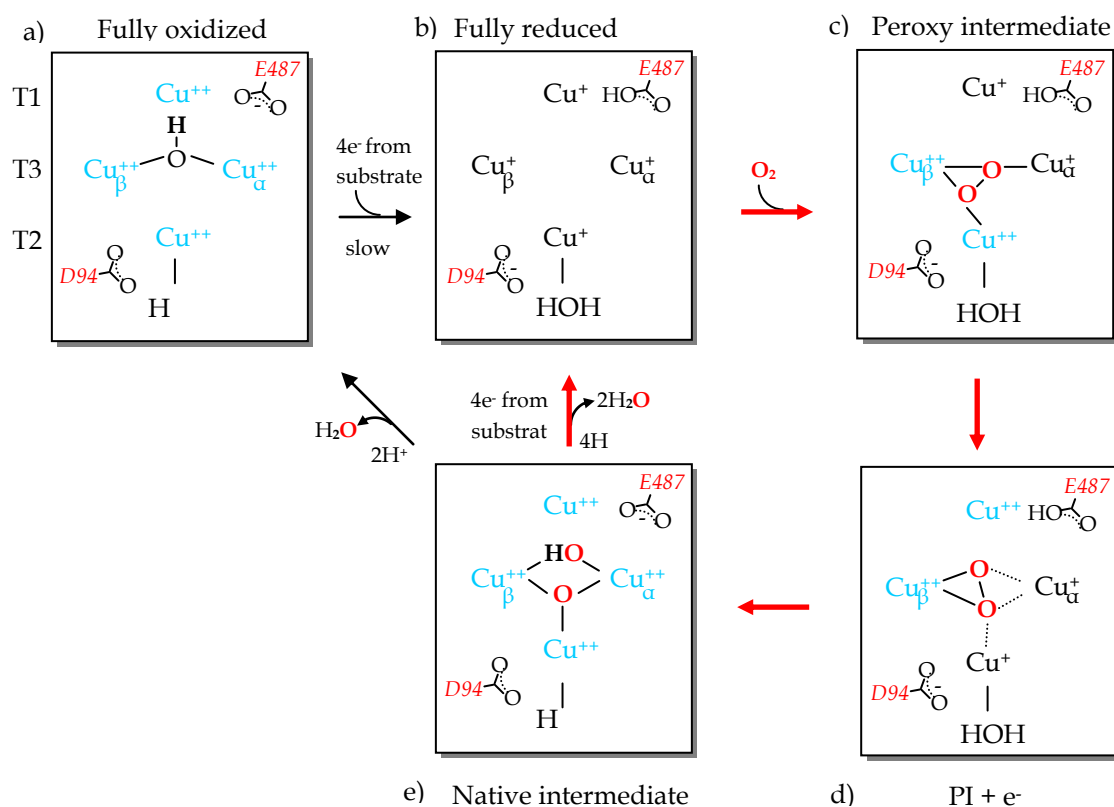

Figure 4. A two 2-electron mechanism of O_2 reduction to H_2O by multicopper oxidases (MCO), adapted from Solomon's group. (Augustine et al., 2010; Solomon et al., 2008). Red arrows indicate steps that take place in the catalytic cycle of multicopper oxidases, while black ones indicate steps that can be experimentally observed but are not a part of the catalytic cycle. a) The fully oxidized MCO is a structure with four copper atoms in their (++) oxidation state and two OH molecules (one is bridging between T3βCu(++) and T3αCu(++) and the second one is bounded to the T2Cu(++) center). The fully oxidized MCO receives, in total, 4e⁻ from its T1Cu(++) center, which are transferred, one by one, over ~13Å to the T2/T3 trinuclear cluster, forming a fully reduced MCO structure b) that can accept an O_2 molecule. O_2 binds preferentially to T3βCu(+) ion and is then reduced by 2e⁻, one from T3βCu(+) and one from T2Cu(+) forming a peroxide intermediate (PI) c) with its 2e⁻ reduced O_2. The driving force for the 2e⁻ reduction is a negatively charged D94 residue (D94 deprotonates by giving away its H⁺ to the OH ligand bound to the T2 center - step a), which lowers the potential of the T3β-T2 edge. After the first 2e⁻ reduction of O_2, one electron is transferred intramolecularly from T1Cu(+) to T2Cu(++) via T1-Cys-His-T3β pathway. This transfer is assisted by an H-bond shunt between the carbonyl oxygen and T1Cu-Cys ligand and Nδ1 on the His483 ligand bounded to the T3βCu, thus forming a PI+e⁻ intermediate d). As a result, the 1e⁻ reduced T2Cu(++) looses its strong bond to peroxide and a H⁺ from E487 is transferred to the peroxide. Moreover the conversion of H_2O to OH, while protonating the D94 residue, lowers the redox potential of the T2Cu center and increases the driving force for another 1e⁻ transfer to peroxide, thus assisting in O-O bond cleavage, producing a fully oxidized native intermediate (NI) with two oxide bridges in the catalytic cavity e). In an excess of

the reducing substrate (RH), NI can be reduced to a fully reduced MCO form by accepting $4e^-$ and $4H^+$ ($3H^+$ will protonate two oxide bridges and release 2 H_2O molecules, $1H^+$ will protonate E487 residue and $1H^+$ will transfer from D94 residue to the OH ligand bounded to the T2Cu center). However in a lack of RH, NI will accept only $2H^+$ which will protonate only one of the oxide bridges, releasing only one H_2O molecule.

The prediction of the intermediate state structures by Solomon's group was based on the **E**xtended **X**-ray **A**bsorption **F**ine **S**tructure (EXAFS) measurements and **C**omputational **C**hemistry (CC) (Hsiao et al., 2006) calculations extended by combined **Q**uantum **M**echanical and **M**olecular **M**echanics (QM/MM) (Ryde et al., 2007) study.

The mechanism presented by Solomon's group (Augustine et al., 2010; Solomon et al., 2008) is based on the Fet3p protein (a multicopper oxidase from *Saccharomyces cerevisiae* that functions in the iron transport, PDB ID: 1ZPU (Taylor et al., 2005)). However, a comparison of the trinuclear cluster and its environment in *Trametes versicolor* with that of other known structures of this enzyme family showed that the trinuclear cluster is structurally highly conserved. Therefore the mechanism of the copper oxidation and dioxygen reduction should be common for all members of the blue multicopper oxidases. The high conservation is observed in copper geometry, for two channels, which accomodate access to molecular dioxygen to and release of water molecule from T2/T3 cluster. The tripeptide His-Cys-His, involved in the **E**lectron **T**ransfer (ET) pathway between T1 copper and T2/T3 trinuclear cluster are also retained in the laccase structure (Piontek et al., 2002).

The mechanism of copper oxidation and dioxygen reduction to water can be divided into 5 steps as shown in Fig. 4.

The first step is initiated by abstraction of a single electron from a reducing substrate (Fig. 4a). In total four single electrons from four reducing substrate molecules are required in the overall process and the laccase stores them as a battery during the first step of lignin depolymerization so that it can use them during the dioxygen reduction. The initial phase of electron abstraction takes place at T1Cu site of a fully oxidized (resting) form of the enzyme (Fig. 4, a), where all copper atoms are at the (++) oxidation state and contains two OH^- molecules. One OH^- molecule, is bridging between T3αCu(++) and T3βCu(++) copper ions and the second one is bounded to T2Cu(++) copper center. The fully oxidized structure of multicopper oxidase is stabilized by hydrogen bindings between a deprotonated glutamic acid (E487), an oxide bridge of T3βCu(++) and T3αCu(++), and a water molecule between the two latter molecules (Augustine et al., 2010). Moreover, there are two highly conserved acid residues in the vicinity of the catalytic pocket, which act together to create a flow of protons from the T3Cu site of the trinuclear cluster to the T2Cu site to drive the reductive cleavage of O-O bond (Augustine et al., 2007). The first one of them, located near the T3 center is an already mentioned, deprotonated glutamic acid residue (E487). It is found to be responsible for donation of H^+ during the reductive cleavage of O-O bond in the **P**eroxide **I**ntermediate (PI) (Fig. 4, d). The second one is a protonated aspartic acid residue (D94), located near the T2 site, which plays a key role in the reaction of the reduced trinuclear center with a dioxygen, and drives an electron transfer from T2Cu center to cleave the O-O bond by deprotonating the T2Cu water ligand (Fig. 4, d) (Augustine et al., 2007).

