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Master Thesis

New Laccases for Lignosulfonate Polymerization

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Statutory declaration

I hereby declare that I am the sole author of this work. No assistance other than that which is permitted has been used. Ideas and quotes taken directly or indirectly from other sources are identified as such. This written work has not yet been submitted in any part.

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Karin Binder

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Abstract

Lignin is the second most abundant natural polymer and the main by-product of the pulp and paper processing industry [1], [2]. This renewable resource has the potential to be applied in a wide variety of biotechnological tasks, such as dispersants, binders and polymer formulations [2].

Laccases are multicopper containing oxidoreductases and are capable of performing one-electron oxidations of a broad spectrum of aromatic and non-aromatic substrates, including lignosulfonate. Their only by-product is water, making them eco-friendly and versatile biocatalysts [3].

Functionalization of technical lignin with laccases leads to bio-polymers with increased molecular weight. The novel fungal laccases MaL1, ToLA and CgL1 as well as bacterial laccases SiLA, SiLA_CBD and SiLA_SBD were recombinantly expressed and characterized for this particular application.

The biochemical characterization of the recombinant laccases MaL1 and SiLA revealed interesting properties: MaL1 and SiLA exhibited similar pH optima at pH 4 and pH 5, respectively. While MaL1 showed a temperature optimum of 50 °C, the bacterial laccase SiLA exhibited an even higher temperature optimum of more than 70 °C. Both showed high stabilities at room temperature over the time period of one week. The good enzyme activities in broad pH and temperature ranges of both laccases could be of high value for a wider range of applications, such as lignin polymerization.

Preliminary results revealed great potential of MaL1 in lignosulfonate polymerization under various conditions, but especially at neutral to alkaline pH-values. SiLA showed a high stability and a higher enzyme activity compared to MaL1, making this enzyme useful for other industrial applications.

Keywords

Laccase, lignosulfonate, heterologous expression, polymerization

Kurzfassung

Lignin ist das zweithäufigste natürlich vorkommende Biopolymer. Diese nachwachsende Substanz ist das größte Nebenprodukt der Papierindustrie und kann in einer Vielfalt von biotechnologischen Anwendungen eingesetzt werden: Zum Beispiel als Dispergiermittel, Bindemittel oder in Polymerformulierungen.

Laccasen sind kupferhaltige Oxidoreductasen, die Oxidationen bei vielen Aromaten und Nicht-aromaten katalysieren. Bei dieser Reaktion wird molekularer Sauerstoff zu Wasser reduziert. Da das einzige anfallende Nebenprodukt Wasser ist, gelten Laccasen als umweltfreundliche Biokatalysatoren.

Die Funktionalisierung von Lignin durch Laccasen resultiert in Bio-Polymeren mit erhöhtem Molekulargewicht. Die neuartigen pilzlichen Laccasen CgL1, ToLA und MaL1 sowie die bakteriellen Laccasen SiLA, SiLA_CBD und SiLA_SBD wurden speziell für diese Anwendung rekombinant exprimiert und charakterisiert.

Die biochemische Charakterisierung der Laccasen MaL1 und SiLA offenbarte interessante Eigenschaften: Beide Enzyme zeigten ein ähnliches pH Optimum von pH 4 im Fall von MaL1 und pH 5 im Fall von SiLA. Die gemessenen Temperaturoptima wiesen jedoch größere Unterschiede auf: Das Optimum von SiLA lag bei über 70 °C und war damit viel höher als das von MaL1 bei 50 °C. Beide Enzyme zeigten eine hohe Stabilität bei Raumtemperatur im Zeitraum von einer Woche. Außerdem konnten Enzymaktivitäten in breiten Temperatur- und pH Bereichen gemessen werden, was für potentielle Anwendungen von hoher Bedeutung ist.

Vorläufige Resultate haben gezeigt, dass MaL1 im neutralen und basischen pH-Bereich Lignosulfonat polymerisierte. Obwohl mit SiLA nicht dieselben Ergebnisse erreicht werden konnten, weisen die hohe Enzymstabilität und -aktivität großes Potential für weitere technische Anwendungen auf.

Schlagworte

Laccase, Lignosulfonat, heterologe Expression, Polymerisation

Inhaltsverzeichnis

List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
BMGY	Buffered glycerol complex medium
CgL1	*Chaetomium globosum* laccase
2,6 – DMP	2,6 – Dimethoxyphenol
Endo H	Endoglycosidase H
Endo Hf	Fusion protein of Endoglycosidase H and maltose binding protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_M	Michaelis-Menten constant
LB	Lennox Bouillion
MaL1	*Melanocarpus albomyces* laccase
MtL	*Myceliophthora thermophila* laccase
MW	Molecular weight
MWCO	Molecular weight cut-off
OD600	Optical Density measured at 600 nm
PCR	Polymerase chain reaction
RCF	Relative centrifugal force
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SiLA	*Streptomyces rochei* laccase
SiLA CBD	*Streptomyces rochei* laccase with cellulose binding domain
SiLA SBD	*Streptomyces rochei* laccase with substrate binding domain
SLAC	Small laccase
ToLA	*Trametes ochracea* laccase
UV/VIS	Ultraviolet/Visible spectrum

V_{max}	Maximal velocity
YPD	Yeast extract peptone dextrose medium

1. Introduction

1.1 Laccases

Laccases (EC 1.10.3.2) are multicopper-containing enzymes belonging to the group of benzenediol:oxygen oxidoreductases. These enzymes perform one electron oxidations of a wide spectrum of substrates. The oxidation is coupled to the four-electron reduction of molecular oxygen to water. By using molecular oxygen as final electron acceptor, the only by-product of the reaction is water. Therefore, laccases are considered eco-friendly catalysts. An enormous biotechnological interest is associated with laccases due to their eco-friendliness and their versatility as biocatalysts [3].

In nature, laccases are most often found in fungi, but also in plants, prokaryotes and insects. Fungal laccases are involved in lignin degradation (delignification) and detoxification. The role of laccases during delignification is not fully understood. Lignin degradation results in by-products which are toxic for the fungal mycelium. Laccases are able to detoxify these low-molecular weight compounds by converting them to non-toxic polymers. In plants laccases are involved in lignin synthesis (lignification) and wound healing. They are also found in plant defense mechanisms, fungal pathogenesis and sclerotization of the cuticle in insects [4], [5], [6].

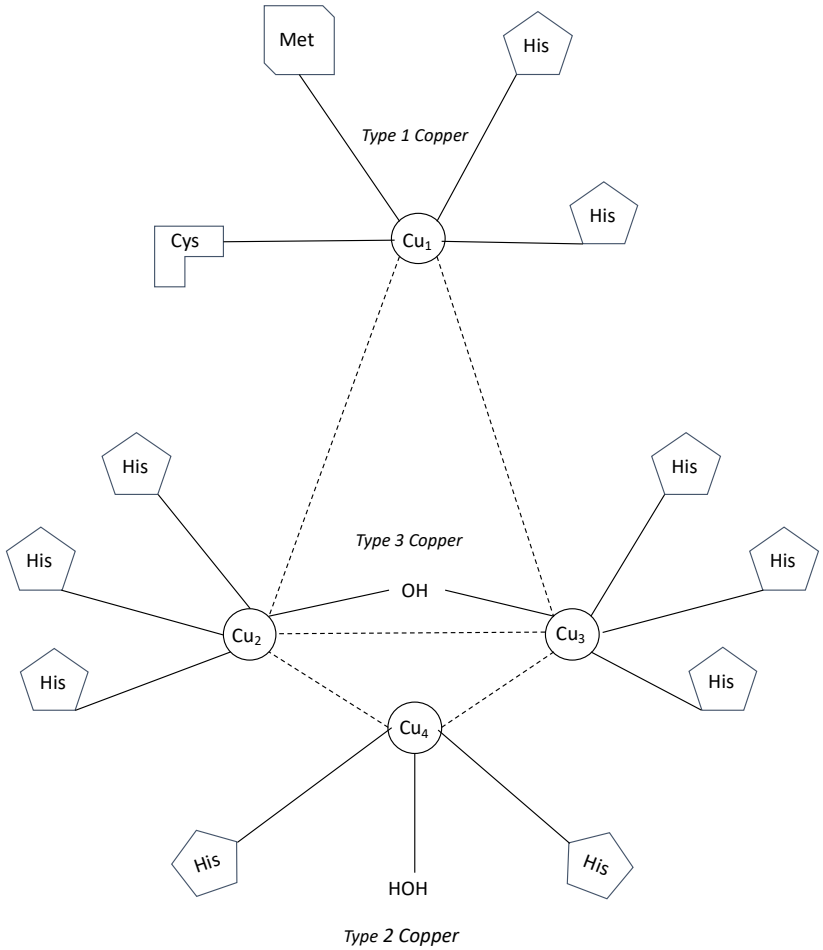

Figure 1: Copper atoms of CotA laccase from *Bacillus subtilis* (adapted from [7])

The active site of laccases contains four copper ions (see figure 1). Three different types of copper, (Type 1, Type 2 and Type 3) are distributed to two sites [8], [9]. Type 1 copper, in its oxidized state, has an absorption maximum at 610 nm. The color shown by this type of copper, also called the paramagnetic "blue" copper, is typical for multicopper proteins. The absorption at 610 nm originates from the intense electronic absorption induced by the covalent copper-cysteine bond. In laccases one Type 1 copper constitutes the first copper site, which is the primary electron acceptor. It is coordinated by one cysteine, one methionine and two histidine residues. Type 2 copper, the paramagnetic "non-blue" copper, does not show any absorption in the visible spectrum. Type 2 copper is coordinated by two histidine residues and can bind one water molecule resulting from the reduction of molecular oxygen. It is located near the type 3 copper pair, a diamagnetic spin-coupled copper-copper pair. Each type 3 copper is coordinated by three histidine molecules. This type of copper ion is characterized by a specific electron absorption at 330 nm, when present in its oxidized form, caused by the hydroxyl bridge between the copper pair. The type 2 copper and the two type 3 copper form a trinuclear cluster where the electrons are transferred to oxygen. At this cluster, the reduction of molecular oxygen (O_2) and the release of water (H_2O) takes place. To sum up, the copper centers of laccases transfer electrons

from the substrate to molecular oxygen without the production of potentially toxic intermediates [8], [9]. Most fungal laccases are organized in a 3-domain structure. Beside these three-domain laccases, two-domain laccases were found in bacteria. They are called small laccases (SLAC). SLAC are lacking domain 2 which is responsible for the formation of trinuclear clusters. SLAC forms homotrimers showing 3-fold symmetry to form a trinuclear cluster and therefore insure an intact catalytic side. These two-domain laccases contain 12 copper ions, which are symmetrically assembled to three active units. The active sites are distributed amongst the individual protein chains. The stability of the trimer is ensured by strong protein-protein interactions [10], [11].

Laccases can utilize a broad spectrum of substrates such as polyphenols, other phenolic substrates (*ortho/para* – diphenols, aminophenols), metals ions and organometallics (ferrocyanide) [3], [5]. This wide range of substrates makes them an ideal biocatalyst for a broad spectrum of biotechnological applications. Laccases are already used in the food industry, pulp and paper industry and forest product industry and, the most important one, bioremediation. Due to their eco-friendliness laccases have gained interest for possible application in the textile industry for bleaching of denims or production of textile dyes. In the pulp and paper industry attempts were made to use laccases in the delignification process during industrial paper production, as lignin in the wood pulp has to be separated from the rest. The idea was to exchange the harmful and polluting chlorine-based reagents to milder and cleaner substances. Laccases are able to oxidize many harmful substances and then releasing less toxic products with a greater bioavailability. These can then get removed physically or by mechanical procedures. Therefore, the use of laccases in enzyme bioremediation is a field of great interest [3].

Fungal Laccases

Fungal laccases are found in a wide variety of species and have been largely characterized. Most of the characterized laccases derive from ascomycetes. Besides their occurrence in phytopathogenic and soil ascomycetes, laccases were found in wood-rotting basidiomycetes. However, not all fungal species produce laccases. Examples for ascomycetes producing laccases include *Melanocarpus albomyces*, fungi from the species *Aspergillus* and *Penicillium,* as well as, some ascomycetes native to freshwater. In case of basidiomycete, laccases are typical for white or brown rot fungi and related lignin degrading fungi groups.

Most fungal laccases are found extracellularly, but depending on their physiological function, the enzyme might be located intracellularly to a small extent. The majority of fungal laccases show a

monomeric structure, however, some exhibit a homodimeric structure. Fungal laccases are glycoproteins and are usually glycosylated to an extent of approximately 10 – 25 % [12].

In comparison to bacterial laccases, fungal laccases have a higher redox potential, which allows them to oxidize a wider array of substrates. The pH-optimum is mostly found in the acidic pH range. Many laccases show a high storage stability for a wide range of pH values [12]–[14].

In this study formerly uncharacterized laccases were chosen for their potential to display certain characteristics. The fungal laccase from *Chaetomium globosum* (CgL1) is closely related to the commercially available laccase *Myceliophthora thermophila* (MtL). MtL is able to utilize a wide range of substrates including lignosulfonate. Therefore, CgL1 was expected to be able to polymerize lignosulfonate under neutral to slightly alkaline conditions [15]. The laccase from *Trametes ochracea* (ToLA) is closely related to a high redox potential laccase. A high redox potential increases the possibility to oxidize a wide array of substrates, which would make this laccase interesting for several industrial applications. The fungal laccase from *Melanocarpus albomyces* (MaL1) showed a high thermal stability and revealed enzyme activities in the neutral to alkaline area [12].

Bacterial Laccases

Laccases were discovered in gram-positive bacteria such as *Bacillus*, *Streptomyces* and *Staphylococcus* and in the gram-negative bacteria *Pseudomonas*, *Enterobacter* and *Proteobacterium* [8].

Laccases from *Streptomyces*, which belong to *Actinomycetes*, are the best identified and characterized group of laccases [10]. Laccases were identified in the *Streptomyces species S. cyaneus, S. coelicolor, S. ipomoea*. Bacterial *Streptomyces* laccases are normally two-domain SLAC laccases and are extracellular enzymes. Bacterial laccases have interesting characteristics such as a broad substrate range, a wide pH range, high stability and a high tolerance to an alkaline environment. Several factors are contributing to the stability of enzymes. These factors include hydrogen bonds and salt bridges, the distribution of charged amino acid residues on the surface, protein packaging and amino acid composition[10], [11]. Additionally, a correlation between proline content and protein thermostability was detected [16], [17]. A high amount of β-strands in the secondary structure of laccases could also lead to the high stability of bacterial laccases [10].

The novel bacterial laccase from *Streptomyces rochei* (SiLA) was chosen because of its similarity to a *S. ipomoea* laccase. The laccase of *S. ipomoea* is able to oxidize phenolic compounds under alkaline

conditions. The enzyme is able to work at high saline concentrations, which makes it attractive for industrial applications [18].

Binding domains

In nature, binding domains are found in enzymes such as cellulases enabling them to utilize bulky substrates. In these enzymes, the binding domain is connected to the catalytic domain via spacers. These binding modules have the ability to improve binding of the enzyme to larger substrates or specific regions of substrates. They increase the amount of active enzyme on the substrate surface and make the targeted bonds more accessible to the catalytic domain by partially disrupting the polymer structure [19].

In this study, the substrate- and cellulose binding modules were fused in-silico to the C-terminal end of SiLA to improve sorption. The fusion proteins were then heterologously expressed in *E. coli*. Spacers were integrated between the binding domain and the catalytic domain to avoid hindrances and to ensure a certain flexibility [20]. The spacer used in SiLA_SBD and SiLA_CBD originally stems from the linker region of 1,4-beta- cellobiohydrolase I from *Trichoderma reesi* [19].

The substrate binding domain (SBD) was derived from the binding module of a polyhydroxyalkanoate depolymerase from *Alcaligenes faecalis.* SBD can bind apolar polyesters such as PHA. The cellulose binding module (CBD) originated from the cellobiohydrolase I from *T. reesei*. CBDs are non-catalytic polysaccharide-recognizing modules. Many CBDs are already identified and revealed a high variation in ligand specificity. They can recognize crystalline and non-crystalline cellulose, chitin, varying glucans, xylan, mannan, galactan, starch and many more [19], [21], [22]. These binding domains can bind to bulky substrates, which could increase the ability of the bacterial laccase SiLA to bind to lignin.