The four one-electron-reduction of four coppers is a slow process, due to the fact that only one electron is moved around the catalytic site at a time and travels from T1Cu(++) to T2/T3(++) trinuclear cluster over a distance of ~13Å (Fig. 11). After receiving one electron on each of T3Cu's, the bond between T3βCu(++), T3αCu(++), and hydroxide is weakened, and hydroxide is being protonated by H^+ from a bulk solution. Since T2/T3 trinuclear center is positioned in a water channel (Fig. 6) (Piontek et al., 2002), the hypothesis of the hydroxide being protonated by H^+ from a water molecule agree with the model (Messerschmidt et al., 1992). As a result, a protonated hydroxide dissociates as a water molecule through T2/T3 water channel leaving the catalytic cavity in a fully reduced form (Fig. 4, b). However, before the catalytic cavity is able to accept a dioxygen molecule, glutamic acid (E487) needs to be protonated by H^+ from the bulk solution and aspartic acid (D94)

needs to be deprotonated. D94 looses its H^+ to the OH ligand bound to the T2 copper center and forms a H_2O molecule (Fig. 4, b). Now a fully reduced MCO is ready to accept a dioxygen molecule, which has a preference to bind to T3βCu(+), due to its geometry. The computational studies have shown that the T3βCu(+) copper ion has a trigonal pyramidal geometry, which is more reactive to bind a fourth ligand than a more energetically stable trigonal planar geometry of T3βαCu(+) (Augustine et al., 2010). When the dioxygen has docked in the catalytic cavity, the redox potential of T3βCu(++) and T2Cu(++) is being lowered by a negative charge from a deprotonated D94 residue. This provides a driving force necessary to reduce the dioxygen by 2 electrons ($2e^-$ are coming from T3βCu(+) and T2Cu(+) copper ions and not from a reducing substrate), thereby forming a PI (Fig. 4, c). The $2e^-$ reduced dioxygen is now tightly bound to the trinuclear cluster as it is coordinated side-on to the T3βCu(++) and also has a strong end-on bonds with both the T2Cu(++) and T3αCu(+) copper ions (Fig. 4, c). This assists to stabilize the peroxide and is facilitated by structural flexibility of the T3α-T2 edge which moves from a distance >5Å in the reduced state to ~4Å in the PI (Augustine et al., 2010).

The next step in the dioxygen reduction to water, based on Fet3p protein, is a rapid, single electron transfer from T1Cu(+) to T2Cu(++) via the T1-Cys[484]-His[483]-T3β pathway (or T1-Cys[453]-His[454]-T3β pathway in *Ganoderma lucidum* CBS229.93, Fig. 5 (Sitarz et al., 2012)), assisted by an H-bond shift (shunt) between the carbonyl oxygen of T1Cu-Cys484 ligand (Cys453 in *G. lucidum*) and Nδ1 of the His483ligand (His454 in *G. lucidum*) of the T3βCu, forming a PI + e^- intermediate (Fig. 4, d). On the contrary, the T1-Cys[484]-His[485]-T3α pathway (and possibly T1-Cys[453]-His[452]-T3α pathway in *G. lucidum*) does not have a comparable H-bond (Augustine et al., 2010; Augustine et al., 2008, Taylor et al., 2005). As a result of this intramolecular transfer, the T2Cu(++) copper center is being reduced and looses its strong bond to peroxide (Solomon et al., 2008). Afterwards, two single electrons from T3αCu(+) and T2Cu(+) are transferred on the antibonding orbitals of the peroxide causing an increase in the energy and making the peroxide structure very unstable. Therefore, in order to lower the bonding order, the O-O bond is cleaved and the proton from E487 is transferred to the peroxide. Additionally, the *pKa* goes down for water molecule and a proton from T2Cu(+)-bound water is transferred to the D94. This conversion of H_2O to OH lowers the redox potential of the T2 copper center and increase the driving force for an electron transfer to peroxide, thus assisting in O-O bond cleavage (Augustine et al., 2007) and decay of PI, producing a fully oxidized **N**ative **I**ntermediate (NI) (Fig. 4, e).
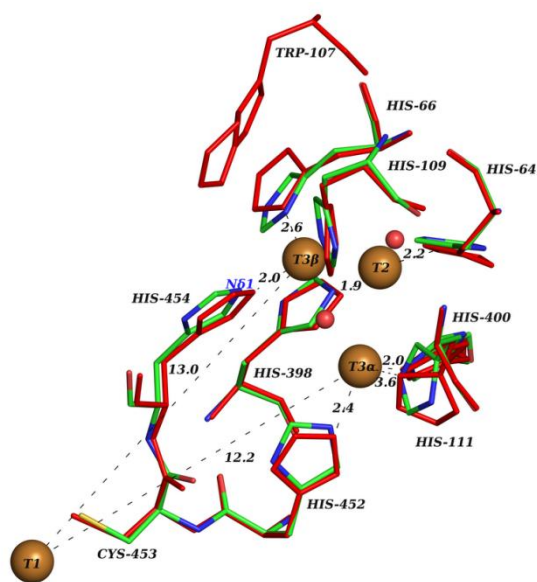
Figure 5. A graphical representation of the three copper centers present in laccases for two superimposed laccases from *Trametes versicolor* (PDB ID: 1KYA) - green sticks, and *Ganoderma lucidum* CBS229.93 - red sticks, respectively. The red colored TRP107 residue belongs to *G. lucidum*'s laccase and can be found in the vicinity of 3.7 Å from the T3β copper center. *T. versicolor*'s laccase lacks this residue in that position. The interatomic distances of residues belonging to *G. lucidum*'s laccase, in the vicinity of each copper site, are shown in black nicked lines and values are given in Ångstrom [Å]. The amino acid nomenclature and their position, according to *G. lucidum* laccase, are written in black. All three copper sites are shown as brown spheres. Two coordinating water molecules are presented as red spheres. Nδ1, colored blue, represents a nitrogen atom from which a proton shifts to the carbonyl of Cys453, when assisting in the electron transfer from T1Cu(+) to T2Cu(++) while the dioxygen is bounded to the T2/T3 copper cluster. The T1-Cys[453]-His[452]-T3β pathway, which enables the electron transfer from T1Cu(++) to T2/T3(++) copper cluster is also shown in the figure by signed single amino acids that form the pathway.

When NI, a fully oxidized form of multicopper oxidase with two bridging oxides is formed, it can either return to a fully reduced form of an enzyme by accepting 4H⁺ (3 H⁺ are needed for a release of two H_2O molecules, 1 H⁺ protonates a deprotonated E487 residue and 1 H⁺ (which is not accounted for the gross reaction) is transferred from a protonated D94 residue to the OH ligand on the T2 copper center) and 4 e⁻ from four molecules of a reducing substrate simultaneously releasing two water molecules, or it can release one water molecule and return to its fully oxidized form. Release of two water molecules is possible due to the flexibility of the T3α-T3β edge which facilitates an increase in the Cu-Cu distance from <3.5Å in NI back to >5Å in the reduced form. Opening of the trinuclear cluster enables the elimination of two water molecules (Augustine et al., 2010) formed probably by protons provided by the bulk solvent in the T2/T3 channel. Therefore the way the reaction follows is determined by availability of a reducing substrate. In excess of it, laccase will return to its fully reduced form and be ready to accept a dioxygen molecule (Augustine et al., 2010). However, if the amount of substrate is insufficient, it will return to its resting state and remain in that form until it can be oxidized again.

Other catalytic models had been presented in the literature over the years. The model of Andréasson, from 1976 studied the kinetics of laccase by mixing the fully reduced enzyme with dioxygen (Andréasson et al., 1976), where the visualization of formed intermediates was performed by EPR and UV-VIS spectroscopy. According to that type of model, which we believe lacks a thorough evaluation; the dioxygen was reduced to water by accepting a total of four electrons. The two first electrons were transferred from T3 copper ions giving a peroxide end-on coordinated to the T3βCu(++). The third electron was rapidly donated from T1Cu(+) to reduce the peroxide end-on coordinate and form an oxygen radical and the last, fourth electron was believed to be transferred from T2Cu(++) to the previously formed oxygen radical, which showed a slow decay in the EPR signal. However, the reaction transfer from T2Cu(++) was too slow to be catalytically significant, and therefore it was proposed that during turnover, the last electron is donated to the oxygen radical from a reducing substrate molecule via the re-reduction of the T1Cu(+). Moreover, the oxidized laccase form had an oxide bride between T3αCu(++) and T3βCu(++) (Andréasson et al., 1976; Bukh et al., 2006; Malmström et al., 1969). This oxygen radical model is still an accepted model (Huang et al., 1999; Messerschmidt et al., 1993).

Another model published in the literature and refined as compared to the previously described dioxygen model, was based on **M**agnetic **C**ircular **D**ichroism (MCD) studies of the long-lived optical intermediate (Clark, Solomon, 1992). The model was proposed by Shin et al. (Shin et al., 1996) and later improved by Quintanar et al. (Quintanar et al., 2005). According to this model, dioxygen was reduced to water by accepting a total of four electrons. The first two electrons were donated from T3 and T2 copper. The peroxide was then reduced to a hydroxide ion via the simultaneous donation of two electrons from T1 and T2 coppers. The optical intermediate arose from the hydroxide ion bridging T3 and T2. The oxide/hydroxide ion bridging the T3 pair derived from the solution, whereas the hydroxide ion or water molecule coordinated to T2 copper ion, in the fully oxidized enzyme was

proposed to originate from the reduced dioxygen (Bukh et al., 2006; Shin et al., 1996). The optical intermediate was formed simultaneously with the oxidation of T1Cu site.