1.2 Heterologous enzyme production

Laccase production, in native hosts, is not able to meet the increasing demand of the market. This is due to the low yields obtained through standard industrial fermentation processes, which are incompatible with the required growth conditions necessary for the particular microorganism. The established hosts for recombinant protein expression are easier to cultivate and the production costs are reduced, because they allow a higher productivity in a shorter time frame. Upscaling of the production process is less complicated in standardized fermentation systems. In addition, it is more convenient to switch the laccase production from pathogenic or toxin-producing species to safer microbial hosts that have the GRAS (generally recognized as safe) status [23].
The heterologous expression of laccases has been established in bacteria, yeast, filamentous fungi plants and insect cells [4], [23].

Laccases, most frequently used in industry, originate from fungi. These laccases can be produced in their original producers, mostly lignolytic fungi. Unfortunately, ligninolytic fungi produce several laccase isoforms simultaneously. These isoforms exhibit similar chemical and physical properties, complicating the purification process. The production of laccases through recombinant production solves this problem. Recombinant laccase production can be further optimized by strong promotor and a signal sequence that leads to secretion of the laccase into the extracellular cultivation medium, simplifying the downstream processing. To increase the yield, further optimal cultivation conditions such as pH and temperature are important [4].

Yeast expression systems

Yeast expression systems provide many advantages in heterologous laccase production compared to non-yeast systems, because yeasts are lower eukaryotes. They offer quick microbial growth, easy gene manipulation and are able to perform eukaryotic-specific post-translational modification. These post-translational modifications include proteolytic processing, formation of disulfide bridges and glycosylation of the recombinant protein. Yeast cultivation is economical, because they achieve high yields, the cultivation medium is inexpensive, and the handling is easy. It is possible to control the level of protein expression in yeast by a variety of strong inducible or constitutive promotors. Other influencing factors are the cultivation conditions, such as media composition, pH and temperature during the expression. Signal sequences are responsible for secretion of the recombinant laccases into the growth medium simplifying the purification and downstream processing. The yeasts of choice for recombinant protein production are *Pichia pastoris* and *Saccharomyces cerevisiae*. The latter is one of

the best characterized experimental systems in biology. Contrary to *P. pastoris*, *S. cerevisiae* has the GRAS (generally recognized as safe) status and therefore its recombinant products can be used in food industry. The GRAS status allows a wider array of genetic manipulations such as directed evolution. In terms of yield and efficient secretory system, *P. pastoris* is preferable to *S. cerevisiae*. Another favorable aspect of *P. pastoris* is the possibility to use one of the strongest and most regulated promotors, the alcohol oxidase (*AOX1*) promotor. This promotor is frequently used [4], [23], [24]. Enzyme expression is induced by the addition of methanol to a cultivation medium lacking methanol as carbon source. The methanol can induce protein expression at a high level [24]. The *AOX1* promotor is regulated so tightly that the growth phase can be uncoupled from the production phase. This enables the accumulation of biomass without accumulating recombinant protein [25]. The methylotrophic yeast *P. pastoris* has a preference for respiratory growth and can therefore be cultured at high cell densities. The high cell density is important for recombinant protein production, because a high concentration of cells is proportional to a higher enzyme yield [24].

Bacterial expression systems

Bacteria are often used in laccase production, because they can be easily modified genetically and are cheap to cultivate. *Escherichia coli* is the most commonly used heterologous expression host for laccases of bacterial and fungal origin. Various approaches have been found to increase expression levels of active recombinant enzyme during expression in *E. coli*. They include using high copy number plasmids with strong promotors (e.g. pET plasmids carrying T7 promotors), elimination of rare codons, the adaptation of the sequence of synthetic genes to the codon bias of the host [26]–[29].

A common problem of heterologous laccase expression in *E. coli* is the formation of aggregates of unfolded protein (inclusion bodies). Factors increasing the recombinant protein expression such as a high copy number of the target gene and a strong promotor system also increase the inclusion body formation as they result in a high metabolic burden for *E. coli* [30].This results in lower yields and complicates the purification. In some cases, it is possible to efficiently refold the recombinant laccases in vitro [4]. A promising approach to considerably increase the volumetric activity of laccases produced in bacteria, is to change conditions during the expression to oxygen-limiting growth conditions. This is achieved by switching from shaking to non-shaking (micro-aerobic). Oxygen-limitation increases the amount of copper incorporated during expression. Microaerobic conditions during recombinant expression lead to the synthesis of a holoenzyme, whereas the expression under aerobic conditions leads to copper-depleted proteins. The copper efflux mechanism, a prevention mechanism of *E. coli* against the accumulation of toxic copper inside the cells, is dependent on the available oxygen. This efflux mechanism is not induced to the same extent when the cells are grown under oxygen-limiting

conditions [31]. By changing the expression to micro-aerobic conditions, a higher accumulation of intracellular copper is promoted as cells, growing under microaerobic conditions, incorporate up to 80-fold more copper compared to cells grown under aerobic conditions [4], [27], [28]. Despite these difficulties, *E. coli* is one of the most favourable expression systems for recombinant enzymes, because expression in eukaryotic hosts (*Pichia pastoris*, *Saccharomyces cerevisiae*) have lower yields and take more time. But *E. coli* cannot perform posttranslational formation of disulfide bridges and glycosylation of the recombinant protein [23].

Laccase production in filamentous fungi, insects and plants

Natural laccase producers are filamentous fungi, plants, bacteria and insects. It is not only possible to recombinantly express laccases in yeast and bacteria but also to use the natural producers for recombinant laccase production [34]. Filamentous fungi possess the ability to secrete large amounts of protein into the growth medium, making them attractive for recombinant protein production. Various filamentous fungi have been used and tested for heterologous protein production. The asexually reproducing filamentous fungi, which have been mostly utilized for laccase production, are mainly *Aspergillus* species, such as *A. oryzae, A. niger,* and *Trichoderma reesei*. [4], [23]. The yields of heterologous laccases produced in filamentous fungi are quite high, but the appropriate tools for genetic engineering are lacking.

Plants are used for the heterologous expression of laccases, as well. They are suitable especially for the production of laccases originally from plant or fungi. The plant species used as expression host are mainly *Arabidopsis thaliana, Nicotiana tabacum, Oryza sativa, Lycopersicon esculentum, Zea mays* and *Saccharum officinarum*. For protein expression in plants, the expression vector is introduced into *Agrobacterium tumefaciens* by electroporation which is used for the transformation of the plants. As plants are eukaryotes, they are able to perform eukaryotic post-translational modifications. Plants are promising expression hosts as many crops are having promotors, targeting sequences and other components that allow high protein production. The main disadvantages of laccase production in plants are the high fermentation costs and the high cultivation time. An additional disadvantage is the low yield of recombinant protein compared to other established hosts such as bacteria and yeasts [35].

Finally, laccases can be produced by insect cells. In insects, laccases play an important role in cuticle sclerotization. The recombinant production of laccases via a baculovirus expression system was already accomplished in insect Sf9 cells [4], [36] but the large scale production is difficult. The recombinant protein production in insects is not widely spread.

1.3 Lignin

Lignocellulosic biomass is mainly composed of the carbohydrate polymers cellulose and hemicellulose and the aromatic polymer lignin. As a component in lignocellulosic biomass, lignin is responsible for strengthening of the cell wall [1]. Additionally, lignin is protecting cellulose and hemicellulose fibres against microbial degradation by forming a complex matrix [1], [37]. Lignin comprises one-third of all non-fossil-based carbon-sources worldwide. It is a greatly irregular polymer found in wood and annual plants [2].

Laccases play an important role in the polymerization and degradation of lignin in fungi and plants [38]. Lignin is synthesized by one-electron oxidations of the three main precursors *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) resulting in phenoxy radicals [38]–[40]. These radicals polymerize with each other by cross-coupling reactions with other radicals formed at the end of the growing lignin [40].

1.3.1 Technical lignin

Technical lignin is the main by-product in the pulp and paper processing industry. It is considered as waste product and most of it is burned for energy production [1], [2], [41]. The estimated amount of extracted technical lignin is approximately 50 million tons (2010) [2]. During the processing of wood to wood pulp, lignin has to be separated from cellulose [39]. Depending on the extraction method, different types of technical lignin such as lignosulfonate or kraft lignin are obtained. Both lignins are residues obtained by chemical pulping processes. Lignosulfonate is sulfonated lignin (see Figure 2). It is removed from wood using a method called sulphite pulping which is based on a cooking process with sulfur dioxide (SO_2) under acidic or neutral conditions [2], [41], [42]. Kraft lignin is obtained during the kraft pulping process using sodium hydroxide (NaOH) and sodium sulphide (Na_2S) at high pH [41]. Lignosulfonate is hydrophilic and water soluble through the introduction of sulfonic acid groups into the lignin structure [42]. Kraft lignin is, in contrast to lignosulfonate, only soluble in alkali solution at high pH [41]. Due to the heterogeneity of technical lignin, only 2% of the waste are applied for value-added production [2].

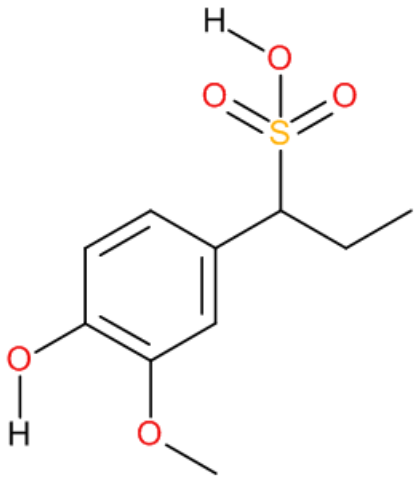

Figure 2: Monomer of lignosulfonate containing a sulfonic acid group

Technical lignin shows a low molecular weight because it is a degradation product obtained during wood processing [2]. In various applications, a larger molecular weight of technical lignin is necessary [43]. Because of the phenolic structure of lignin, laccases are able to enzymatically modify its structure. Laccases generate radicals which can cross-link with each other, forming a lignin material of higher molecular weight [38], [43]. These modifications of technical lignin by laccases is strongly dependent on the type of lignin, reaction conditions (pH, lignin concentration, oxygen availability and temperature) and the redox potential of the laccase [44]. Different laccases have varying abilities to polymerize lignosulfonate and other technical lignin. Therefore, it is important to find suitable laccases for potential applications. This method of valorising a main by-product of the wood processing industry is a very sustainable approach which can lead to valuable products such as dispersants, binders, surfactants, resins, polymer formulations and many more [38].

2. Aim

Lignosulfonate is sulfonated lignin, a major by-product of the pulp- and paper industry. It is produced through delignification of wood by a method called sulphite pulping [1], [2], [41]. Only a small percentage of the extracted lignosulfonate is used for conversion into value-added materials [2]. As the only by-product of the laccase-catalyzed reaction is water, laccases are considered “green biocatalysts”. They are able to oxidize the phenolic structure of lignosulfonates and enzymatically polymerize lignin under eco-friendly conditions. Functionalization of technical lignin with laccases leads to bio-polymers with increased molecular weight. These polymers could be applicable to bio-based paper-coating formulations and resins [43].

The aim of this work was to characterize new laccases and to assess their potential to polymerize lignosulfonate under neutral to alkaline conditions. In addition, properties potentially favorable for other industrial applications were evaluated. For this purpose, newly designed fungal and bacterial laccases were recombinantly expressed in *Pichia pastoris* and *Escherichia coli.* The expressed enzymes were purified and biochemically characterized regarding their pH optimum, temperature optimum and stability. As a final step, these enzymes were characterized with lignosulfonate over a wide pH range.

3. Materials and Methods

Materials

All bulk chemicals were of analytical grade and either purchased at Sigma-Aldrich or Carl Roth if not stated otherwise. The plasmids were designed by Dr. Doris Ribitsch and produced by GenScript. ToLA_Strep, CgL1_Strep, MaL1_Strep were optimized for *Pichia Pastoris* and produced in PICZαB plasmids, containing a Zeocin selection marker. The other genes (SiLA_woSP, SiLA_SBD, SiLA_CBD) were produced in pET-26b(+) plasmids, containing a kanamycin selection marker and were codon-optimized for *Escherichia coli*. All designed enzymes had a Strep-tag attached to the C-terminus.

Media preparation

Table 1 shows the recipes of the different media and their components.

Table 1: preparation of the different used media and media components

LB-Medium (Lennox Bouillion)	
20 g/L	LB Broth
Nutrient Agar plates	
20 g/L	GranuCult Nutrient agar (Merck)
YPD (yeast extract peptone dextrose medium)	
1%	Yeast extract (Bacto Yeast extract, BD)
2%	Peptone (Tryptone/Peptone aus Casein)
2%	Dextrose (glucose)
Yeast extract and peptone were dissolved in water and autoclaved at 121 °C for 20 minutes. The appropriate amount of sterile 20% dextrose solution was added after the autoclaved media had cooled down to 40 °C.	
YPD plates	
1%	Yeast extract
2%	Peptone
2%	Dextrose (glucose)
2%	Agar (Bacto Agar, BD)

Yeast extract, peptone and agar were dissolved in water and autoclaved at 121 °C for 20 minutes. The appropriate amount of sterile 20% dextrose solution was added after the autoclaved media had cooled down to 40 °C and the agar plates were poured under sterile conditions.	
BMGY (buffered glycerol complex medium with 0.1 M phosphate buffer)	
10 g	Yeast extract
20 g	Peptone
698 mL	ddH_2O
Autoclaved for 20 minutes at 121 °C	
Cooled to room temperature. The following solutions were added	
100 mL	100 mM Potassium phosphate buffer pH 6
100 mL	10x YNB
2 mL	500x B
100 mL	10x GY
10x YNB (13,4% yeast nitrogen base with ammonium sulfate without amino acids)	
134 g	Yeast nitrogen base (YNB) with ammonium sulfate and without amino acids (BD)
or	
34 g	YNB without ammonium sulfate and amino acids
100 g	Ammonium sulfate
1000 mL	ddH_2O
autoclaved for 20 minutes at 121 °C solution stored at 4 °C	
500x B (0,02% biotin)	
20 mg	Biotin
100 mL	ddH_2O
was filter sterilized	
10x D (20% dextrose)	
200 g	D-Glucose
1000 mL	ddH_2O
autoclaved for 20 minutes at 121 °C	

10x GY (10% glycerol)	
100 mL	100% Glycerol
900 mL	ddH_2O
autoclaved for 20 minutes at 121 °C	
100 mM potassium phosphate buffer pH 6	
Stock solution A: 1 M $K_2HPO_4*3H_2O$ Stock solution B: 1 M KH_2PO_4	
13.2 mL	Stock solution A
86.8 mL	Stock solution B
850 mL	ddH_2O
pH 6 was confirmed and filled up to 1000 mL autoclaved for 20 minutes at 121 °C	

3.1 Heat shock transformation of *Escherichia coli*

Heat shock transformation was performed to incorporate plasmids carrying the gene of interest into *E. coli*. This is essential for either recombinant laccase production in *E. coli* BL21-Gold(DE3) or plasmid proliferation in *E. coli* XL10.

All steps were carried out under sterile conditions using a biosafety cabinet (ESCO Class II BSC, Airstream). LB medium was preheated to 42 °C. The competent cells (XL10 or BL21-Gold(DE3)) were put on ice for 5 minutes. Then 10 μL of plasmid was added and incubated on ice for 20 minutes and mixed every 5 minutes by inverting the tubes. The tubes were heated to 42 °C for 45 seconds and put on ice for 2 minutes. Afterwards 900 μL of preheated LB medium was added to each tube and incubated for one hour at 37 °C and 300 rpm (Thermomixer comfort, Eppendorf). Then 100 μL was put directly on a plate and the rest centrifuged (Centrifuge 5417R, Eppendorf) for 3 minutes at 20,800 rcf (relative centrifugal force). After centrifugation, 800 μL of supernatant was discarded, and the pellet resolved in the remaining 100 μL and plated. The plates were incubated overnight at 37 °C (Heratherm Incubator, Thermo scientific™).

3.2 Plasmid purification

E. coli cells containing the proliferated plasmids were lysed and the plasmids separated and purified using the PureYield Plasmid Midiprep System (Promega Corporation). An overnight culture with

100 mL LB medium containing one transformed XL10 *E. coli* colony and 40 µg/mL kanamycin (bacterial laccase) or 100 µg/mL zeocin (fungal laccase) was prepared. The culture was incubated for 16 to 21 hours at 37 °C (Innova 44, New Brunswick) and 150 rpm until an optical densitiy of 2 to 4 at 600 nm (OD600) was reached (DR 3900 photometer, Hach). The cells were harvested by centrifugation (Centrifuge 5920 R, Eppendorf AG) for 20 minutes at 3,428 rcf and the supernatant was discarded.

The cell pellet was resuspended in 3 mL Cell Resuspension Solution. Afterwards 3 mL of Cell Lysis Solution was added, and the mixture inverted 3 to 5 times and incubated for 3 minutes at room temperature. Following that, 5 mL Neutralization Solution was added and the tube inverted 5 to 10 times for mixing purposes. The lysate was centrifuged for 30 minutes at 3,428 rcf.

In the meanwhile, a column stack was assembled by placing a blue PureYield Clearing Column on top of a white PureYield Binding Column. After centrifugation the supernatant was transferred into the clearing column and a vacuum applied until all liquid had gone through both columns. In the next step the blue clearing column was removed. The white binding column was washed by adding 5 mL Endotoxin Removal and 20 mL Column Wash Solution. Vacuum was applied for 30 seconds until the membrane was dry and the ethanol odor was gone.

The plasmids were eluted using the Eluator Vacuum Elution device. The binding column was placed onto the elution device and on the vacuum manifold. Then 500 µL of nuclease free water was added dropwise onto the surface of the binding column until the whole surface was wetted. After an incubation of 1 minute the vacuum was applied for another minute.

Finally, the plasmid concentration was measured using the nanodrop photometer (NanoPhotometer, Implen GmbH) where nuclease free water was used as blank.

3.3 Plasmid linearization

For transformation of the *Pichia pastoris* strain (KM71H) the purified plasmids needed linearization.

Table 2: summary of substances needed for plasmid linearization

1 µg	Plasmid
2 µL	SacI-HF®
2 µL	10x HF cutting buffer
X µL	Nuclease free H_2O
Σ= 20 µL	

Nuclease free water and 1 µg of plasmid were mixed with 2 µL of the SacI-HF® restriction enzyme (New England Biolabs Inc.) and 2 µL of 10x HF cutting buffer (New England Biolabs Inc.) and incubated for two hours at 37 °C and 300 rpm (see Table 2). After that, the mixture was heated to 65 °C for 20 minutes at 300 rpm to stop the reaction and inactivate the restriction enzyme.

Linearization of the plasmid was confirmed by agarose gel electrophoresis. Therefore, 2 µL of the linearized plasmid and 2 µL of the circular plasmid were each mixed with 2 µL of 6x loading dye (Fermentas), respectively and loaded on an 1 % agarose gel, as well as a 1 kBp Marker (Generuler 1 kb plus, Thermo Scientific™) and run with 80 V. For details see chapter 3.11.2 Agarose gel electrophoresis.

3.4 Electroporation of *Pichia pastoris*

The transformation of *Pichia pastoris* was carried out by electroporation. By applying an electrical field to the cells, the permeability of the cell membrane increased and the linearized plasmid was able to enter the cell and integrate into the host chromosome [45].

Table 3: preparation of 1 M sorbitol

1 M Sorbitol	
182 g	Sorbitol
1000 mL	ddH_2O
autoclave for 20 minutes at 121 °C Store at 4 °C	

For the transformation of *P. pastoris* electroporation was performed. Therefore, 500 µL 1 M Sorbitol (see Table 3) and the electroporation cuvettes (peqlab) were chilled on ice. Aliquots of electrocompetent KM71H *P. pastoris* cells were thawed on ice.

Under sterile conditions, 10 µL of the linearized plasmid was added to the thawed cells and mixed gently by flicking the tube, because electrocompetent cells are very fragile.

The cell/DNA mixture was added to the electroporation cuvette and tapped gently on the counter to move the cells to the bottom. Excess moisture was removed from the outside of the cuvette using a kimwipe (Kimtech™). Cells were shocked with an electroporator (Bio-Rad Laboratories, Inc.) set to "Fungi Pic".

Immediately after the electroporation the cuvette was removed from the chamber and 500 µL ice-cold 1 M Sorbitol was added to the cuvette and the Sorbitol/cell/DNA mixture was poured back to the sterile 50 ml tube. The tubes were incubated in an angular position on a tray for 30 minutes at 28 °C

and 150 rpm (Multitron Pro, Infors HT). Afterwards 500 µL of prewarmed (28 °C) YPD was added and incubated for another two hours.

Afterwards, 100 µL of cell suspension, of a concentrated aliquot and of the negative control (w/o DNA) were plated onto YPD-Zeocin100 plates and incubated for 3 to 5 days at 28°C until colonies formed.

3.5 Colony PCR

Colony PCR was performed of freshly transformed *P.pastoris* KM71H cells to screen for clones containing the insert DNA, with primers specific to AOX (Table 4). Alcohol oxidase (AOX) is the key enzyme which enables *P. pastoris* to ultilize methanol as carbon source and the *AOX1* promotor is part of the inserted plasmid [46]. This promotor is often used in *P. pastoris* expression systems as it is one of the strongest and most regulated promotors. It is induced by methanol and induces protein expression [4], [24] .

Table 4: PCR primers

3AOX_Agowa.Fw (IDT)	5'-GCAAATGGCATTCTGACATCC-3'
5AOX_Agowa.Rev (IDT)	5'-GACTGGTTCCAATTGACAAGC-3'

Colonies were picked using a sterile toothpick and resuspended in 25 µL nuclease free water (nfH_2O; Promega) under sterile conditions. Afterwards the cell suspension was heated to 95 °C for 10 minutes, cooled down on ice and centrifuged for 1 minute at 20,800 rcf. First, 44.5 µL of the mastermix (described in Table 5) were mixed with 5 µL of the supernatant and 0.5 µL Phusion polymerase were added to the mixture. A negative control without supernatant was prepared simultaneously to the other samples to check the performance of the PCR.

Table 5: Preparation of 1x 45 µL Mastermix

24.5 µL	Nuclease free water
10 µL	5x Buffer HF (New England Biolabs)
5 µL	2 mM dNTP's (Fermentas)
2.5 µL	AOX 3' Agowa Primer 1:10
2.5 µL	AOX 5' Agowa Primer 1:10
0.5 µL	Phusion Polymerase (Finnzymes)

The gene of interest was amplified in a PCR cycler (SuperCycler 8800, Agilent Technologies Inc.) with an appropriate program (Table 6).

Table 6: PCR Procedere

Fast heating to 98 °C
Start Cycle (30 times)
Denaturation 98 °C – 10 s
Annealing 58 °C – 20 s
Elongation 72 °C – 45 s
End Cycle
Elongation 72 °C – 7 min
Temperature 8 °C

PCR products were visualized by agarose gel electrophoresis. 12 μL of the PCR samples were mixed with 2 μL 6x loading dye. Of this mixture 10 μL were loaded onto a 1% agarose-gel as well as a 1 kb marker and run with 80 V. For details, see chapter 3.11.2 on Agarose gel electrophoresis.

3.6 Laccase expression

3.6.1 Laccase expression in *Pichia pastoris*

The KM71H clone, containing the insert, was then grown under defined cultivation conditions and the recombinant laccase production induced by methanol.

Freshly plated clones were necessary for the expression experiment. The preculture was prepared by inoculating the clones in 1 L baffled flasks, which were covered with a layer of sterile cotton fixed with a rubber band and aluminum foil. Before the inoculation, 100 mL BMGY broth was mixed with 100 μL Zeocin stock solution (100 μg/mL) (Alfa Aesar). The culture was incubated (Infors HT – Multritron) at 28 °C and 150 rpm for about 16-18 hours (overnight) until an OD600 between 2 and 6 was reached.

The cells were harvested by centrifugation for 15 minutes at 3428 rcf. The cell pellet was resuspended in 1/10 of the original volume (=10 mL) and transferred in a 100 mL baffled flask. For the induction of protein expression, 0.1 mM $CuSO_4$ (10 μL, 0.1 M) and 0.5 % methanol (50 μL, 100 %) were added to the mixture. Additionally, 50 μL of 100 % methanol was added twice a day for further induction. Once a day before the addition of methanol, a 300 μL sample was taken. After centrifugation, the supernatant was stored at -20 °C. After 72 hours the expression culture was centrifuged for 20 minutes at 3,428 rcf. The supernatant was then stored at -20 °C.

3.6.2 Laccase expression in *Escherichia coli*

The *E. coli* host strain BL21-Gold(DE3) was cultivated under defined conditions and the protein expression induced with IPTG.

Table 7: Preparation of antibiotic- and IPTG stock solutions

Kanamycin stocks (50 mg/mL)	
0.5 g	Kanamycin
10 mL	ddH_2O
the solution was filtered through a 0.22 μm PES filter under sterile conditions Stored in aliquots at -20 °C	
Isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 M	
2.38 g	IPTG
10 mL	ddH_2O
the solution was filtered through a 0.22 μm PES filter under sterile conditions Stored in aliquots at -20 °C	

The freshly transformed *E. coli* host strain BL21-Gold(DE3), containing the plasmid of interest, was pre-cultured overnight in 30 mL LB containing 24 μL kanamycin (40 μg/mL stock solution, Table 7) at 37 °C and 150 rpm (innova44, New Brunswick).

The corresponding amount of pre-culture was taken to obtain a starting OD600 of 0.1 in a total volume of 200 mL LB media, which had 160 μL kanamycin (40 μg/mL stock solution) added, in sterile 500 mL shake flasks. The cells were grown in liquid culture at 30 °C and 120 rpm until an OD600 of 0.6 was achieved. After reaching an OD600 of 0.6, 0.25 mM CuSO4 (500 μL, 0.1 M) and 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (10 μL, 1 M) were added to the culture and the temperature was reduced to 25 °C. Further incubation was either done under aerobic or micro-aerobic conditions.

Aerobic expression:

For aerobic expression the incubation was continued at 25 °C and 120 rpm for 24 hours.

Micro-aerobic expression:

For micro-aerobic expression the incubation at 25 °C and 120 rpm was continued for 4 hours and then changed to micro-aerobic conditions by switching off the shaking function and further incubation for 20 hours.

After 24 hours, the cells of the aerobic and micro-aerobic expression were harvested by centrifugation for 30 minutes at 4 °C and 3,428 rcf and the cell pellets were stored at -20 °C.

3.7 Deglycosylation

Proteins in *P. pastoris* supernatant are normally glycosylated when secreted. If the glycosylation pattern is not homogenous this may lead to problems when trying to identify them using SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) as it leads to smearing in the gel instead of clear protein bands. Deglycosylation was important for the examination and interpretation of the protein expression. As the protein expression was rather low proteins were subsequently precipitated by acetone precipitation.

First, 61.2 µL of fermentation supernatant was mixed with 6.8 µL 10x Denaturation buffer (New England Biolabs), heated to 100 °C for 10 minutes and cooled down on ice. After that, 6.8 µL 10x G5 Reaction buffer (New England Biolabs) and 1 µL Endoglycosidase H (Endo H) (New England Biolabs) were added to the mixture and incubated for 2.5 hours at 37 °C.

For the subsequent acetone precipitation, 227 µL of pure acetone was added to each tube and incubated for a minimum of 3 hours or overnight at 4 °C (on ice). The tubes were centrifuged for 10 minutes at 20,800 rcf and 4 °C. The supernatant was removed and the precipitate was left to dry at room temperature.

The precipitate was dissolved in 20 µL 2x Lämmli buffer and analyzed by SDS-PAGE. For details see chapter 3.11.1 on SDS-PAGE.

3.8 Cell lysis

E. coli cannot secrete enzymes to the supernatant. For purification- and timepoint analyzation-purposes it was necessary to disrupt the cell membrane to release the enzyme.

3.8.1 Cell lysis buffer

Cell lysis by a specific cell lysis buffer was used to lyse the cell pellets of the *E. coli* expression timepoints.

The cell lysis buffer (CellLytic B Cell Lysis Reagent – 10 x concentrate, Sigma) was diluted 1:10 in 0.1 M Sodium-phosphate buffer pH 7. The cell pellet was dissolved in 200 μL of the diluted cell lysis buffer and incubated for 7 minutes at 26 °C and 600 rpm. The tubes were centrifuged for 5 minutes at 20,800 rcf and 4 °C.

The soluble protein was located in the supernatant, whereas the inclusion bodies can be found in the remaining cell pellet. The cell pellet was dissolved in 100 μL 2x Lämmli buffer and heated to 100 °C for 10 minutes and 5 μL of this mixture was loaded onto the gel. 10 μL from the soluble protein was mixed with the same amount of 2x Lämmli buffer and proceeded according to chapter 3.11.1 SDS-PAGE.

3.8.2 Sonification

This method was used for cell lysis of the *E. coli* cell pellets from the expression subsequent to ÄKTA purification as the required amount of Cell lysis buffer would be quite high.

The cell pellet was resuspended in the respective amount of binding buffer (Buffer W) (5 mL /g cell pellet) in a falcon. The cell lysis procedure was carried out on ice. The sonifier (Digital sonifier, BRANSON) was set to send pulses of 10 seconds with 60 % amplitude. The sonification was repeated 5 times with 2-minute breaks in between where the ultrasonic probe was removed from the resuspended cells as it would heat up the cell solution and harm the enzyme. The solution was centrifuged for 15 minutes at 4 °C and 20,800 rcf. The supernatant was filtered through a 0.22 μm PES filter prior to ÄKTA purification, to remove any eventually remaining cells.

3.9 Buffer exchange using PD-10 desalting column

PD-10 desalting columns (GE Healthcare, Life sciences) utilize a stationary phase containing Sephadex G-25 to separate molecules according to their difference in size in a process called gel filtration. These columns are designed for rapid clean-up of proteins and other larger biomolecules. They have other applications such as desalting, buffer exchange and removal of low-molecular weight compounds [48].

The column was assembled with an adapter and a collection tube. The cap was removed first and then the sealed end of the column was cut at the notch. The column with an adapter was filled with 25 mL equilibration buffer and the flow-through discarded. 2.5 mL of sample was added to the column. For smaller volumes, equilibration buffer was added to adjust the volume to 2.5 mL after the sample had entered the packed bed completely. For elution 3.5 mL buffer was added and the eluate was collected.

3.10 Purification of laccases

The Strep-Tactin XT purification system (Figure 3) is based on affinity chromatography using optimized streptavidin (Strep-Tactin) as stationary phase (Strep-Tactin XT, 1 mL cartridge, IBA GmbH) to bind and purify recombinant proteins. Proteins are fused to engineered short sequences (Strep-tag, sequence: WSHPQFEK) which serves as purification tags and are capable of binding strongly and with high selectivity to the biotin binding pocket of the engineered streptavidin. This enables purification under mild conditions. For elution, biotin is added to the buffer. The column is regenerated by applying NaOH [49].

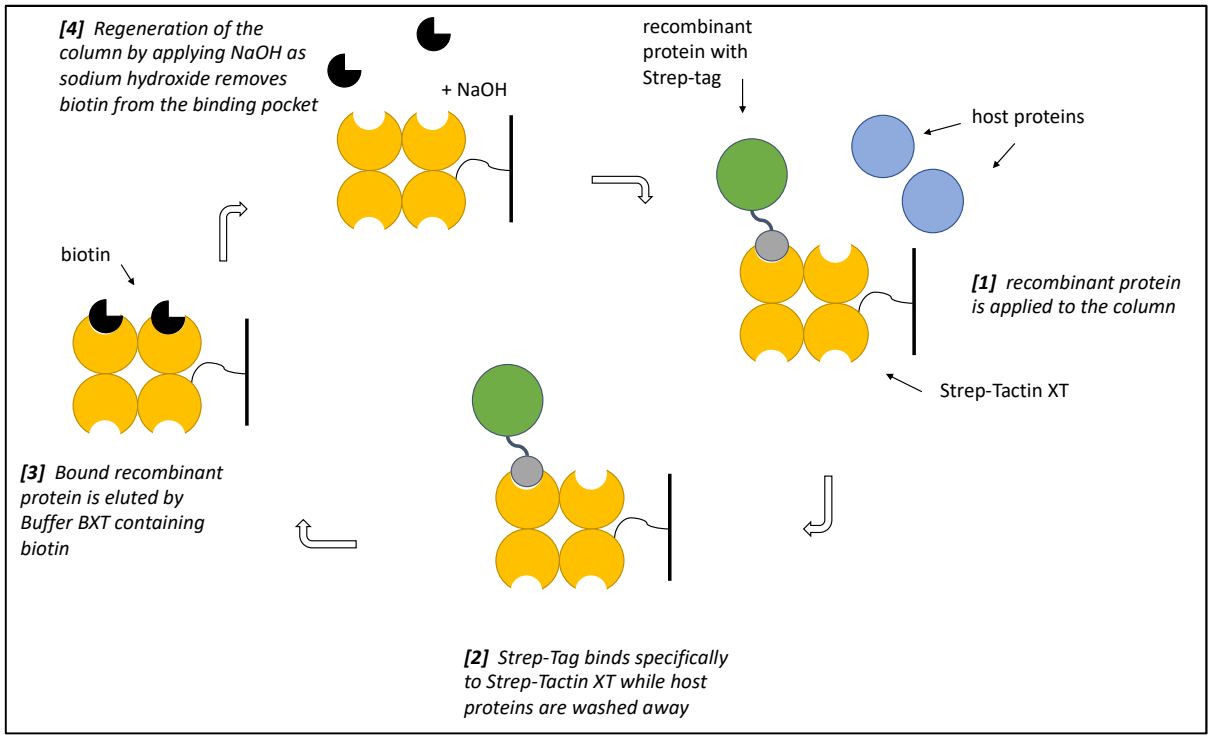

Figure 3: Principle of Strep-Tactin XT purification cycle

Preparation of the used buffers:

Buffer W:	100 mM Tris/HCl pH 8.0
	150 mM NaCl
Buffer BXT (elution buffer):	100 mM Tris/HCl pH 8.0
	150 mM NaCl
	50 mM biotin

3.10.1 Gravity column purification

Gravity column purification was performed for the purification of enzyme from *P. pastoris* supernatant.