In summary, all available models presented in the literature (Andréasson et al., 1976; Augustine et al., 2010; Chalupský et al., 2006; Messerschmidt et al., 1993; Quintanar et al., 2005; Rulišek et al., 2005; Shin et al., 1996; Solomon et al., 2008; Solomon et al., 2001) have their own interpretation of the intermediate structures formed during the oxygen reduction to water. We strongly believe that a described here model, which is based on an H-bond shunt is the one that gives the most reasonable explanation to what really happens in the catalytic pocket during the redox reaction . However, the three major steps that are independent of the intermediate structures seem to be common for all models. Those are (Witayakran , Ragauskas, 2009):

1. T1 Cu(++) in its resting state is reduced by accepting electrons from the reducing substrate
2. Electrons are internally transferred ~13 Å from T1 copper to T2/T3 trinuclear cluster
3. Molecular oxygen is activated and reduced to water at the T2/T3 trinuclear center.

Oxidation of non-phenolic substrates - laccase/mediator-catalyzed oxidation

Laccases have a lower redox potential (0.5 - 0.8 V) than ligninolytic peroxidases (>1 V) and it was initially thought that laccases would only be able to oxidize phenolic substrates which represents less than 20% of lignin (Galli, Gentilli, 2004; Kersten et al., 1990). Phenols are typical laccase substrates because their redox potential, ranging from 0.5 to 1.0 V vs. NHE (**N**ormal **H**ydrogen **E**lectrode), is low enough to allow electron abstraction by T1 copper (Giardina et al., 2010). However, the range of substrates oxidized by laccase can be increased in presence of a mediator, either a synthetic or a natural one.

The mediator is intended to promote or facilitate enzyme action by increasing its oxidation potential (Giardina et al., 2010). It is a small molecule that acts as an electron shuttle: once it is oxidized by generating a strongly oxidizing intermediate, a so-called mediator. The oxidized mediator diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size and steric hindrance may not directly enter into the active site of the laccases. In other words; enzyme and a reducing substrate do not need to interact in a direct manner. In reactions where the substrates have a higher redox potential than laccase, typically 0.5-0.8 V vs. NHE, or the substrate is too large to dock into the active site of the laccase, the mediator can catalyze the oxidation (Desai, Nityanand, 2011). Therefore, the use of a mediator is suitable for laccase applications when dealing with depolymerization of non-phenolic substrates of lignin which constitutes 80% of the lignin polymer and may have a higher redox potential than laccase itself.

There are two types of mediators, the artificial and native catalysts. In general, mediators should be low molecular compounds able to generate stable radicals (in its oxidized form) to avoid the inactivation of laccase. Moreover, their reactivity should allow recycling without degeneration. In addition for industrial use, an ideal mediator should be available at low costs and should be environmentally safe (Cañas, Camarero, 2010).

Artificial mediators

The two most commonly used artificial mediators are 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Bourbonnais, Paice, 1990) and 1-hydroxybenzotriazole (HBT) (Call, Mücke , 1997; Hirai et al., 2006; Kawai et al., 2002). They oxidize the non-phenolic substrates but follow different mechanisms.

Laccase/mediator-catalyzed oxidations of the major non-phenolic substrates (representing 80-90 % of lignin) (Kawai et al., 2004), can proceed via three different mechanisms depending on the structure of the oxidized mediator (Galli, Gentilli, 2004). The mediators containing a N-OH structural feature, such as N-hydroxybenzotriazole (HBT), violuric acid (VA), 3-hydroxyanthanilic acid (HAA), and N-hydroxyphthalimide (HPI), favor a radical **H**ydrogen **A**tom **T**ransfer (HAT) pathway (Fig. 6a), while 2,2'-azinobis-(3-ethyl-benzylthiozoline-6-sulphate) (ABTS) reacts via an **E**lectron **T**ransfer (ET) pathway (Fig. 6b). 2,2,6,6-tetramethyl-1-piperidine-N-oxyl (TEMPO), and its analogues follow an **I**onic **O**xidation pathway (IO) (Fig. 6c). Examples of mediator-substrate oxidation mechanisms are depicted in Fig.7.
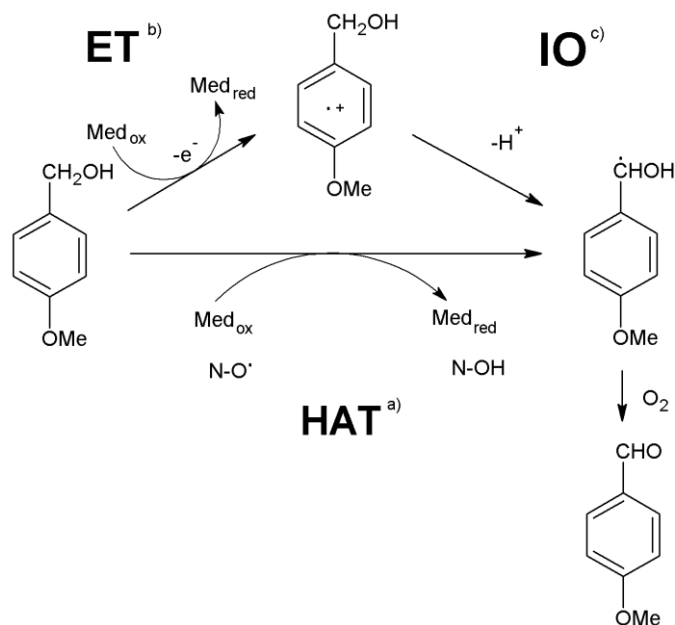

Figure 6. Schematic representation of the mediator-substrate oxidation catalyzed by laccases via three different routes: a) the radical **H**ydrogen **A**tom **T**ransfer (HAT) route, b) the **E**lectron **T**ransfer (ET) route, and c) the **I**onic **O**xidation (IO) route. Med_{ox} represents oxidized mediation and Med_{red} represents a reduced mediator.

Degradation of one of the examples studied by the **L**ignin **M**ediator **S**ystem (LMS) is a non-phenolic β-O-4 model compound, representing the major substructure in lignin. In this model the dimer: 1-(4-ethoxy-3-methoxyphenyl)-1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)propane, in a coupled enzyme/HBT system, can undergo four types of reaction; β-ether cleavage, C_α - C_β cleavage, C_α - oxidation, and aromatic ring cleavage. The coupled enzyme/HBT system catalyzes 1 e⁻ oxidation of the substrate to form β-aryl radical cation or benzylic (C_α) radical intermediates. However, the electron density of the aromatic ring is affecting the reaction. Substrates containing electron-donating groups favor aromatic ring cleavage products. The β-aryl radical cation is converted to the product via an aromatic ring cleavage, and the benzylic radical is cleaved at the C_α - C_β bond similar to a Baeyer-Villiger reaction. The β-ether cleavage of the β-O-4 lignin substructure is caused by reaction with the C_α -peroxy radical intermediate produced from the benzylic radical (Kawai et al., 2004; Wong, 2009).

Mediators of natural origin

Mediators of natural origin are classified into two groups. The first one represents compounds that are produced by fungi as secondary metabolites, while the second group is based on the origin of the compound.

The first discovered secondary metabolite produced by *Pycnoporus cinnabarinus* was 3-hydroxyanthranilic acid. This compound was believed to mediate oxidation of non-phenolic substrates and synthetic lignin catalyzed by laccase (Eggert et al., 1996). However, Li et al. (Li et al., 2001) showed that this secondary fungal metabolite generates cinnabaric acid during the oxidative coupling, which is simply unable to mediate the oxidation of non-phenolic compounds due to the lack of a substituent group that can be further oxidized to phenoxy radical by laccase. There are however examples of secondary metabolites produced by fungi that are able to oxidize polycyclic aromatic hydrocarbons (PAH); those are: 4-hydrobenzylic alcohol (Johannes, Majcherczyk, 2000) and 4-hydroxybenzoic acid, which was also used for fungicide degradation and detoxification (Murayama et al., 2007).

The second group of natural mediators represents phenolic compounds related to the lignin polymer. These phenolic compounds are presented in herbaceous plants either as secondary metabolites, precursors for lignin biosynthesis or they can originate from lignin degradation (Cañas, Camarero, 2010). Examples are as follows: acetosyringone, syringaldehyde (delignification of paper pulps (Camarero et al., 2007), lipid removal (Gutiérrez et al., 2006), dye decolorization(Murugesan et al., 2009), dehalogenation of pesticides (Torres-Duarte et al., 2009)), vanillin, acetovanilline, ferulic acid, and *p*-coumaric acid (anthracene and benzo[a]pyrene transformation (Cañas et al., 2007)).

In general terms, natural mediators follow the **H**ydrogen **A**tom **T**ransfer (HAT) oxidation mechanism (d'Acunzo, Galli, 2003), and are generally regarded as the most efficient non-phenolic degraders in laccase catalyzed reactions. The term "natural", is not only the definition for the natural origin of these compounds, but also defines their role in nature, being the most likely the usual (true) mediators of laccases activities during the biodegradation of lignin polymer carried out by laccase catalyzed reactions (Cañas, Camarero, 2010).

THE STRUCTURE OF FUNGAL LACCASES

Fungal laccases in general belong to extracellular, monomeric proteins of approximately 60-70kDa, with an acidic isoelectric point (pI) around 4.0 (Giardina, Faraco, 2010). However, glycosylation can increase their molar weight with 10 to 45%, e.g. as shown for *Rhus vernicifera* laccase (Reinhammar, 1997).

The molecular architecture of laccases has been sequentially arranged in three tightly associated cupredoxin-like domains, each of which has a greek key β-barrel topology, and is strictly related to that of small copper proteins such as azurin and plastocyanin (Murphy et al., 1997). The laccases are also members of the multi-copper oxidase family, housing enzymes such as *Rhus vernicifera* laccase (Nitta et al., 2002), ascorbate oxidase (AOx) (Messerschmidt et al., 1992), ferroxidase from *Saccharomyces cerevisie* (Fet3p) (Taylor et al., 2005), bilirubin oxidase (Cracknell et al., 2011), and mammalian ceruloplasmin (CeuO) (Zaitsev et al., 1996). In addition, specific domains contain catalytically important copper atoms, which function in the oxidation mechanism (a detailed description in section; Reduction of dioxygen to water).

The observations of a 3D crystallographic structure of *Trametes versicolor* laccase (PDB ID: 1KYA chain C) (Fig. 7), revealed that the four catalytically important copper ions are situated in domain 1 and 3. The T1 copper site is located in domain 3 with one copper (T1Cu) lying in a shallow

cleft on the surface of the enzyme, while the T2 and the T3 copper sites, having one (T2Cu) and two (T3αCu and T3βCu) copper atoms, respectively, which form a trinuclear copper cluster (T2/T3) are positioned at the interface between domains 1 and 3, which also provide ligand residues for the coordination of the copper atoms. This position of the copper atoms fits very well with the superimposed structure of *Ganoderma lucidum* laccase (Sitarz et al. 2012) and is also in agreement with the first X-ray structure of *Coprinus cinerreus* laccase (Ducros et al., 1998). Additionally, the superimposed laccases from *T. versicolor* and *G. lucidum* have two hypothetical water channels, which are located between domains 1 and 3, and 3 and 2. A similar discovery was also described by Piontek et al. (Piontek et al., 2002) for a different strain of *T. versicolor* (PDB ID: 1GYC). However, by comparing the two investigated and superimposed laccases; from *Trametes versicolor* (PDB ID: 1KYA) and *Ganoderma lucidum* CBS229.93, it is clear that they differ in their topology (Fig. 7 a, b, c). One of the inryguing differences in the 3D structure of *G. lucidum* laccase, in respect to its performance during lignin depolimerization, is an existence of an additional α-helix in the vicinity of the trinuclear copper site. The presence of this α-helix results in an additional residue (TRP107) in the vicinity of T2/T3 copper site (Fig. 5). The model for *G. lucidum*'s laccase was predicted by using a comparative protein modeler - 3D-JIGSAW, where the crystallographic structure of *T. versicolor* was used as a template. Additionally, the 3D structure from *T. versicolor* had more than 80% homology on the conformational level with the laccase from *G. lucidum* (Sitarz et al., 2012).
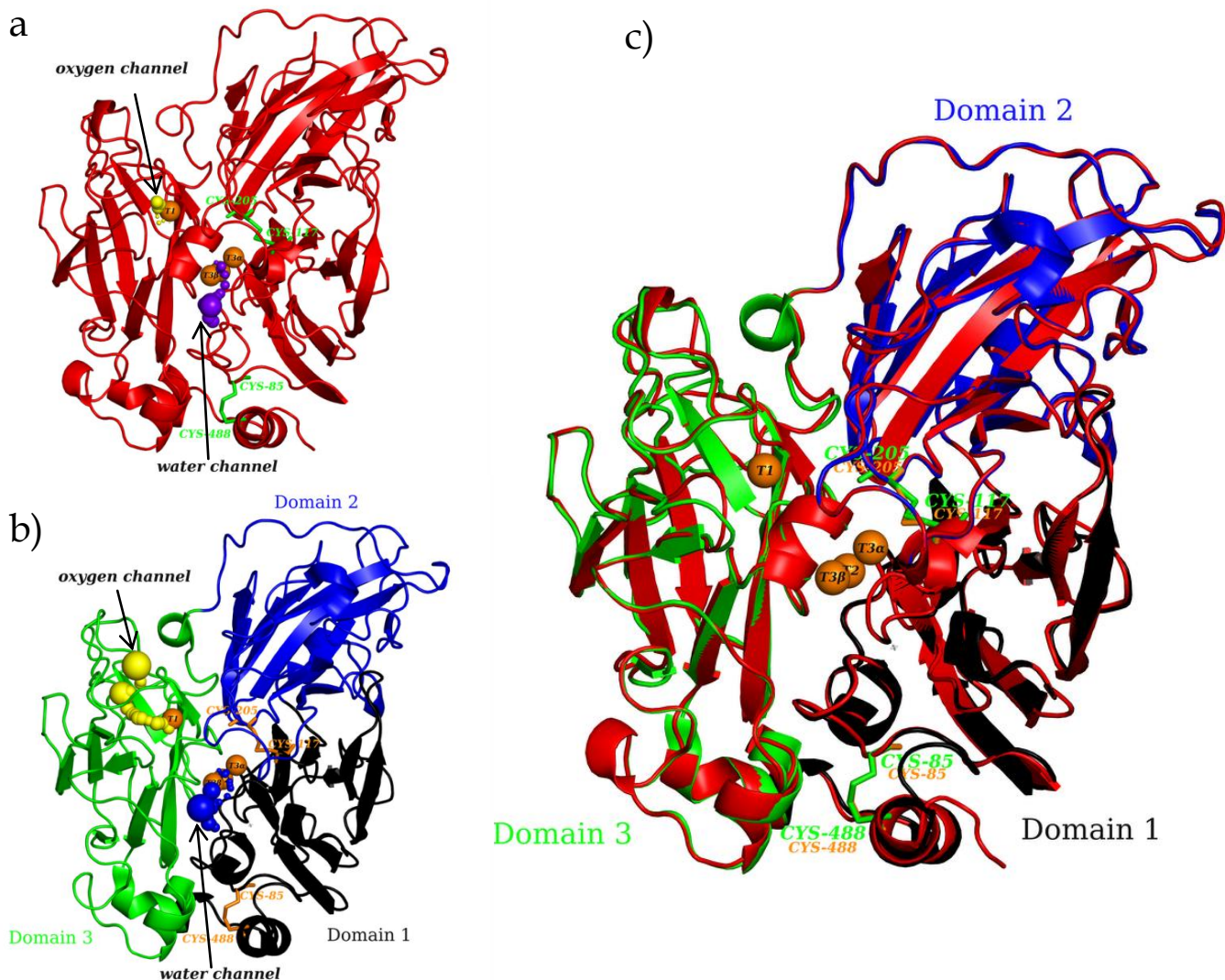
Figure 7. A graphical representation of two superimposed laccase structures from *T. versicolor* (PDB ID: 1KYA chain C) and *G. lucidum* CBS229.93, illustrated using the PyMol Molecular Graphic System Version 1.5 Schrödinger, LLC. a) The red colored 3D structure of *G. lucidum* laccase was predicted by submitting its full amino acid sequence (Sitarz et al., 2012) to the 3D comparative protein modeler - 3D-JIGSAW (available at: http://bmm.cancerresearchuk.org/~3djigsaw/). The green sticks represent cysteine residues responsible for formation of disulphide bridges. b) The three colored 3D structure of *T. versicolor* that was a base for prediction of 3D structure for *G. lucidum* laccase. Brown spheres represent four copper ions, which play an important role in catalysis. They are located in domain 3 (T1Cu) and between domain 3 and 1 (T2Cu, T3αCu and T3βCu). The orange sticks represent cysteine residues that form disulphide bridges. Domain 1 (residues 1-131 and 476-499) is colored black, domain 2 (residues 132-300) is colored blue, and domain 3 (residues 301-475) is colored green. The range of amino acid residues that are a part of each domain was predicted according to (Aleksandrov, Shinddyalov, 2003) and found at www.pdb.org under *T. versicolor*'s sequence. The yellow and blue small spheres that float through the two laccases are the oxygen and water channel, respectively. Both channels were calculated using CAVER - a plug-in for PyMol (Medek et al., 2007). c) Two superimposed laccases from *G. lucidum* (a) and *T. versicolor* (b) with their potential position of disulphide bridges. The color coding is identical as in point a and b.