The top cap was removed first and then the lower cap at the outlet of the column (Gravity flow, Strep-Tactin XT Superflow, High capacity column; IBA GmbH). The storage buffer was removed, and the column equilibrated with 2 mL Buffer W.

The supernatant was loaded onto the column by applying 12 x 0.5 mL and the flow-through was collected. The column was washed five times with 1 mL Buffer W and the wash fractions were collected individually. To elute the bound protein 6 x 0.5 mL Buffer BXT was added and the eluate was collected in 0.5 mL fractions.

To regenerate the column 2 x 4 mL of freshly prepared 10 mM NaOH was added. The NaOH was immediately removed by adding 2 x 4 mL Buffer W. The column was overlaid with 2 mL Buffer W and stored at 4 °C.

3.10.2 ÄKTA purification

To purify the enzyme from *E. coli,* ÄKTA purification was performed as it showed to be effective and lead to high yields.

The sample preparation for sample loading was performed according to the chapter 3.8.2 Sonification. The ÄKTA system (ÄKTA pure, GE Healthcare) was washed with ethanol and water prior to sample loading (Table 8).

Table 8: Different steps of ÄKTA purification

System wash with ethanol	External pump: 20 mL; 3 mL/min Rest of system: Inlet A, 50 % Inlet B 8 CV; 3 mL/min
System wash with water	External pump: 15 mL; 3 mL/min Rest of system: Inlet A, 50 % Inlet B 8 CV; 3 mL/min
System equilibration with buffer W	External pump: 15 mL; 2 mL/min Rest of system: Inlet A, 50% Inlet B 10 CV; 3 mL/min
Column equilibration with buffer W	Inlet A: 4 CV; 1.5 mL/min
Sample Application	External pump: 1 mL/min

	Sample volumes were always defined before starting the procedure
Washing with buffer W	External pump: 25 mL; 1 mL/min
Elution with buffer BXT	External pump: 30 mL; 1 mL/min
Regeneration with 10 mM NaOH	External pump: 50 mL; 1.5 mL/min
Column equilibration with buffer W	Inlet A, 50% Inlet B: 15 CV; 1.5 mL/min
System wash with water	External pump: 15 mL; 3 mL/min Rest of system: Inlet A, 50% Inlet B 15 CV; 3 mL/min
System wash with ethanol	External pump: 15 mL; 2 mL/min Rest of system: Inlet A, 50% Inlet B 20 CV; 2 mL/min

After washing, the system and column were equilibrated with buffer W. After sample loading the unbound substances were washed away and the recombinant protein retained in the column. The bound protein was then eluted in 2 mL fractions, which were collected automatically by the fraction collector. To detect the fractions containing the protein of interest an SDS-PAGE was performed. The determined fractions containing the protein of interest were pooled together and concentrated using a centrifugal concentrator (Vivaspin 20, Sartorius AG) with a molecular weight cut-off (MWCO) of 10,000 Da at 3,428 rcf and 4 °C for 15 to 20 minutes.

The buffer was exchanged to 0.1 M Tris/HCl pH7 buffer as storage buffer using PD-10 desalting columns (GE Healthcare) and the proteins were stored at -20 °C until further use.

3.11 Electrophoresis

3.11.1 SDS-PAGE

SDS-PAGE was performed to visualize proteins and to separate them according to their size.

Table 9: Composition of Lämmli buffer, running buffer, staining solution and destaining solution

2x Lämmli buffer (for 16 mL)	
ddH_2O	9.4 mL
0.5 M Tris/HCl buffer pH 6.8	1.2 mL
100% Glycerol	1.9 mL
20% SDS	2.0 mL
1% Bromophenol blue (merck)	1 mL
Stored in 969 µL aliquots at -20 °C 31 µL β-mercaptoethanol were added to each aliquot when used	
Running buffer 10x (Bio-Rad)	
Tris	25 mM
Glycine	192 mM
SDS	0.1%
pH 8.3 diluted to 1x with water	
Coomassie staining solution	
H_2O	600 mL
Ethanol	300 mL
Acetic acid	100 mL
Coomassie Brilliant blue G 250 (VWR)	0.125% (or 1.25 g/L)
Destaining solution	
H_2O	600 mL
Ethanol	300 mL
Acetic acid	100 mL

Samples were mixed with 2x Lämmli (Table 9) in a 1:1 ratio and heated to 99 °C at 300 rpm for 10 minutes. The samples were cooled on ice and centrifuged shortly due to condensate formation on the tube lid.

Stainfree precast 4 – 15% polyacrylamide gels with 15 wells and 15 μL/well (4-15 % Mini PROTEAN TGX Stain-Free Protein Gels; Bio-Rad Laboratories, Inc.) were used. The gel was loaded with 10 – 12 μL of the prepared samples alongside 5 μL of pre-stained protein marker (peqGOLD Protein Marker IV, VWR International, LLC) and the gel was run in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Inc.) filled with 1 x Tris/Glycine/SDS (TGS) running buffer at 150 V for approximately 35 minutes.

The protein bands in the stainfree gel were visualized by a UV-induced reaction induced by the imaging system (ChemiDoc MP, Bio-Rad Laboratories, Inc.) and stained if necessary, with Coomassie brilliant blue method. The gel was incubated with Coomassie-blue staining solution for 1 hour and followed by two destaining steps with destaining solution for 1 hour each to remove the stained background and make the protein bands visible.

3.11.2 Agarose gel electrophoresis

To verify successful transformation or linearization of a plasmid, agarose gel electrophoresis was performed.

A 1 % agarose gel was prepared by either mixing 0.4 g or 1 g agarose with 40 mL or 100 mL 1x TAE buffer respectively and heated until the agarose was melted. After the agarose was cooled down to less than 60 °C, 4 or 10 μL SYBR safe (DNA gel stain, Invitrogen AG) was added. The mixture was poured into a gel tray and a comb was placed into the gel. This led to either a small gel with 8 slots or a big one with 20 slots.

After setting, the gel (still in the gel tray) was transferred to the horizontal DNA electrophoresis cell (Bio-Rad Laboratories, Inc.) and filled with 1x TAE buffer. Samples were mixed with the corresponding amount of 6 x loading dye (Fermentas). After sample loading, the DNA ladder was loaded additionally (Generuler 1 kb Plus, Thermo Fisher Scientific Inc.). The voltage was set to 80 V and then left until the ladder reached the middle of the gel. The DNA bands were visualized by stimulation with UV-rays using an imaging system (ChemiDoc MP, Bio-Rad Laboratories, Inc.).

3.12 Protein characterizations

3.12.1 Bradford Assay

The Bradford method was used for a rapid determination of protein concentration of purified laccases. This assay was performed in 96-well microtiter plates with bovine serum albumin as standard. Starting from a 20 mg/mL bovine serum albumin standard (Fermentas), 8 standards were prepared by dilution with MQ water. The prepared standards had a concentration of 0.25 mg/mL, 0.2 mg/mL, 0.125 mg/mL, 0.1 mg/mL, 0.0625 mg/mL, 0.05 mg/mL, 0.03125 mg/mL and 0.025 mg/mL. Triplicates of enzyme, blank or standard (3 x 10 µL) were added to a microtiter plate. MQ water and the storage buffer of the enzyme were used as blanks. Different dilutions of the enzyme were prepared. After that, 200 µL of Bradford reagent, diluted 1:5, (Protein Assay – Dye Reagent Concentrate, Bio-Rad) was added to each well and incubated at 23 °C for 5 minutes at 300 rpm. The absorbance was measured at 595 nm using a spectrophotometer (Synergy HT, BioTek Instruments, Inc.). The protein concentration was calculated by from the linear equation generated from the BSA standard curve (see Equation 1).

$$c = \frac{\left(A_{595nm} - A_{B,595nm}\right) - d}{k} * f$$

c = protein concentration of the sample [mg/mL]

A_{595nm} = absorbance of the protein at 595 nm

$A_{B,595nm}$ = absorbance of the buffer blank at 595 nm

d = intercept of the calibration curve

k = slope of the calibration curve

f = dilution factor of the protein solution

Equation 1: Calculation of protein concentrations from BSA standard curve

3.12.2 ABTS activity assay

ABTS assays were performed to determine laccase activity. The enzymes were diluted in buffer where they showed their highest measured enzyme activity (pH optimum). All measurements were carried out in triplicates. First, 150 µL of buffer were added to a 96 well plate and mixed with 20 µL of the enzyme or pre-diluted enzyme. Finally, 50 µL of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

(ABTS; Alfa Aesar) was added to each well. The absorbance was measured immediately at 420 nm for 2.5 minutes with a measuring point every 10 seconds using a spectrophotometer. A blank was measured for every measurement. As blank, 170 µL of buffer were mixed with 50 µL ABTS.

The enzyme activity was calculated according to the formula (Equation 2) below, which gave values in µmol/mL/min, also termed unit per mL (U/mL), or the amount of enzyme that catalyzes the reaction with 1 µmol substrate per minute. This can either be calculated per mL (volumetric activity) or mg enzyme (specific activity). The slope of the linear section of the kinetic curve was calculated and used for activity calculation. Following the first linear section the graph flattens, as the concentration of un-oxidized substrate runs low. Only the linear section in the beginning is applicable to the activity calculation.

$$a = \frac{k}{L * \varepsilon} * f$$

a = enzyme activity [U/mL]

k = slope [min^{-1}]

L = vial thickness = 0.5785 cm

ε = extinction coefficient of the used buffer [mM^{-1} cm^{-1}]

f = dilution factor (11 *x, as 20 µL of enzyme or enzyme dilution are always in a reaction mixture of in total 220 µL)

Equation 2: Calculation of enzyme activity

3.12.2.1 pH optimum

The pH optimum was one of the first characteristics determined for each enzyme. This information was important for the choice of buffer used for the other characterizations. The buffers were prepared to a molarity of 0.1 (Table 10).

Table 10: used buffers for the pH-optimum

pH	**used buffer**	**Extinction coefficient [mM^{-1} cm^{-1}]**
3	Citrate Buffer, 100 mM	34.683
4	Sodium-Acetate Buffer, 100 mM	33.115
5	Sodium-Acetate Buffer, 100 mM	31.332

6	Sodium-Phosphate Buffer, 100 mM	21.103
7	Sodium-Phosphate Buffer, 100 mM	12.07
8	Sodium-Phosphate Buffer, 100 mM	5.2507

The assays were performed according to chapter 3.12.2 ABTS assays. For each assay a different buffer was applied in triplicates, starting with Citrate Buffer 100 mM pH 3 until Sodium-Phosphate buffer 100 mM pH 8. The blank of each triplicate was the respective buffer for each assay. The extinction coefficient of each specific buffer (Table 10) was used during the calculation of the specific enzyme activity.

3.12.2.2 Temperature optimum

Enzyme activities were measured at different temperatures to determine the optimal temperature for enzyme activity.

The time scan was carried out at 420 nm with a scan time of 300 seconds and a cycle time of 19 seconds and a delay of 0 seconds. The spectrophotometer (Hitachi U-2900, Metrohm Inula GmbH) itself was able heat to 40 °C and afterwards the waterbath (Julaba EO) was used to reach higher temperatures. Autozero was performed with an empty cuvette.

The empty cuvettes (for triplicates and blank), which were measured simultaneously, were placed into the spectrophotometer to equilibrate to the current inside temperature. This was repeated for every reaction temperature.

The enzyme was prediluted in storage buffer and the last dilution step was done with preheated buffer. Therefore, 900 µL of the buffer of the pH-optima were heated to the wanted temperature for 3 to 4 minutes. In the meanwhile, 250 µL of 10 mM ABTS was added to each cuvette to equilibrate it to the appropriate temperature, as well. After 3 to 4 minutes, 100 µL of the prediluted enzyme was added for the final dilution step and then 850 µL was added to each cuvette and the measurement was started. The calculation itself was carried out similar to the described ABTS assay (Equation 2). The difference was that the vial thickness (L) in this measurement was 1 cm and the dilution factor (f) was also different. The dilution factor in this case was 1.29 * the final dilution of the enzyme. The calculation was carried out for specific temperatures in the range from 20 °C to 70 °C in 10 °C steps.

3.12.2.3 Kinetic measurement

To determine kinetic variables of the enzymes with the substrate ABTS, such as the maximal velocity (V_{max}) or the Michaelis-Menten constant (K_M), the enzyme activity with different ABTS concentrations

was measured. These values enable important information about the affinity of the enzyme to the substrate. K_M, the Michaelis constant, is equal to the substrate concentration at half-maximal velocity. This value is a gauge of the enzyme substrate affinity. Under reaction conditions enzymes show a maximum velocity (V_{max}). This is the limiting velocity value to the increasing substrate concentrations and this value is reached only theoretically when the whole enzyme would have been saturated by an infinite amount of substrate [50] [51]

The different ABTS concentrations (see Table 11) were prepared by dilution of a 30 mM stock solution. The assays were performed at optimal pH and were carried out in triplicates.

Table 11: ABTS concentrations prepared from the ABTS stock solution for kinetic assays

[S] ABTS conc. [mM]	0,5	1	5	10	15	20	25	30

The enzyme activities were calculated as described in 3.12.2 ABTS assays. These activities were converted to specific activities, which is the enzyme activity per mg enzyme. The true ABTS concentrations in each well was calculated considering the additional dilution factor, as 50 µL of ABTS were diluted to a total volume of 220 µL by buffer and enzyme.

The enzyme activity in U/mg [reaction rate, V] was plotted against the final ABTS concentrations [substrate concentration, S] in mM to obtain the Michaelis-Menten plot. This plot was used for a general overview of the reaction.

V_{max} and K_M was calculated from the linear equation (see Equation 3) of the Hanes-Woolf plot, where [S/V] was plotted against [S]. This plot was chosen over the Lineweaver-Burke plot because the measurement values did not fit to the double reciprocal model as most of the values were at rather low substrate concentrations.

$$\frac{S}{V} = \frac{K_M}{V_{max}} + \frac{1}{V} * S$$

S = substrate concentration [mM]

V = reaction rate [U/mg]

K_M = substrate concentration at half-maximal velocity [mM]

V_{max} = maximal velocity [U/mg]

Equation 3: linear equation of Hanes-Woolf (HW) plot

The y-intercept is the quotient from K_M divided through V_{max} and the slope is corresponding to $1/V_{max}$. The Hanes-Woolf plot is a linearized model which enables the calculation of the kinetic parameters from the linear parameters as intercept and slope. This model converts the original Michaelis-Menten equation into a different format which can be graphed as a linear plot. [51]

3.12.2.4 Enzyme stability

This method was performed to determine the stability of laccases at certain temperatures over a defined period of time. The enzyme was incubated at a room temperature. ABTS activity assays were performed after 10 minutes, 1 hour, 2 h, 4 h, 6 h, 24 h, 48 h, 72 h and 96 h. The assays were executed according to chapter 3.12.2 ABTS assays.