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In addition, the tertiary structure of basidiomycete laccases is stabilized by a total of two disulfide bridges between domain 1 and 2, and between domain 1 and 3 (Fig. 7 a, b, c). The two disulphide bridges are formed between CYS85 and CYS488, and between CYS117 and CYS205, and are present in the exact same place for both investigated laccases. The position and number of disulfide bridges is not conserved through out fungal multicopper oxidases, but they can still be arranged into two groups; basidiomycetes with two and ascomycetes with 3 disulphide bridges (Hakulinen et al., 2002).

With respect to the amino acid sequence, all laccases comprise a polypeptide chain of approximately 520-550 amino acids (Fig. 8), where 20-22 residues is the N-terminal signal peptide (Smith et al., 1997), which is not a part of the mature protein. In rare cases a propeptide (Berka et al., 1997) and a C-terminal signal peptide (Hakulinen et al., 2006) have been observed. In addition, fungal laccases are different to other members of the multi-copper oxidase family, by the presence of a set of four, R1-R4 (Fig. 8, Table 1), ungapped sequence regions, containing conserved residues of one cysteine and 10 histidines, that are involved in binding of four copper atoms (Thurston, 1994). Eight out of ten histidine residues appear in a highly conserved pattern of four HXH motifs (Fig. 8) in the enzyme. An X in this motif represents an undefined residue. Moreover, the HXH motifs are separated from one another by segments of between 25 and 175 residues (Kumar et al., 2003) and are likely to be brought close in a composite catalytic apparatus by an aspect of protein folding.
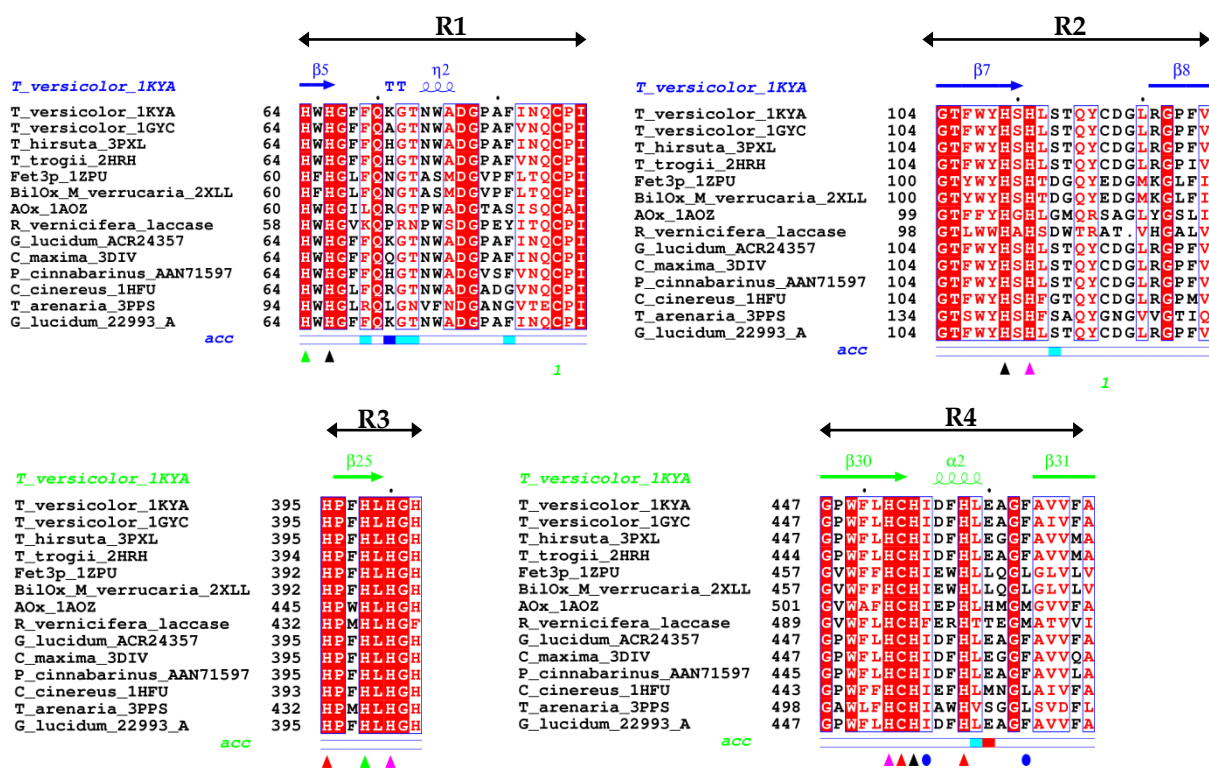

Figure 8. Selected alignment parts of randomly chosen enzymes, belonging to the multicopper oxidases family, which show the copper binding domains with their conserved amino acids that take part in coordination to four copper atoms. Amino acids in red boxes indicate fully conserved residues and triangles below the red boxes point out to 11 fully conserved amino acids coordinating to the four copper atoms. All 11 residues are positioned within sequence segments (R1-R4), which are ungapped for fungal laccases and allow them to be distinguished within a broader class of multicopper oxidases. Red, green, pink, and black triangles indicate coordination to the T1Cu, T2Cu, T3αCu, and T3βCu copper ions, respectively. The blue circles indicate the residues positioned 4Å axial to the T1 copper ion. Note that the axial, non-coordinating isoleucine residue (455I) is almost invariable while the residue at position 463 varies among members of the multicopper oxidase family (F, L, M). The secondary structures (α-helices and β-sheets) above the alignment are based on a crystallographic structure of *Trametes versicolor* (PDB

ID: 1GYC) (Piontek et al., 2002). α-helices and β-sheets colored black indicate domain 1 (residues 1-131, and 476-499); α-helices and β-sheets colored green indicate domain 3 (residues 301-475). Domain 2 (residues 132-300) is not presented here due to the lack of residues serving a function in catalysis. The domain coloring in this figure corresponds to the coloration code of domains in Fig. 6. Alignment of sequences was made using ClustalW 2.0 software (Goujon et al., 2010; Larkin et al., 2007) and ESPript for final output (Gouet et al., 1999). The signal peptides were cleaved off prior to alignment using SignalP software (Emanuelsson et al., 2007).

In addition to the R1-R4 sequence segments, laccases have four loop regions, which have been identified on basis of their 3D structure (Larrondo et al., 2003). They were designated I-IV, and are involved in substrate binding. When comparing the 3D model *G. lucidum* and the 3D structure of *T. versicolor*, the loops and general 3D structure are almost perfectly superimposed (Fig. 9).
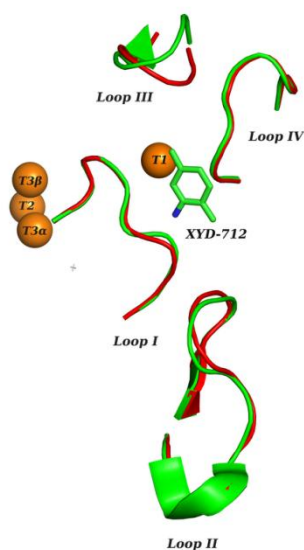
Figure 9. A graphical representation of superimposed substrate binding loops for laccases from *Trametes versicolor* (PDB ID: 1KYA chain c) and *Ganoderma lucidum* CBS229.93. The red coloring represent loops belonging to *G. lucidum*, while green coloring represents loops belonging to *T. versicolor*. Brown spheres represent four copper ions, which play an important role during reduction of oxygen to water, and a green colored residue, namely XYD712, is a substrate - 2,5-xylidine oxidized by the enzyme.

The slight conformational difference is however observed in loop II and III, where *T. versicolor* contains a fragment of a β-strand. The range of amino acids forming a loop in *G. lucidum*'s laccase was chosen according to the laccase from *T. versicolor* (PDB ID: 1KYA) (Larrondo et al., 2003), since these two 3D structures carry 80% homology. The amino acids responsible for substrate binding are presented in Table 2.

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Table 2. Copper signature sequences in fungal laccases.

Region of consensus	Consensus motifs[a] in signature sequences of basidiomyceteous laccases	Residue (coordinating copper)
R1	^{64}**H**W**H**G-F/L-FQ-X-GTNWADG-X$_2$-F/G-X-NQCPI^{87}	^{64}H(T2Cu), ^{66}H(T3βCu)
R2	^{104}GTFWY**H**S**H**-L/F-S/G-TQYCDGLRGP-F/MV^{125}	^{109}H(T3βCu), ^{111}H(T1Cu)
R3	^{395}**H**PF**H**L**H**GH^{402}	^{395}H(T1Cu), ^{398}H(T2Cu), ^{400}H(T3αCu)
R4	^{447}GPWF-L/F-**H**C**H**I-D/E-F**H**L-E/M-X-G-F/L-A-V/I-V-X-A^{468}	^{452}H(T3αCu), ^{454}H(T3βCu), ^{458}H(T1Cu)

Loop sequence motif [b]			
Loop I	Loop II	Loop III	Loop IV
^{159}GPAFPLGAD^{166}	^{261}ANPNFGNVGFTGGIN^{275}	^{332}FNGTNFF^{338}	^{386}ATAAAPGAP^{393}

[a] The start position of the signature sequence is shown with respect to *T. versicolor* (PDB ID:1KYA c) laccase (Bertrand et al., 2002).
[b] The range of amino acids forming a loop in *G. lucidum*'s laccase was predicted based on *T. versicolor* (PDB iD: 1KYA c) laccase (Larrondo, 2003).

The active site of fungal laccases

The copper sites in laccases are very similar to ascorbate oxidase, ceruloplasmin and billirubin oxidase, and are categorized into three centers, in which the copper atoms can be distinguished according to their characteristic **E**lectronic **P**aramagnetic **R**esonance (EPR) and UV/visible signals (Dwivedi et al., 2011). Moreover, in a resting state of an enzyme, all four copper atoms are in the (++) oxidation state as previously shown in Fig. 4.

T1 copper center (blue copper center)

T1 copper (e.g. paramagnetic 'blue' copper) has a characteristic absorbance at 614 nm and can be detected by EPR. The charge transfer transition SCys→Cu(++) is responsible for the deep blue color of the enzyme (Lee et al., 2002). The T1Cu ion has a trigonal coordination (Fig. 10), with two histidines and one cysteine as conserved equatorial ligands (Claus, 2004; Leontievsky et al., 1997). In addition two non- (or weakly) coordinating residues has also been observed in vicinity (4Å) of the T1Cu(++) center (Rodgers et al., 2009).
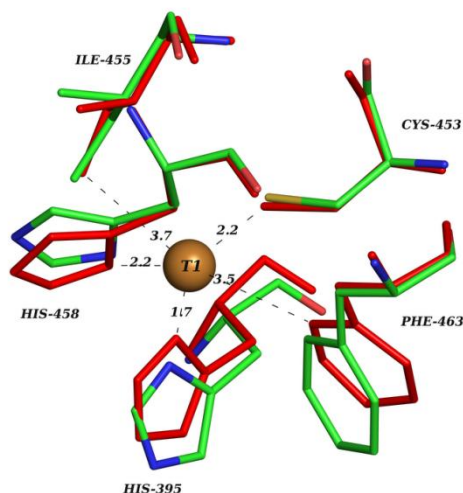
Figure 10. A graphical representation of T1 copper site for two superimposed laccases from *Trametes versicolor* (PDB ID: 1KYA) - green sticks, and *Ganoderma lucidum* CBS229.93 - aquamarine sticks, respectively. The interatomic distances [Å] are based on the location of residues belonging to *G. lucidum*'s laccase. The amino acid nomenclature and their position according to *G. lucidum* laccase are written in black. PHE-463 and ILE-455 are non-coordinating amino acids and the dashed lines do not represent the binding to the copper ion. T1 copper site is shown as a brown sphere.

The latter weakly coordinating amino acid residues comprise a methionine that can be found in the *Rhus vernicifera* laccase, AOx and CueO. It has been suggested that the methionine residue is a fourth ligand, but since the electron donating ability of the thioether group is considerably lower compared to the sulphur in cysteine and the imidazole nitrogen, the coordinating property may be low (Gray et al., 2000). In addition a non-coordinating phenylalanine or a leucine may be found in this position in several fungal laccases. The lack of the fourth coordinating axial ligand in fungal laccases may be an advantage considered as an important factor determining the higher redox potential values (E_0), displayed by laccases (Fig. 11), in comparison with the other multicopper oxidases (Giardina et al., 2010; Gray et al., 2000). It seems like phenylalanine is determining a higher redox potential of T1Cu center (Eggert et al., 1998). Therefore it is also expected that the redox potential of *G. lucidum's* laccase will also be high, due to the presence of a non-coordinating PHE-463 residue in the vicinity of the T1Cu center (Fig. 10). Additionally, all residues of T1Cu center are aligned sterically to a high degree for the two superimposed, previously mentioned laccases, although small changes can be observed. How those small changes affect the redox potential and the electron abstraction from a reducing substrate will remain the topic of the further study of *G. lucidum*.
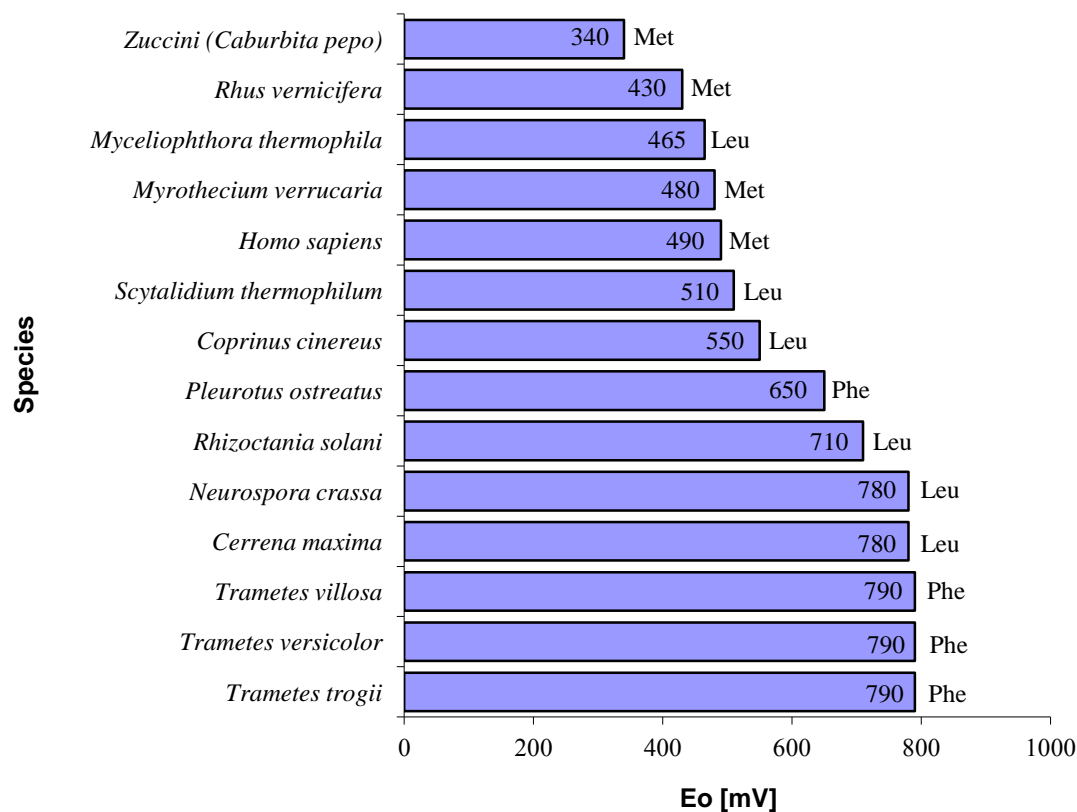
Figure 11. Diagram showing potentials of the T1 copper site of some oxidoreductases. The values in the boxes represent the redox potential and the amino acid residues mentioned, represent residues in the axial position of the T1 copper. *Zuccini (Caburbita pepo), Myrothecium verrucaria, Homo sapiens,* and *Trametes villosa* (Alcalde, 2007; Kumar et al., 2003), *Rhus vernicifera* (Alcalde, 2007; Yaropolov et al., 1994), *Myceliophthora thermophila* (Alcalde, 2007; Alcalde et al., 2002; Kumar et

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al., 2003), *Scytalidium thermophilum* (Alcalde, 2007; Kumar et al., 2003; Xu et al., 1995), *Coprinus cinereus* (Alcalde, 2007; Kumar et al., 2003; Schneider et al., 1999), *Pleurotus ostreatus,* and *Trametes trogii* (Garzillo et al., 2001; Morozova et al., 2007), *Rhizoctania soalni* (Alcalde, 2007; Kumar et al., 2003; Wahlethnet et al., 1996), *Neurospora crassa* (Alcalde, 2007; Piontek et al., 2002), *Cerrena maxima* (Koroleva et al., 2001; Morozova et al., 2007), *Trametes versicolor* (Alcalde, 2007; Alcalde et al., 2002).