3.13 Substrate screening

The characterization of the enzymes was carried out with ABTS as substrates. Laccases are, however, able to utilize different substrates. For a rapid testing of a broad spectrum of substrates, wavelength scans of laccases with various substrates were performed. This was helpful to get a general overview of the substrate range.

Several stock solutions of various substrates (see Table 12) were prepared and diluted using different buffers. First, 200 µL of the substrate solution was added to UV-VIS suitable microtiter plates (Microplate UV-VIS, 96/F; Eppendorf) and 20 µL pure enzyme or a dilution, depending on the protein concentration, was added to the substrates. As blank, 20 µL of buffer was added to one well containing substrate. Additionally, 220 µL of pure buffer were measured. After that, a wavelength scan was performed.

Wavelength scans were performed to measure the absorbance of the enzyme/substrate mixtures in a range from 200 to 800 nm in 1 nm steps using a spectrophotometer and UV-VIS suitable microtiter plates (Microplate UV-VIS, 96/F, with film bottom; Eppendorf).

Table 12: Phenolic substrates used for substrate screening: Stock solutions of each substrate were prepared with specific solvents and/or buffer. The stock solutions were diluted to their final concentration in their final buffer.

Substrate	**Stock solution**	**Final concentration**	**Final buffer/pH***
Catechol	10 mM in buffer pH 5*	2 mM	pH 5
Syringaldazine	10 mM in methanol	0.05 mM	pH 6
2,6-Dimethoxyphenol	10 mM in buffer pH 4*	0.1 mM	pH 4
Guaiacol*	100 mM in MQ water	20 mM *	pH 4
Sinapic acid	10 mM (dissolved in 200 µL methanol + 1.8 mL buffer pH 4*)	0.1 mM	pH 4
Ferulic acid	10 mM (dissolved in 1 mL ethanol + 1 mL buffer pH 4*)	0.1 mM	pH 4
Vanillic acid	10 mM in MQ water	0.1 mM	pH 4
Gallic acid	10 mM in buffer pH 4*	0.1 mM	pH 4

* pH 4: Sodium-Acetate Buffer, 100 mM

pH 5: Sodium-Acetate Buffer, 50 mM

pH 6: Sodium-Phosphate Buffer, 100 mM

3.14 Lignosulfonate polymerization

Laccases are able to oxidize phenolic substrates while reducing molecular oxygen to water. This leads to an oxygen consumption which can be measured. The oxidized substrate loses a single electron and forms a free radical. This free radical can undergo nonenzymatic reactions such as polymerization [52]. Therefore, polymerization could be pursued by measuring oxygen consumption.

Lignosulfonate was dissolved overnight in water to a concentration of 10 %, 13.75 % or 15 % dry weight. The next day, the pH was set to a certain pH, depending on the assay specifications. Glass vials were equipped with immobilized oxygen sensor spots, which can monitor the oxygen content during the measurement. Sensor calibration was performed in empty glass vials with a 2-point calibration using atmospheric air as the 100 %-point and pure nitrogen as 0 %-point. Therefor the oxygen sensor of the apparatus (FirestingO2, PyroScience GmbH) superimposed the immobilized oxygen sensor spot in the glass vial. The program was set to "% air sat" and to a fixed temperature of 22.5 °C. The measurement was continuous with a measuring point every 10 seconds. After the calibration, 1.5 mL lignosulfonate solution was added to each vial and after the air saturation was back to 100 %, 150 µL of pure enzyme or prediluted enzyme was added to each vial except for the negative control which only consists of lignosulfonate. The oxygen saturation of the polymerization process of lignosulfonate was then continuously measured for 24 hours.

4. Results and Discussion

The aim of this thesis is the development of the novel laccases from *Chaetomium globosum* (CgL1), *Trametes ochracea* (ToLA), *Melanocarpus albomyces* (MaL1) and *Streptomyces rochei* (SiLA) for lignin polymerization. For improvement of the enzyme binding to lignin, SiLA was C-terminally fused to the cellulose binding module of cellobiohydrolase I from *Hypocrea jecorina* (SilA-CBM) and the substrate binding domain from a polyhydroxyalkanoate depolymerase from *Alcaligenes faecalis* (SilA-SBD). The expression conditions were optimized and improved to increase the enzyme yield. The biochemical characterization of the expressed and purified enzymes SiLA and MaL1 revealed the optimal reaction conditions for the oxidation of the substrate ABTS and the high stability of both enzymes at room temperature. Furthermore, the enzymes were tested with a wide range of phenolic substrates which revealed the ability of both enzymes to utilize a broad array of substrates.

4.1 Expression and purification

Fungal laccases

The eukaryotic genes coding for CgL1, ToLA and MaL1 were codon-optimized for expression in *P. pastoris*. Strain *P. pastoris* KM71H was transformed by electroporation with SacI linearized plasmids. The resulting clones were screened using colony PCR. Agarose gel electrophoresis was employed to determine copy numbers of the insert (see appendix). The clones showing the strongest bands in the agarose gel were chosen for expression.

The expression of CgL1 according to the standard protocol (3.6.1) and under varying expression conditions was not successful. The cultivation was conducted under various conditions (see appendix) such as varying temperatures (20 °C and 28 °C), varying copper concentrations (0.1 mM, 0.5 mM and 1 mM) and an increased expression duration of 96 hours. They all led to the same result. SDS-PAGE of CgL1 expression at 28 °C (see Figure 4) did not show any band at the theoretical molecular weight of 67 kDa and no enzyme activity on ABTS was detected after 72 hours of expression. However, SDS PAGE showed a faint band at approximately 150 kDa, which could result from a homodimer of CgL1 as the doubled molecular weight is 135 kDa without glycosylation. This was indicated by the increasing intensity of the band over the expression period. Presumably, the enzyme was forming a dimer at some point during the expression. This is not uncommon since several fungal laccases also exhibit a homodimeric structure. In this case, the enzyme was probably composed of two identical subunits where each subunit has the molecular weight of a monomeric laccase [12]. In case of CgL1 it could be that the subunits were not correctly assembled. Due to the missing activities, CgL1 was not further investigated.

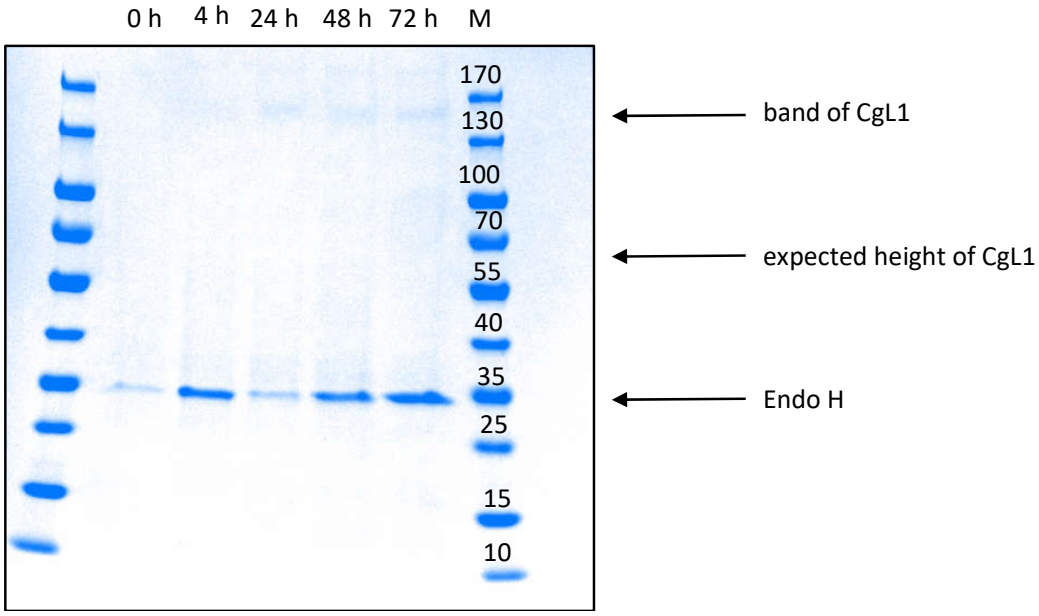

Figure 4: CgL1 expression at 28 °C. SDS-PAGE of deglycosylated culture supernatants obtained from expression of CgL1 at 28 °C and different time points. Lane M shows the peqGold protein marker IV in kDa.

Expression of ToLA was successful and activity on ABTS was measurable after 72 h of induction. Furthermore, the appropriate band was found in the SDS-PAGE gel of the conducted deglycosylated ToLA expression timepoints.

Expression at 20 °C (see Figure 5) led to a higher yield than at 28 °C (see appendix) resulting in 0.016 U/mL on ABTS compared to 0.002 U/mL for expression at 28 °C. The strongest visible band in the SDS PAGE resulted from Endo H used for deglycosylation but faint bands of ToLA at 53 kDa were visible.

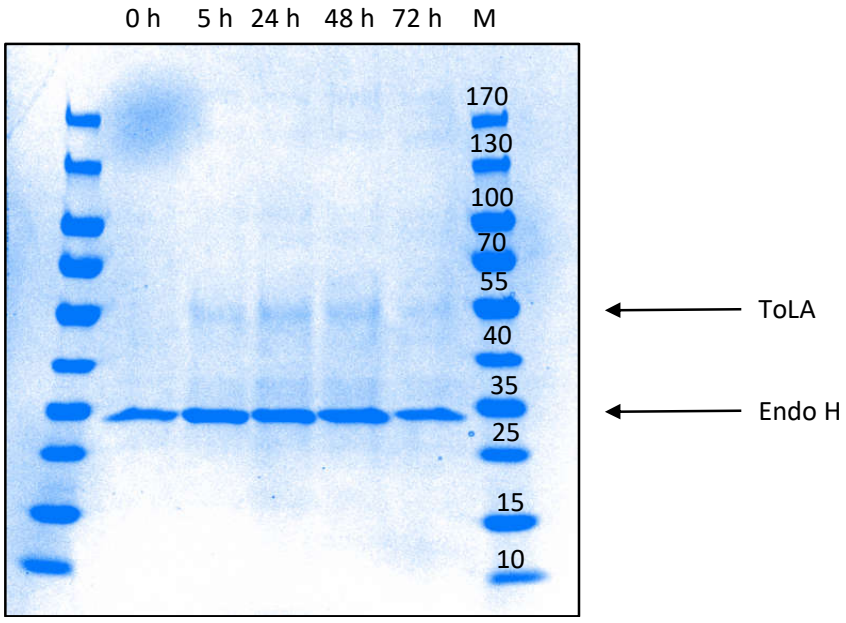

Figure 5: ToLA expression at 20 °C. SDS-PAGE was conducted of deglycosylated ToLA expression time points collected during expression at 20 °C. Lane M shows the peqGold protein marker IV in kDa.

ToLA obtained from the expression at 20 °C was purified by gravity column purification. The purification was not successful. The SDS-PAGE of the purification fractions (see appendix) showed that the band of ToLA was neither visible in the supernatant nor in the collected fractions. Activity assay of the fractions (see Figure 6) indicated, however, the presence of active enzyme. The color of ABTS changed to green after 48 h of incubation with most fractions. Elution fraction 3 contained most of the active enzyme, according to the intensity of the color change.

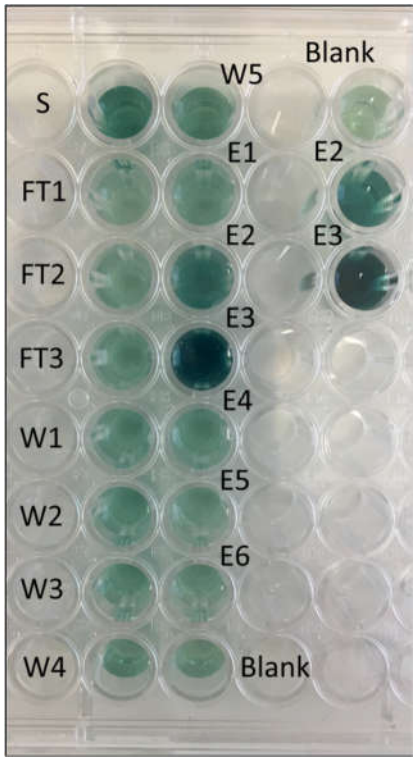

Figure 6: ABTS assays of the ToLA purification fractions after 48h of incubation. S: culture supernatant. FT1 – FT3: flow-through fractions. W1 - W5: wash fraction from purification. E1 - E6: elution fractions. Blank B: buffer W. The three wells on the right contained the repeated assays for blank, E2 and E3.

Heterologous expression of the fungal laccase MaL1 at 28 °C was successful. SDS-PAGE of samples from different timepoints (see Figure 7) showed a band at 70 kDa corresponding to the theoretical molecular weight of Mal1 and enzyme activity was measured after 72 hours of induction. The highest enzyme activity of MaL1, after 72 hours of expression, was 0.26 U/mL with ABTS.

The strong band of the gel from the expression timepoints (see Figure 7) stems from Endo H. The band of glycosylated MaL1 is visible at 100 kDa. Among all tested fungal laccases, MaL1 was the only laccases where deglycosylation was not necessary for visualization of the protein band by SDS-PAGE as the enzyme which indicates a homogenous glycosylation pattern.

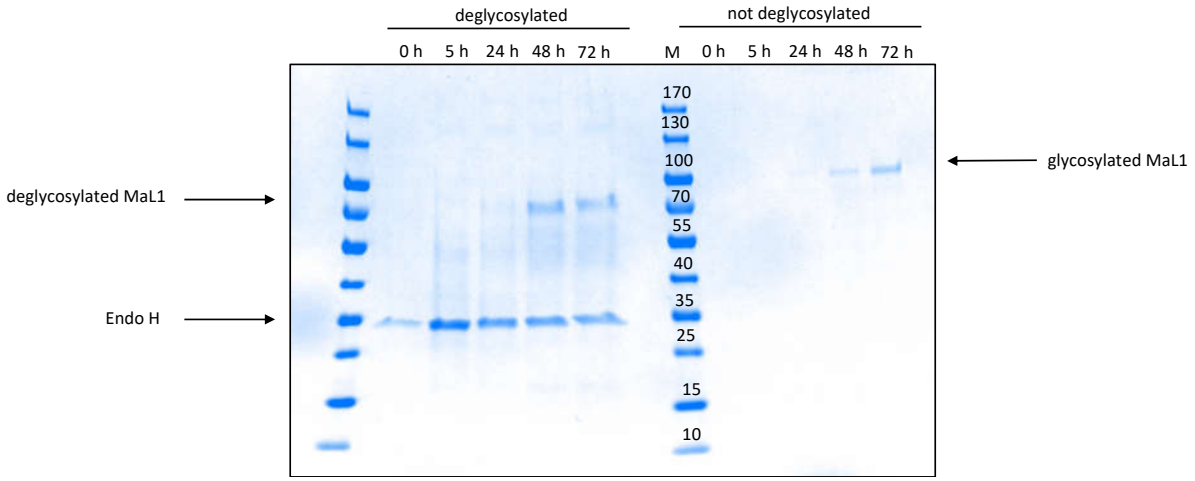

Figure 7: MaL1 expression at 28 °C. SDS-PAGE was conducted of deglycosylated and not deglycosylated MaL1 culture supernatants from expression time points collected during expression at 28 °C. Lane M shows the peqGold protein marker IV in kDa.

The gravity column purification of MaL1 was not successful. The strep-tag fused to the enzyme did not bind to the column and the enzyme was washed out during the sample loading and the subsequent washing, as evident by the activity on ABTS of the various fractions (see Figure 8). This could have happened due to several reasons. According to 3D-models of the enzyme (PDB 1GW0), the strep-tag was hidden in the enzyme and therefore not able to bind to the affinity column. It was also possible that the tag could have been removed by the host during the expression. This could have not been detected on SDS-PAGE as it is not sensitive enough to detect the absence of the fused tag comprising only a few amino acids. If the strep-tag was located inside the enzyme a purification under denaturing conditions could help the strep-tag to bind to the column. MaL1 was secreted to the supernatant during expression. Therefore, it was not necessary to remove a high amount of impurities, because other proteins normally will not secrete into the supernatant as well. The only other substances in the supernatant were residual media components and other low-molecular substances. Therefore, the supernatant was concentrated using Vivaspin20 and then purified with PD10 columns. These columns can remove low molecular weight substances and exchange the buffer. This approach resulted in an almost pure enzyme solution.