The second amino acid that can be found in the vicinity of the T1Cu site is a non-coordinating ILE-455 (Fig.10). Moreover, due to its high redox potential (0.5 to 0.8 V (Xu, 1996b) vs. NHE), T1Cu is the primary electron acceptor site where substrate oxidation takes place and HIS-458, occurring in fungal laccases, is believed to be the entrance door for the electrons abstracted from a reducing substrate during their transfer to T1Cu(++).

T2 copper site (normal copper center)

T2 copper confers no color (so called: paramagnetic 'non-blue' copper), but can still be detected by an electronic paramagnetic resonance (Claus, 2004; Leontievsky et al., 1997). Moreover, it is strategically proximal to the T3 coppers and coordinates with two histidine residues and one oxygen atom as an OH^- ligand, forming a trigonal coplanar configuration (Fig. 5) (Xu et al., 2000).

T3 copper site (coupled binuclear copper center, containing two Cu atoms)

T3 copper center is a pair of copper atoms, T3αCu and T3βCu that give a weak absorbance in the near UV spectrum (330 nm) and it does not have any EPR signal (Claus, 2004; Leontievsky et al., 1997). Each of the T3 coppers coordinates with three histidine residues and a bridging oxygen atom, having a distorted tetrahedral geometry (Fig. 5) (Xu et al., 2000).

T2/T3 copper site (trinuclear copper center)

The T2/T3 copper center is a complex domain comprising both the T2 and T3 copper sites. It contains three copper atoms, and is localized ~13 Å from the T1Cu site (Reinhammar, Malmstrom, 1981). The T2/T3 copper site carry out the reduction of molecular oxygen using the electrons transferred from the T1Cu site.

Fig. 5 illustrates two superimposed trinuclear centers of *Trametes versicolor* (PDB ID. 1KYA) and *Ganoderma lucidum* CBS229.93. The T3 copper ions are symmetrically connected to six imidazole side chain residues from histidines and T2 copper is connected to two imidazoles. The geometry of this structure is a trigonal planar. The conserved residues of *G. lucidum* and *T. versicolor* are well aligned to each other. Moreover, one additional residue; TRP-107, which is colored red, is present only in the *G. lucidum*'s laccase and correlate to the observed α-helix from Fig. 6 that is positioned in the vicinity of the catalytic coppers. However, further evidence will require a future elucidation of the crystallographic structure of *G. lucidum*'s laccase.

The trinuclear center has access to the solvent through a water channel (Fig. 6), leading to the T2 and T3 copper atoms. The water channel leading to the T3 coppers contain residues like Asp and Glu, which are capable of donating protons (Bento et al., 2006), which form a hydrogen network with the water molecules in the channel and therefore allow for the fast access of dioxygen and also an effective release of water after performing a ping-pong reaction (Bukh et al., 2006).

WHAT IS A GOOD LACCASE? FOCUS ON DESIRED FEATURES OF AN INDUSTRIAL OXIDOREDUCTASE

Laccases have been used for a large number of applications spanning from technical solutions such as biobleaching, bioremediation, biosensors, pulp and paper production, opening of biomasses for biofuel production, organic synthesis and to functional food ingredients (Table 2.). Given the large number of different applications and the diversified desire of the specific processes may not be fulfilled by a single good laccase discovered in nature or even optimized by a strong and focused molecular evolution strategy.

The present laccases have a number of drawbacks with respect to redox potential and the high dependency of added mediators to accommodate the oxidation of substrate with high redox potential and polymers containing bulky substrates not fitting the active clefts of substrate binding sites of the natural laccase. These present deficiencies are detailed below using the opening of ligning-cellulose biomass as the example.

Resistance of laccase to inactivation by the free radicals.

Investigations of the laccase-mediated biomass depolymerization process have shown that redox mediators drive these enzymes towards the oxidation of non-phenolic substituents such as aliphatic alcohols. The mediators therefore play an important role in the biocatalysis process, and a desirable enzymatic solution without the mediators has so far not been developed by molecular tools. Although the mediator stimulates the catalysis, the laccase is sensitive to the free radicals of the mediator, which subsequently inactivate the laccase and reduce the rate of oxidation of the non-phenolic lignin structures. According to Li et al. (Li et al., 1999) an effective laccase mediator should be able to effectively oxidize non-phenolic lignin at a high rate. In addition one laccase mediator may interact efficiently with one laccase but in contrast do not perform at optimal rate with other laccases. For example, the mediator, violuric acid inactivates the laccases from *Trametes villosa*, *Pycnoporus cinnabarinus*, *Botrytis cinerea* and *Myceliophthora thermophila* with much higher rate than that of N-hydroxybenzotriazole (HBT). If violuric acid free radical generated by the laccases could be consumed by substrate fast enough, then inactivation of laccases may be avoided and oxidation of the substrate would mainly depend on k_{cat} of violuric acid (Li et al., 1999).

High k_{cat} for an effective laccase mediator system (LMS) with high redox potential.

The k_{cat} is dependent upon the electron transfer from a substrate to the T1 copper, which is dependent on both: the difference in the redox potential between the laccase and a substrate, and the affinity between laccase and the substrate.

Increase of the redox potential value of T1Cu of laccases of more than 0.7 V.

It has been experimentally shown that laccase from a thermophilic phaeoid mould *Myceliophthora thermophila* has a redox potential too low to efficiently oxidize 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propan-1,3-diol in the presence of 1-HBT or violuric acid. Since an effective laccase mediator system (LMS) must have a redox potential high enough to oxidize non-phenolic lignin, then laccase should also have redox potential high enough to make the oxidation of an effective laccase

mediator kinetically possible (Li et al., 1999). Xu et al. suggests a few factors that may influence the redox potential of the T1Cu site in fungal laccases (Xu et al., 1996b). The first is the solvent accessibility previously reported for the iron-sulfur proteins (Langen et al., 1992). The second factor may be an internal hydrogen bonding to the ligands at the T1Cu site, which could change the electron density at the copper center. The third factor may be contributed by the dialectric anisotropy around the T1Cu site. However, Xu et. al. excludes as if the difference in potentials at the oxidized T1Cu site was attributed to the ligand change or a structural distortion (Xu et al., 1996b).

To sum up, a good laccase should ideally comprise; broad substrate specificity, a reaction mechanisms not dependent on a mediator, a high redox potential to accommodate oxidation of the major substrates (non-phenolic substituents such as aliphatic alcohols), tolerance to radicals, organic solvents and shearing forces and it should additionally have a high turnover, high t½, broad pH and temperature regimes.

Laccase optimization by molecular evolution

A growing number of diversity screenings have currently gained a large number of novel laccases, although none with superior performance on biomass delignification. Further optimization by directed evolution techniques is therefore necessary to satisfy the needs from the industry. One of the main targets for improvements is the T1Cu site, which defines the redox potential of the laccases. A higher redox potential at the primary electron acceptor site is desired in order to oxidize a range of recalcitrant polymers in the biomass. Generally different regions of the laccase polypeptide chain have been subjected to specific modifications. They involve:

1. **Replacement of aromatic amino acids residues with non-aromatic side chains have been carried out to make laccases less vulnerable to free-radicals and to prevent their inactivation (Li et al., 1999).**

2. **Improvements of the substrate binding site to accommodate a better docking of the reducing substrate.**

   Based on predicted modeling, specific amino acid residues involved in hydrophobic protein-ligand interactions were selected and substituted by mutation (Galli et al., 2011; Mohamad et al., 2008). By this method, the *Trametes versicolor* laccase became more active towards bulky phenolic substrates. In addition the modeling also revealed that PHE-162 was a critical residue located in the T1Cu site, and that substitution with an apolar alanine residue resulted in increased oxidation of bulky substrate, such as bisphenol A (BPA). Moreover a double mutation in positions PHE162ALA and PHE332ALA showed a cooperative effect, resulting in 98% oxidation of BPA in only 5 h. Optimization of the T1Cu redox potential can, in part be correlated to the axial ligand's hydrophobicity, which is presumed to increase the redox potential of the primary electron acceptor site. This hypothesis is true for laccase from *Bacillus subtilis* (CotA) (Durão et al., 2006), *E.coli* (CuO) (Sakurai, Kataoka, 2007) and *Pseudomonas aeuroginosa's* azurin (Karlsson et al., 1989; Pascher et al., 1993), where mutation of an axial Met → Leu resulted in increase in redox potential by as much as 100 mV, and 60 mV for the later two organisms, respectively.