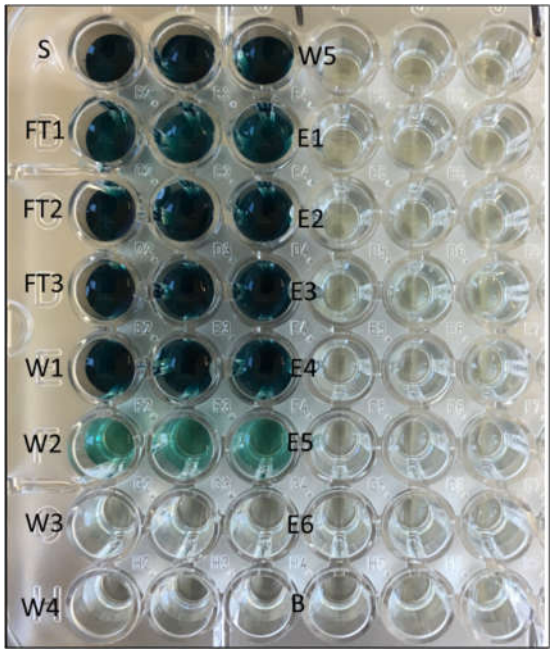

Figure 8: ABTS assays of the MaL1 purification fractions. S: culture supernatant. FT1 – FT3: flow-through fractions. W1 - W5: wash fraction from purification. E1 - E6: elution fractions. Blank B: buffer W.

In summary, out of the fungal laccases CgL1 (see Figure 4), ToLA (see Figure 5) and MaL1 (see Figure 7) only Mal1 could be successfully expressed and was neither forming homodimers nor getting degraded by proteases.

Bacterial laccases

SiLA was codon optimized for expression in *E. coli* and C-terminally fused to the cellulose binding module of cellobiohydrolase I from *Hypocrea jecorina* (SilA_CBM) and the substrate binding domain from a polyhydroxyalkanoate depolymerase from *Alcaligenes faecalis* (SilA_SBD).

The expression timepoints of all bacterial laccases revealed that the micro-aerobic or non-shaking approach at 25 °C was more effective compared to the aerobic or shaking approach as the protein yields were higher. In case of SiLA the cell pellets exhibited an intense blue color, when cultivated under micro-aerobic conditions in comparison to the cell pellets obtained from the aerobic approach. The homeostasis mechanism of *E. coli* (copper efflux and copper-sensing systems) is dependent on the available oxygen. By changing the expression to micro-aerobic conditions, a higher accumulation of intracellular copper is promoted. The increased amount of intracellular copper accounts for the intensely blue color of the laccase [31], [32].

The most pronounced bands in the gel (see Figure 9) were at 38 kDa and 50 kDa. SiLA was expected at a height of 34 kDa. The band at 38 kDa probably represented SiLA. This was indicated by the increasing intensity of this band over the expression period. The difference in molecular weight was probably due to incomplete denaturation during the heating process with Lämmli buffer. The band at 50 kDa was visible from the start of the expression before induction with IPTG. This band either represented *E. coli* specific proteins or recombinant protein, which was not folded properly. The band fully disappears after 24 hours of induction.

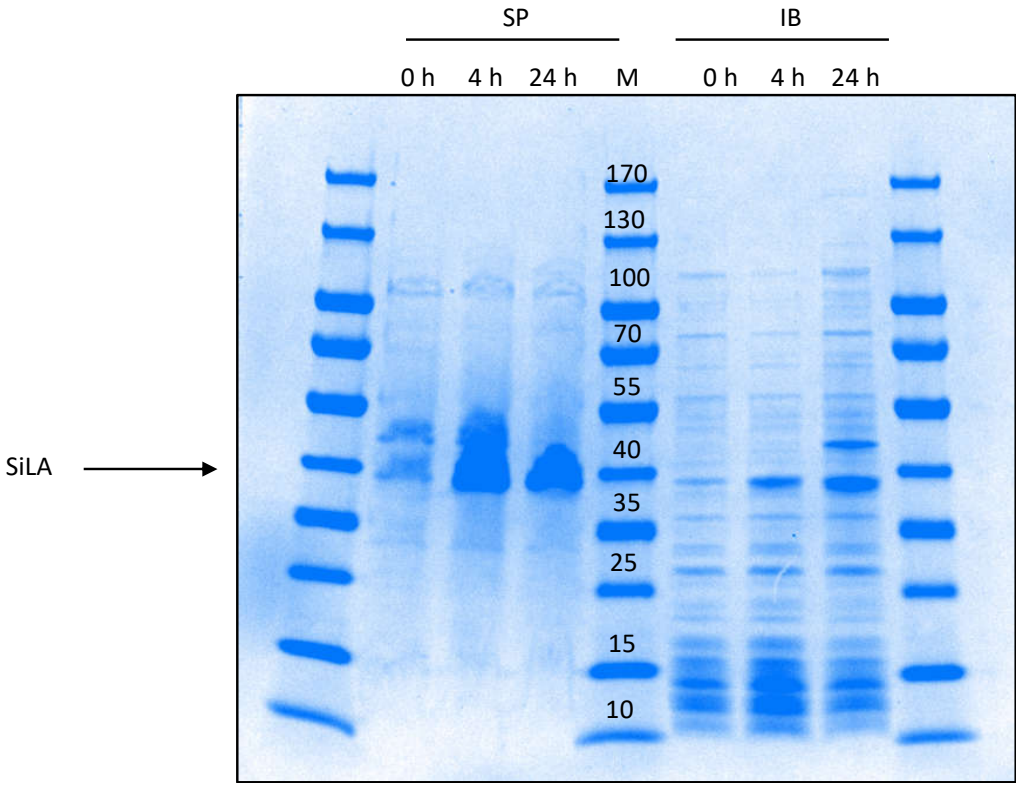

Figure 9: SDS-PAGE of cell lysates at expression timepoints of SiLA. The expression timepoints were conducted during the micro-aerobic expression at 25 °C. The gel consists of two parts. SP: cleared cell lysis; IB insoluble cell fraction containing including bodies. The lanes indicate the timepoints in hours. Lane M shows the peqGold protein marker IV in kDa.

In case of the heterologously expressed bacterial laccases SiLA_CBD and SiLA_SBD, the yield was increased by decreasing the temperature during expression from 25 °C to 20 °C. The expression timepoints at 20 °C showed stronger bands compared to the bands of expression timepoints at 25 °C. The protein concentration per g cell pellet (see Table 13), conducted from the expression at 20 °C, showed 2.3-fold increase (SiLA_SBD) and a 3-fold increase (SiLA_CBD) of enzyme yield for the expression at 20°C.

In case of SiLA_CBD, the amount of partially folded enzyme present in inclusion bodies was decreased by decreasing the cultivation temperature from 25 °C (see appendix) to 20 °C (see Figure 10). The most pronounced bands were at 40 kDa and 50 kDa. The band at 40 kDa showed increasing intensity over the expression period, representing SiLA CBD, which has a calculated molecular weight of 40 kDa. The second band at 50 kDa was visible from the start of the expression before induction. This band could represent recombinant enzyme, which was not properly folded. This could have happened because of the cellulose binding domain fused to the enzyme, increasing the hydrophobicity of the enzyme.

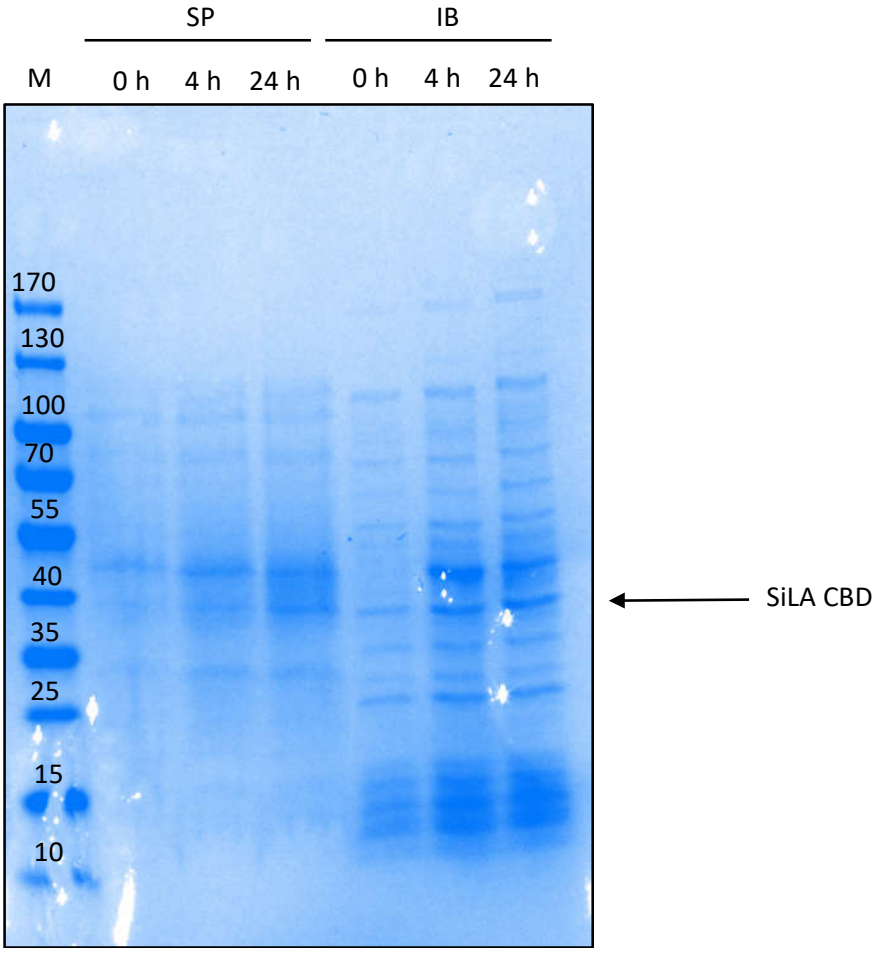

Figure 10: SDS-PAGE of expression timepoints of SiLA_CBD. The expression timepoints were taken during the micro-aerobic expression at 20 °C. SP: cleared cell lysate. IB: insoluble cell fraction. The lanes indicate the timepoints in hours. Lane M: peqGold protein marker IV [kDa].

In case of SiLA_SBD, the most pronounced band (see Figure 11) was at 45 kDa. This band represented SiLA_SBD as it has a calculated molecular weight of 42.8 kDa. The expression timepoints at 20 °C showed an increased intensity compared to the bands of expression timepoints at 25 °C. The lowered temperature and therefore the lowered growth of the host cells had a positive impact on the production and proper folding of the recombinant protein.

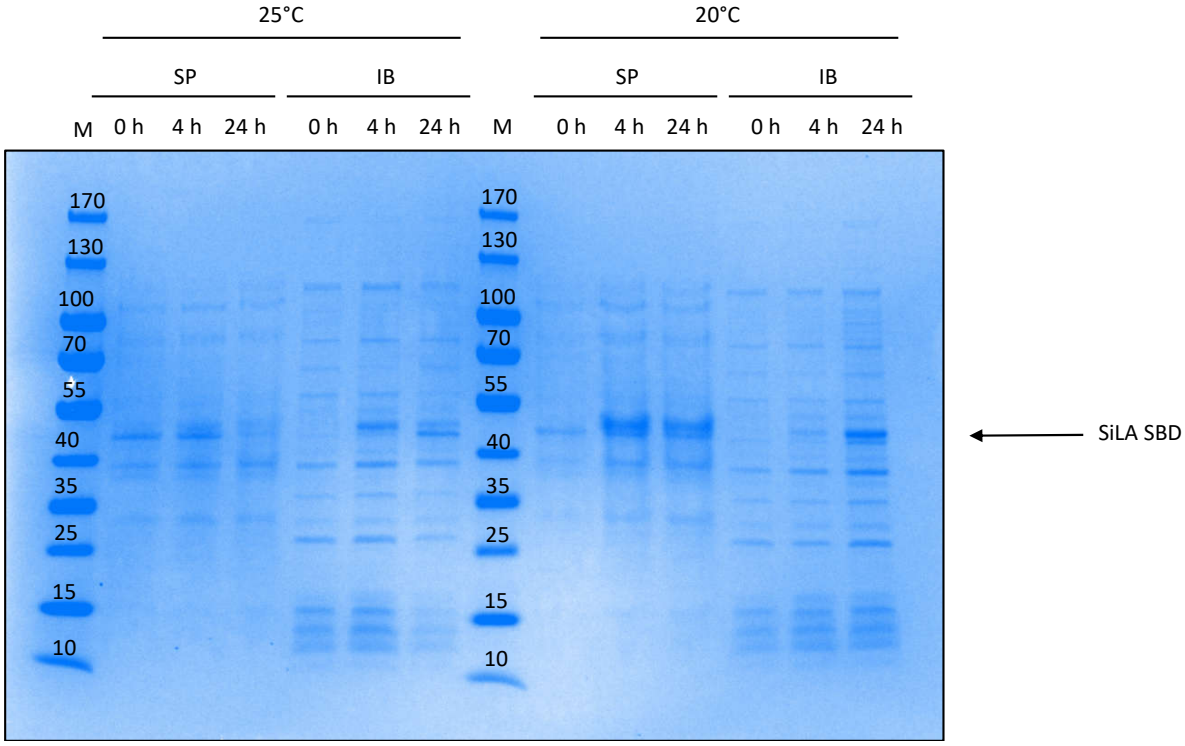

Figure 11: Comparison of micro-aerobic expression of SiLA_SBD at 25 °C and 20 °C. The expression timepoints were taken during the expression at 20 °C and the expression at 25 °C. SP: cleared cell lysate. IB: insoluble cell fraction. The lanes indicate the timepoints in hours. Lane M: peqGold protein marker IV [kDa].

The expression of SiLA was the most successful out of the heterologous expressions of the bacterial laccases SiLA (see Figure 9), SiLA_CBD (see Figure 10) and SiLA_SBD (see Figure 11). The respective protein band of SiLA shows a higher intensity as SiLA_CBD and SiLA_SBD. The protein concentration of SiLA per gramm cell pellet (see Table 13) was 6.8-fold and 16.8-fold higher compared to SiLA_SBD and SiLA_CBD respectively. The reason for that lies in the more difficult expression of fusion proteins. The cell pellet of SiLA showed an intensely blue color whereas the other displayed a beige color. SiLA retained the intensely blue color after purification. The SiLA expression at 25 °C achieved a high yield while the yield of SiLA_CBD and SiLA_SBD was low at that temperature. The lower temperature resulted in a 2.3-fold increase (SiLA_SBD) and a 3-fold increase (SiLA_CBD) compared to 25 °C.

Table 13: Comparison of enzyme production under micro-aerobic conditions

	Pellet [g]	Laccase concentration after purification [mg/mL]	Specific activity [U/mg]	Purified enzyme per [g] pellet [mg/mL/g]
SiLA – 25 °C	3.02	6.1	2.2	2.02
SiLA_SBD – 25 °C	2.03	0.26	0.91	0.13
SiLA_SBD – 20 °C	2.45	0.73	0.62	0.30
SiLA_CBD – 25 °C	1.8	0.07	1.52	0.04
SiLA_CBD – 20 °C	5.5	0.67	2.28	0.12

Summary of all expressed enzymes

Out of all expressed laccases, Mal1 and SiLA were particularly promising. CgL1 was transformed twice and the expression revised several times under different conditions. It was impossible to detect any enzyme activity after 72 or 96 hours of induction. This was probably caused by dimerization of the enzyme during expression as several fungal laccases of varying genera exhibit a homodimeric structure, where the enzyme is composed of two identical subunits [12]. It was possible that the subunits were not correctly assembled. The expression of ToLA was successful at a temperature of 28 °C and the enzyme had an activity of 0.00253 U/mL after 72 hours of expression. It was possible to increase the enzyme activity to 0.0158 U/mL by reducing the expression temperature to 20 °C. SDS-PAGE of the purification fractions and the supernatant did not show a band corresponding to ToLA. Presumably, the laccase was partially degraded by proteases present in the supernatant. The delayed reaction of the remaining enzyme in the purification fractions (see Figure 6) showed that the purification using the Strep-Tactin XT system in principal would have been successful. MaL1 expression was successful right away. Therefore, the expression conditions were not varied. MaL1, located in the supernatant of the expression culture, reached enzyme activities up to 0.5 U/mL. The main issue was the purification, as the strep-tag of the enzyme was unable to bind to the engineered streptavidin (Strep-Tactin XT) during sample loading. It will be crucial to find a purification technique, but as MaL1 was secreted into the supernatant during cultivation it was already pure to some extent. The supernatant was partly purified by Vivaspin20 and PD10 as a big part of the impurities was removed. This enzyme is one of the two most promising enzymes in this study.

In contrast to the fungal laccases, it was possible to express and purify all three bacterial enzymes. Two different expression methods, aerob and micro-aerob, were tested. The micro-aerobic approach worked significantly better, as the enzyme concentration and the activity were considerably higher. It was possible to increase the amount of expressed SiLA CBD and SiLA SBD by decreasing the temperature of the expression to 20 °C. Further characterizations were performed with MaL1 and SiLA as the purified amount of SiLA CBD and SiLA SBD were too low for all biochemical assays.

4.2 Characterization

The other focus of this master thesis was the biochemical characterization of these enzymes and the characterization with lignosulfonate. The polymerization of lignosulfonate for the production of novel materials is a possible application of these laccases.

pH-Optimum

The pH optima of SiLA and MaL1 (see Figure 12) were determined, as this information was important for further characterizations. The optimal pH value was implemented for every ABTS assay. The data showed that the pH optima of MaL1 and SiLA were located at pH 4 and pH 5, respectively. In case of MaL1, the difference of specific enzyme activity between pH 3, 4 and 5 were only slight, with values of 1.96, 2.06 and 2.01 U/mg respectively. The specific activity of SiLA was significantly higher compared to MaL1. This was not surprising, as bacterial laccases are known for their high enzyme activity. Additionally, the affinity chromatography purification of SiLA resulted in a purer enzyme solution than the enzyme solution of MaL1 purified by Vivaspin and PD10 desalting columns. The protein concentration of MaL1 may include impurities, which were not removed during purification and lead to a higher value as the actual MaL1 content.

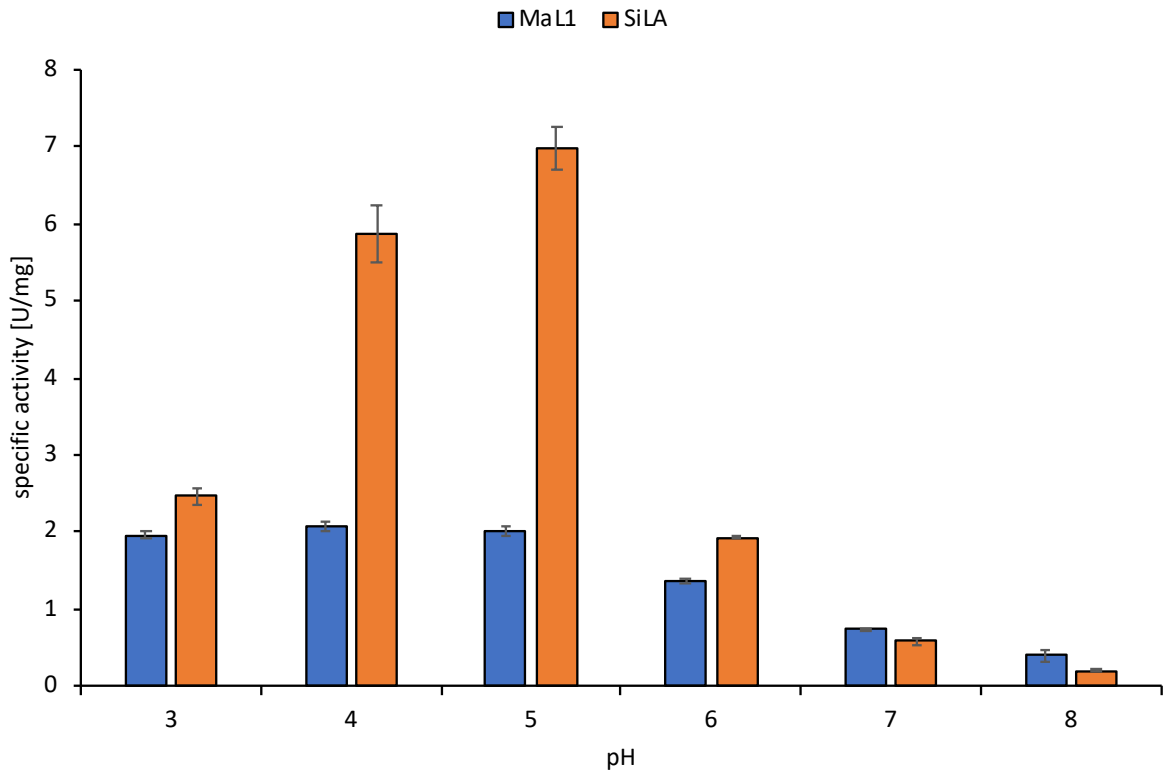

Figure 12: Enzyme activities of MaL1 and SiLA at different pH values ranging from pH 3 to 8.

Temperature Optimum

The temperature optima of MaL1 and SiLA, measured in the temperature range from 20 °C to 70 °C, were 50 °C and 70 °C, respectively (see Figure 13). The enzyme activities measured at the optimum showed a 2.5-fold increase (MaL1) and a 4.3-fold increase (SiLA) compared to room temperature. The temperature optimum of Mal1 was explicit, whereas in the case of SiLA, it was inconclusive. SiLA exhibited the highest activity at 70 °C which that was the highest measured measuring temperature due to the limitation because of the water bath. It was conceivable that SiLA would be more active at higher temperatures and therefore it was unclear if the optimum was at 70 °C.

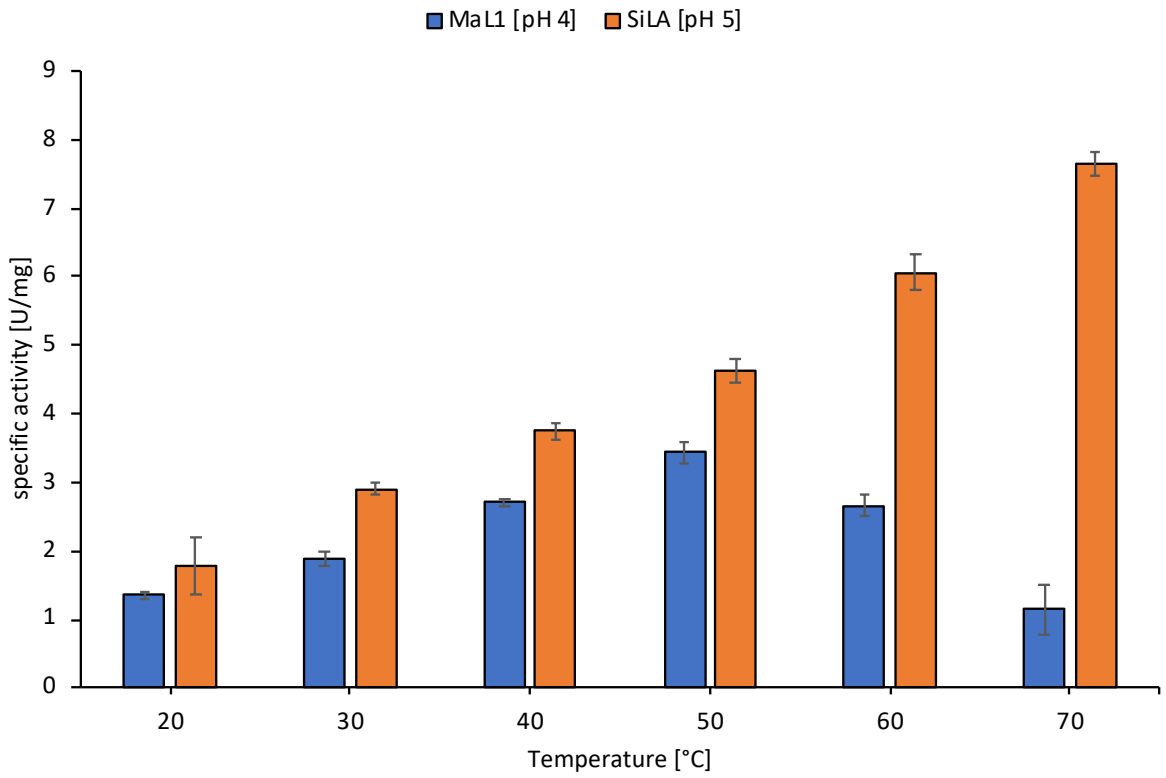

Figure 13: Enzyme activities of MaL1 and SiLA at different temperatures ranging from 20 °C to 70 °C in 10 °C steps.

Enzyme kinetics

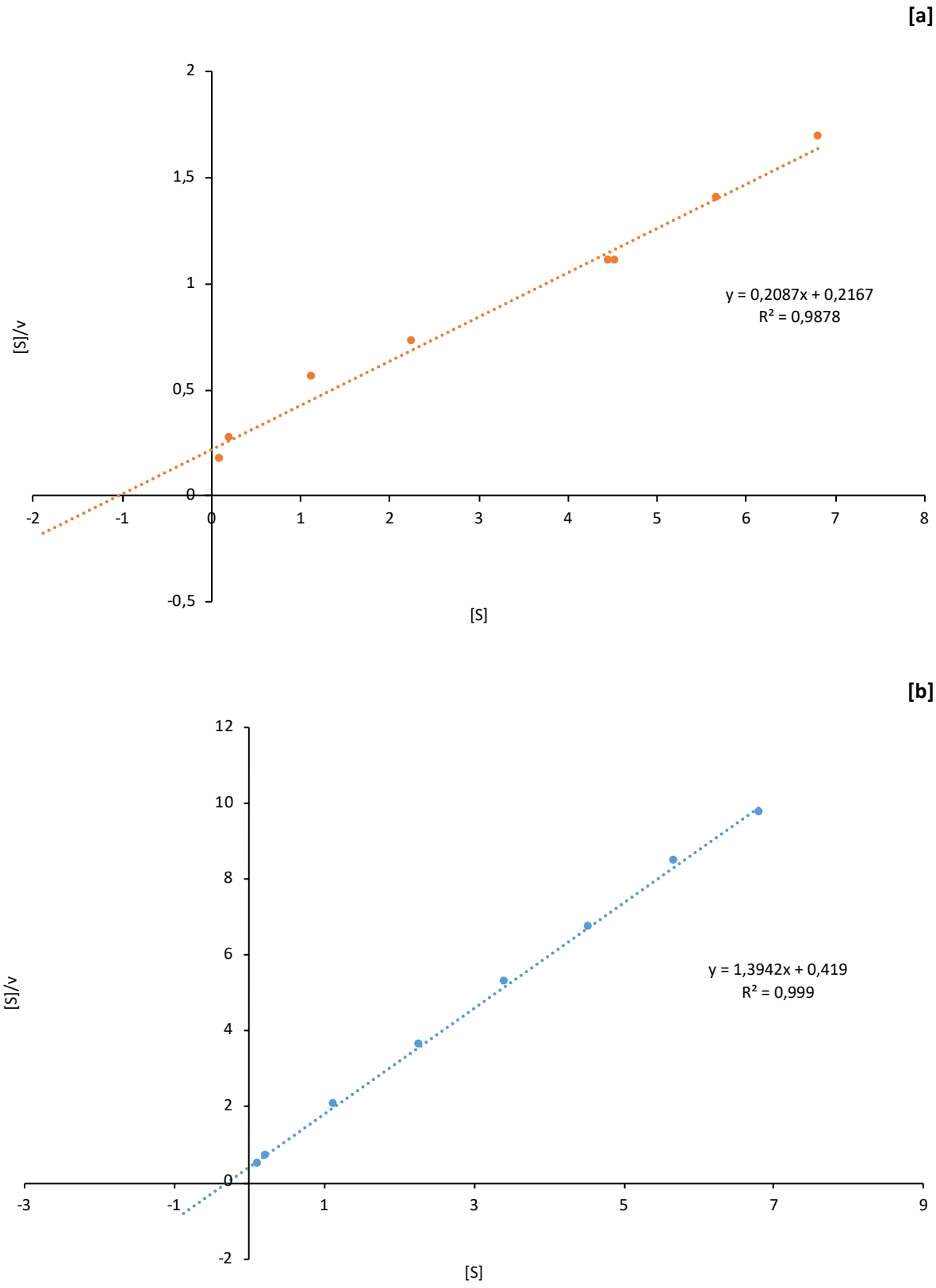

Figure 14: Hanes-Woolf plots of SiLA [a] and MaL1 [b] plotted with data from of activity assays conducted with ABTS concentrations ranging from 0.5 mM to 30 mM.

The Michaelis-Menten kinetic parameters were determined at 20 °C in Sodium-phosphate buffer pH 4 (MaL1) and 5 (SiLA) and calculated for ABTS as substrate with concentrations ranging from 0.5 mM to 30 mM (see Figure 14). The Hanes-Woolf plot was chosen for the calculation (see Equation 3) of V_{max} and K_M. The V_{max} values of MaL1 and SiLA were 0.72 U/mg and 4.79 U/mg, respectively, whereas the calculated K_M values were 0.3 mM (MaL1) and 1.04 mM (SiLA). The higher V_{max} value of SiLA fits to the generally higher enzyme activity of this bacterial laccase.

Table 14: K_M and V_{max} of MaL1 and SiLA on ABTS at 20 °C in Sodium-phosphate buffer pH 4 (MaL1) and 5 (SiLA)

	V_{max} [U/mg]	**K_M [mM]**
MaL1	0.72	0.3
SiLA	4.79	1.04

Enzyme stability

The activity of enzymes decreases over time. In industrial applications, such as lignosulfonate polymerization, it is necessary that enzyme activity during usage stays high. Therefore, the enzyme stability was determined (see Figure 15). MaL1 showed a high stability at room temperature for 144 hours undiluted in storage buffer (Tris/HCl pH 7), as well as, diluted in 100 mM Sodium-acetate buffer pH4. The enzyme activity of MaL1 was decreasing to a similar extent during both experiments. During the period of 144 hours the enzyme in storage buffer retained 54% of its original activity while the diluted enzyme retained 37 % of its original activity. The enzyme activity after 144 hours was 0.6 U/mg (in storage buffer) and 0.3 U/mg (prediluted in buffer pH 4). A main difference was the lower initial enzyme activity at the beginning in the case of prediluted MaL1 with 0.7 U/mg instead of 1 U/mg. This could have happened due to a slight dilution error or the enzyme was not fully used to the temperature. MaL1 showed a slightly better stability in storage buffer as compared to in a dilution in buffer pH 4.

In case of SiLA the difference in stability between enzyme in storage buffer and diluted in 100 mM Sodium-acetate buffer pH 5, was more obvious. The enzyme showed a higher stability when it was undiluted and in storage buffer as in the 1:100 dilution with buffer pH 5. Both experiments started with a similar initial enzyme activity of 4.2 U/mg (diluted) to 4.3 U/mg (undiluted). During the period of 168 hours the enzyme in storage buffer retained 47 % of its original activity. The final activity at the end was 2 U/mg which was half of the initial activity. The diluted SiLA showed a steep decrease of activity in the same time period, with a final activity of 0.1 U/mg or 3 % of the initial activity.

When stored in storage buffer SiLA retained 50 % of its enzyme activity over the course of 7 days at room temperature while SiLA diluted in pH 5 buffer already lost 50 % of enzyme activity after 24 hours.