3. **Alternation of C-terminus tail.**

   A C-terminal protruding tail is 13-14 amino acids long in the amino acid sequences of ascomycetes *M. albomyces* (Hakulinen et al., 2002), *M. thermophila* (Bulter et al., 2003; Zumárraga et al., 2008) and up to 16 amino acids long in basidiomycete *Pleurotus ostreatus* (Autore et al., 2009). In ascomycetes this tail is generally cleaved off by proteolysis at a conserved cleavage site (Asp-Ser-Gly-(Leu/Val/Ile)) to produce an active form of an enzyme. Analysis of the 3D structure of the laccase from *Melanocarpus albomyces* has shown this C-terminal extension as a plug that blocks the solvent channel, thus leading to the hypothesis that its cleavage is required to favor the entrance of oxygen and the subsequent exit of water molecules (Hakulinen et al., 2002). Moreover, creating a C-truncated version of ascomycete *Mycelophthora thermophila* laccase, by introducing a stop codon at the processing site, leads to a 10-fold decrease in catalytic efficiency of the enzyme. It seems that C-terminal tail exerts a strong influence during processing steps, which affect the mature protein activity. According to this theory, the C-terminal tail forms a loop interacting with the active site to prevent binding of copper ions during processing. It is also assumed that a change of two consecutive amino acids (GlyGlu to AspLys) in *M. thermophila* C-terminal tail might contribute to a higher grade of tightness between the C-terminus and the main enzymatic core, which would affect the protein folding and the final mature protein (Zumárraga et al., 2008). In any case, the closure of the tunnel, leading to the trinuclear copper site, certainly affects the function of the trinuclear copper site. Whether this feature is possible among basidiomycete laccases, which have different C-terminal residues, is not known. However, going in that direction, Festa et al. (Festa et al., 2008) have obtained a more stable and substrate specific laccase from basidiomycete by mutagenesis of C-terminal tail of laccase in position PRO494THR, simultaneously allowing a higher accessibility of water molecules to the T1Cu site by increasing mobility of loops that form the reducing substrate binding site, and possibly leading to an increased activity of laccase. Another confirmation of C-terminal end significance on performance and stability of laccase from basidiomycetes was shown in experiments of Autore et al. (2009), who decisively ruled out the belief that four C-terminal amino acids act as a plug that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster. Experiments were performed on a mutant of *Pleurotus ostreatus*, designed according to the results of aforementioned Zumárraga. In case of *P. ostreatus*, the C-terminal tail has a positive effect on the specific activity to K_M ratio towards the phenolic substrates such as 2,6-dimethoxyphenol (DMP), and syringaldazine (SGZ), but not ABTS.

4. **Improved activity and temperature regimes:**

   The first study on directed evolution of laccase was carried out by Butler et al. (Bulter et al., 2003), where functional expression of a thermophilic laccase was performed in *S. cerevisiae.* The laccase gene was subjected to ten rounds of directed evolution and screening. The laccase activity was hereby increased by 170-fold and in addition it also developed a higher temperature tolerance.

5. **Improved tolerance to organic solvents:**

   Another directed evolution study from the group of Alcalde was carried out on the laccase from *Myceliophthora thermophila.* The designed laccase was tolerant to high concentrations of organic solvents such as methanol, ethanol (30%), acetonitrile (20%) and dimethylosulfoxide

(Alcalde et al., 2005; Bulter et al., 2003). Specific mutants GLU182LYS gave better stability in organic media, or increased the redox potential and resistance against denaturation under external factors SER280ASN, or modified the channel leading to the trinuclear center, which might affect the transit of O_2 to the active pocket ASN552HIS, or enhanced substrate binding proximal to the T1Cu and increased the tolerance to co-solvents LEU429VAL.

6. **Improved catalysis and superb stability:**

The PM1 laccase from basidiomycetes has recently been improved by directed evolution by the group of Alcalde (Maté et al., 2010). The final winner laccase contained 15 different mutations; five in the yeast α-factor signal sequence and ten in the mature protein. The laccase activity was improved by 32,000-fold. The winner laccase was also very tolerant to temperature, pH range and organic solvents. Mutations in the signal sequence (yeast α-factor) enhanced functional expression, whereas the mutations in the mature protein improved its catalytic capacities by altering the interactions with the surrounding residues (Maté et al., 2010; Sayut, Sun, 2010).

CONCLUSIONS AND PERSPECTIVES

No naturally occurring laccase combines the desired attributes of being robust and active over a range of demanding parameters such as high temp, pH, and organic solvents. In order to carry out an efficient biocatalyse of non-phenolic substrates the laccase need a strong mediator. To decrease the cost of operations and to create a sustainable process the industry search for laccases with higher stability, enhanced activity, lower production costs, and improved laccase mediators systems (LMS) of natural origin that are environmentally friendly. Other improvements like directed evolution of the laccase enzymes has currently been implemented. Some ideas of Laccase without mediator, better substrate binding, tolerance to organic solvents, etc., one of the examples, as a troublesome region of the laccase can be the C-terminal tail. Depending on the organism, the activity of the enzyme can be enhanced or can drop dramatically when this region is modified. Overall, the laccases have a wide range of applications, and they can be tuned for even more.

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Table 3. Laccase modifications and their potential applications in the industry

Organism	Mutant	Modification	Expression host	Desired characteristics	Industrial application	Ref.
Laccase tuned by directed evolution and rational design						
Myceliophthora thermophila	MtL T2	A(n20)P, S3I, E86G, A108V, N303S, F351L, T366M, Y403H, S450P, N454K, L489L, L536F, Y552N, H(c2)R	*S. cerevisiae* (BJ5465)	Robust at high temp. 170 fold increase in activity 22 fold increase in k_{cat}	decontamination, pulp bleaching, bioremediation (PAH)	(Bulter et al., 2003; Zumárraga et al., 2007)
Myceliophthora thermophila	MtL 6C9	A(n20)P, **P(n20)H**, S3I, E86G, A108V, N303S, F351L, T366M, Y403H, S450P, N454K, **S462S, L476V**, L489L, L536F, Y552N, H(c2)R,	*S. cerevisiae* (BJ5465)	3,5 fold increase in stability in 20% acetonitrile and 30% ethanol mixture	organic chemistry	(Alcalde et al., 2005)
basidimycete PM1	OB-1	V[α10]D, N[α23]K, A[α87], V162A, H208Y, S224G, A239P, D281E, S426N, A461T	*S. cerevisiae* (strain type n.d.)	34.000 fold increase in activity, highly robust to temp., organic solvents, and acidity	pulp biobleaching, food processing, textile treatment, organic synthesis, biofuels, bioelectrochemistry,	(Sayut, Sun, 2010), (Maté et al., 2010)
Improvements in the reduction potential of T1 copper center						
Trametes versicolor	F162A/ F332A	F162A, F332A	*Yarrowia lipolytica* (Po1g)	98% consumption of bisphenol A (BPA) in 5 h	bioremediation, biofuel cells, paper and pulp bleaching	(Galli et al., 2011)
Trametes versicolor	F162A	F162A	*Yarrowia lipolytica* (Po1g)	99%, 63%, 78%, and 45% consumption of 2-*t*-Bu-phenol, 3,5-di- *t*-Bu-phenol, BPA, and trimeric substrate, respectively	bioremediation, biofuel cells, paper and pulp bleaching	(Galli et al., 2011)
Alterations in the C-terminus of laccases						
Pleurotus ostreatus	3M7C	L112F, P494T (C-terminal loop)	*S. cerevisiae* (W303-1A)	higher activity, and increase in stability	immobilization (stain removal)	(Festa et al., 2008)
Pleurotus ostreatus	POXA1bΔ4	truncation of 4 amino acids on the C-terminus 529PLKA533	*S. cerevisiae* (W303-1A)	higher specific activity to K_M ratio towards DMP, and syringaldazine but not ABTS	acidic bio-processes (xenobiotic transformation), organic synthesis, enzymatic polimerization	(Autore et al., 2009)
Melanocarpus albomyces	Tr(L559G) and Tr(delDSGL$_{559}$)	truncation of 4 amino acids on the C-terminus 556DSGL559, or L559G	*Trichoderma reseei* RutC-30	20 fold lower expression levels, unstable protein, reduced or no activity on ABTS and syringaldazine for Tr(L559G) and Tr(delDSGL$_{559}$), respectively	n.d.	(Andberg et al., 2009)
Melanocarpus albomyces	Sc(L559G) and Sc(delDSGL$_{559}$)	truncation of 4 amino acids on the C-terminus 556DSGL559, or L559G	*S. cerevisiae* (strain type n.d.)	inactive Sc(delDSGL$_{559}$) mutant, 11 fold increase in the inhibition constant (K_i) of NaN$_2$ for ABTS as substrate, narrower pH opt., and decreased thermal stability for Sc(delDSGL$_{559}$)	n.d.	(Andberg et al., 2009)
Stachybotrys chartarum	M254F/E346V, E348Q	elongation of C-terminus end by covalently linked carotenoid-binding peptides YGYLPSR, SLLNATK, KASAPAL, IERSATAPPP, CKASAPALC	*Aspergillus niger* (strain type n.d.)	4-6 fold increased specific activity, 2-3 fold increased bleaching ability of paprika and tomato stains	bleaching carotenoid-containing stains (paprika and tomato), detergent and textile applications	(Aehle et al., 2002; Janssen et al., 2004;)

n.d. not determined

Patent application pending