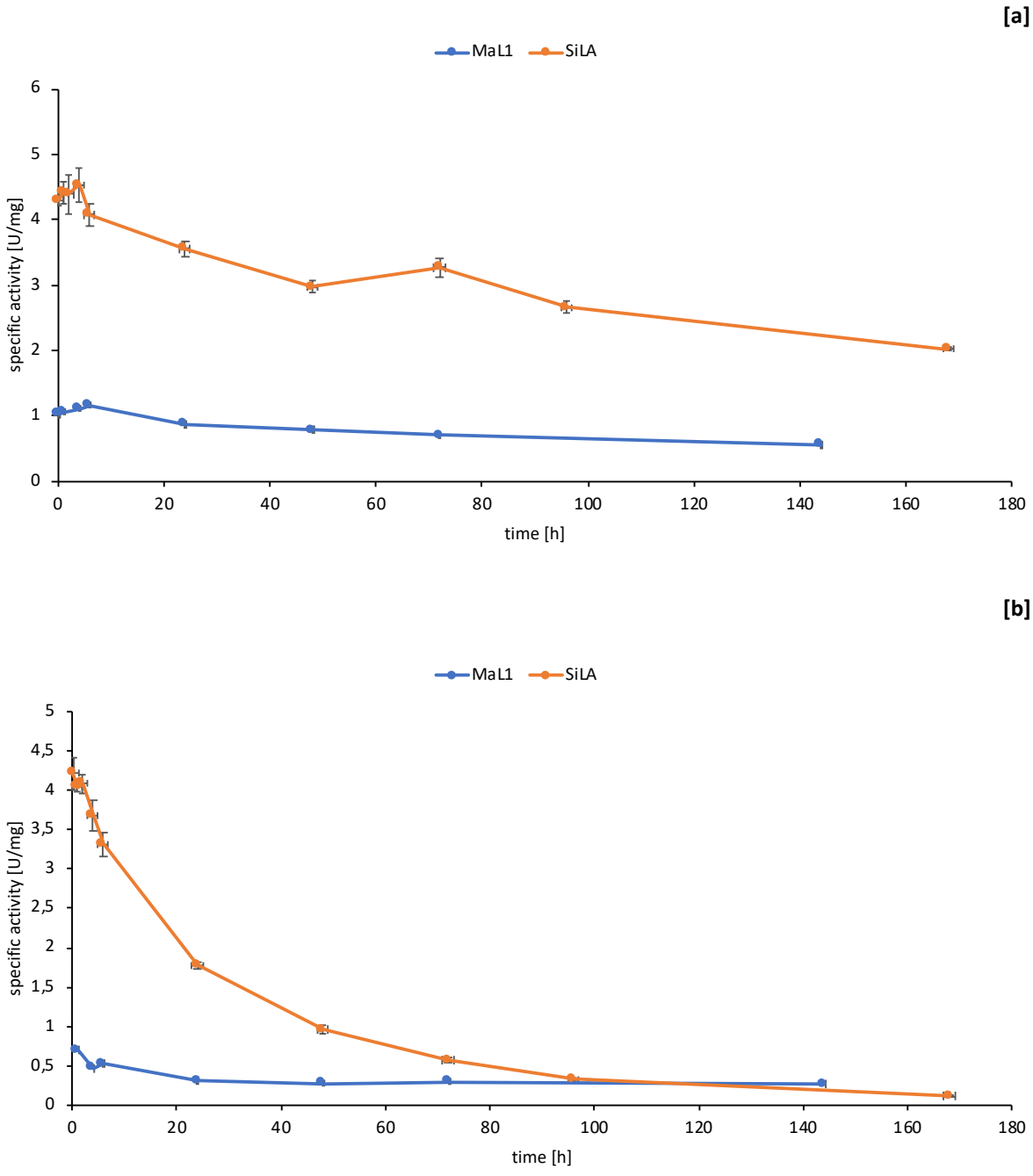

Figure 15: Stability of MaL1 and SiLA at room temperature. enzyme activity of [a] undiluted MaL1 and SiLA in storage buffer Tris/HCl pH 7 and [b] prediluted enzymes in the buffer of their respective pH optima, pH 4 for MaL1 and pH 5 for SiLA.

Substrate screening

Different laccases are able to utilize varying substrates. The utilization of non-phenolic and phenolic substrates by laccases depends on their redox potentials. Therefore, a wide variety of phenolic substrates (see Table 15) were screened with MaL1 and SiLA to determine the substrate specifity of these enzymes. As a reference, the laccase the *Myceliophthora thermophila* laccase (MtL) which is commercially available and can utilize a wide spectrum of substrates [15] was used.

MaL1 and SiLA were able to utilize the substrates 2,6-DMP, syringaldazine, guaiacol (for all three see Figure 16), sinapic acid and ferulic acid, but to a different extent. MaL1 showed a higher activity than SiLA with the substrates 2,6-DMP and syringaldazine. Both enzymes were unable to utilize the substrates catechol, vanillic acid and gallic acid. The reference laccase MtL was able to utilize catechol and gallic acid but was unable to utilize vanillic acid.

Table 15: Ability of MaL1 and SiLA to oxidze various phenolic substrates

Substrate	***MaL1***	***SiLA***	***MtL***
2,6-DMP	+++	+	++
Syringaldezine	++	+	+++
Catechol	-	-	+++
Guaiacol	++	++	+++
Sinapic acid	+++	+++	++
Ferulic acid	+	+	+++
Vanillic acid	-	-	-
Gallic acid	-	-	++

For the substrates showing a high activity, quantitative substrate assays will be necessary. Although MaL1 is a fungal laccase and SiLA a bacterial laccase they showed a similar oxidation pattern, hinting a similar redox potential. They were able to utilize the same substrates, but to a different extent. This could be due to the lower redox potential of the T1 copper of the similar laccase *Streptomyces ipomoea* [53].

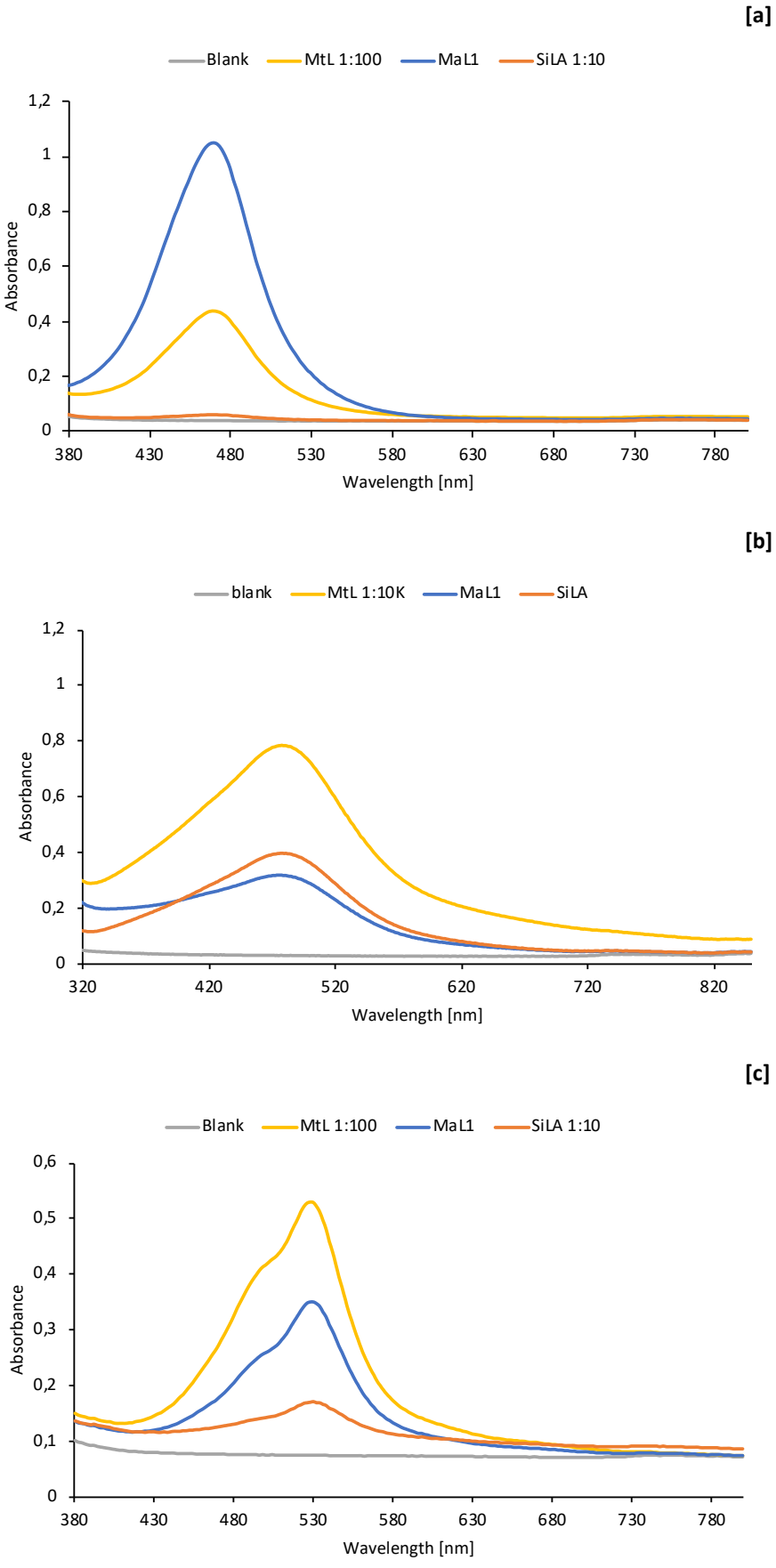

Figure 16: Oxidation of [a] 2,6-DMP; [b] Guaicacol; [c] Syringaldazine with MtL diluted 1:100, MaL1 and SiLA

Lignosulfonate Polymerization

Initial trials of lignosulfonate polymerization with MaL1 and SiLA were conducted. During these polymerizations, the O_2 consumption was monitored for roughly 24 hours using firesting.

The pH optima of MaL1 and SiLA (see Figure 17) with lignosulfonate were determined. The optimal pH-values for lignosulfonate polymerization with MaL1 (see Figure 17 [a]) were in the neutral region (pH 6 to 8) and in the acidic region (pH 3.4) indicating two pH optima. The lignosulfonate polymerization under neutral to alkaline conditions with MaL1 showed higher oxygen consumptions compared to the other pH values. This could be especially interesting for industrial applications as technical lignin, such as kraft lignin, is only soluble at alkaline pH.

Contrary to the clear results for MaL1, the lignosulfonate polymerization of SiLA (see Figure 17 [b]) proceeded less obvious results. The figure showed the highest oxygen consumption during polymerization under acidic conditions (pH 3.4 and pH 4). MaL1 was able to polymerize lignosulfonate to a higher extent as SiLA which is shown by the higher oxygen consumption.

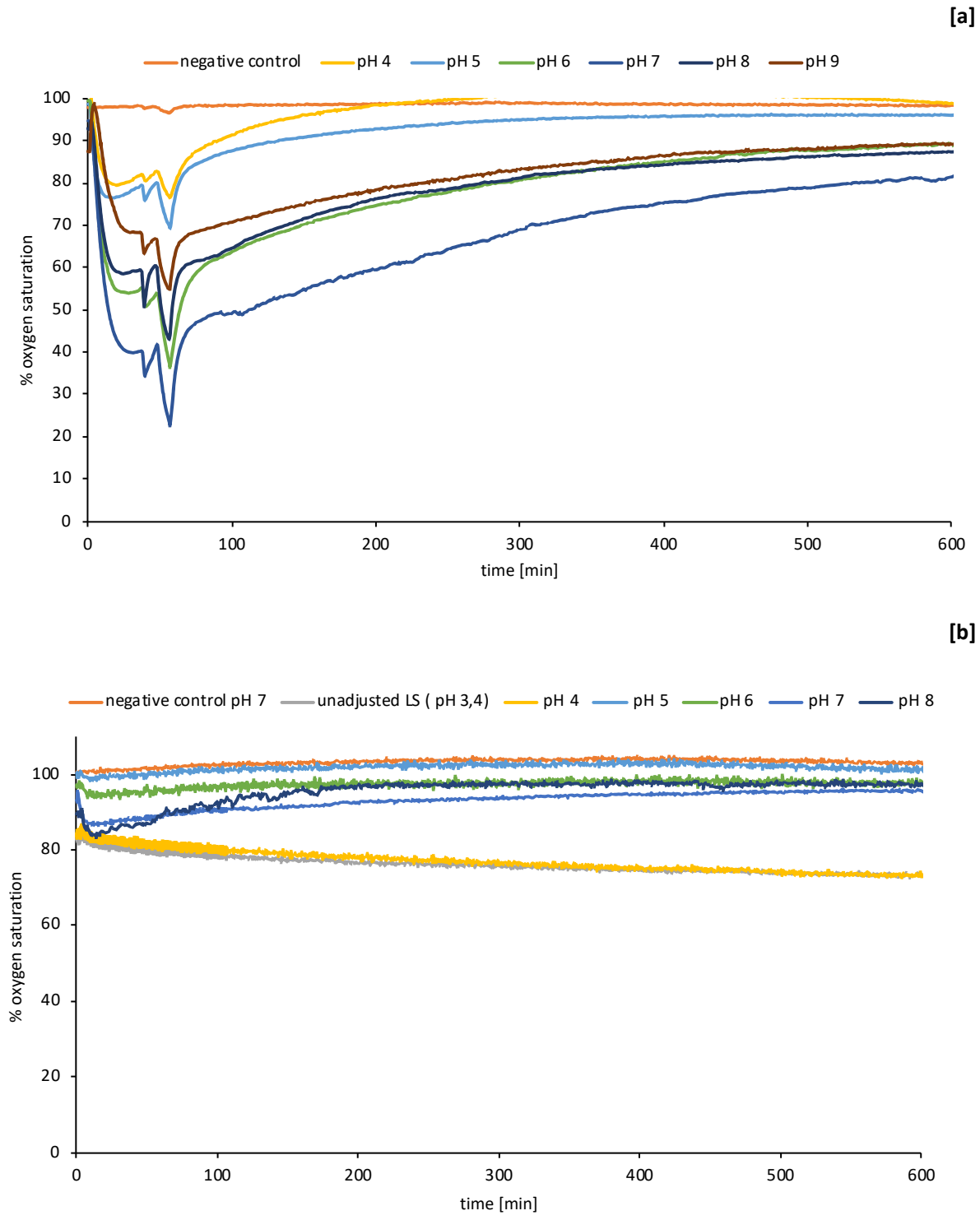

Figure 17: pH optima of [a] MaL1 and [b] SiLA on lignosulfonate. O_2-consumption of [a] MaL1 and [b] SiLA during lignosulfonate polymerization at different pH-values. In [a] the stirrer changed between fast and slow stirring between 40 and 70 minutes

5. Conclusion and Outlook

The aim of this thesis was to develop newly designed laccases (CgL1, ToLA, MaL1 and SiLA) for polymerization of lignosulfonate. These laccases were chosen for their high redox potential or their high activities under alkaline conditions. The bacterial laccase, SiLA, was chosen as bacterial laccases often showing a higher enzyme activity and stability compared to fungal and plant laccases. It was possible to express three out of the four enzymes (ToLA, MaL1 and SiLA). CgL1 was inactive after expression, probably due to dimerization, not unusual for fungal laccases [12]. As these dimerized fungal laccases normally show enzyme activity it was possible that, in case of CgL1, the subunits were not correctly assembled or something other had gone wrong. Possibly, due to protease degradation, ToLA could not be purified. Therefore, it was only possible to purify and characterize MaL1 and SiLA. Because of the high enzyme activity of SiLA, two variants of SiLA, containing a substrate binding domain and a cellulose binding domain, were designed. The expression at 25 °C led to low yields because of the stressed *E. coli* cells having troubles to properly fold the bigger and more hydrophobic enzymes. It was possible to increase the amount of expressed enzyme of both variants by decreasing the expression temperature to 20 °C. This led to a 4-fold increase of expressed enzyme, because the lower temperature led to a slower growth of *E. coli* and decreased the stress of the cells. Unfortunately, it was impossible to properly purify MaL1 using affinity chromatography, as the strep tag was either unable to bind to the column due to steric hindrance or it was cleaved off during expression. The tag is only a few amino acids long and its cleavage is impossible to detect with SDS-PAGE. One of the main advantages of expression in *P. pastoris* was the secretion of the expressed enzyme into the supernatant. Therefore, there are fewer impurities. For further applications and characterizations, it will be important to overcome this issue as it falsifies the enzyme concentration measurement, the actual protein concentration of MaL1 is lower than the measured concentration.

The second part of this thesis was the characterization of the MaL1 and SiLA using ABTS as substrate. The amount of SiLA_SBD and SiLA_CBD was too low and the characterization with lignosulfonate had priority. SiLA and MaL1 exhibited similar pH optima of pH 5 and pH 4, respectively. The fungal laccase MaL1 showed a temperature optimum of 50 °C. In contrast, the highest activity of SiLA was measured at 70 °C. The actual temperature optimum of SiLA could lie above this temperature, because activity at higher temperatures was not measurable with the experimental setup. The stability of both enzymes was measured at room temperature in storage buffer and in dilution with the buffer of their respective pH optimum. The thermal stability, when stored in Tris/ HCl pH 7 storage buffer, was quite similar between MaL1 and SiLA. Both enzymes exhibited a residual enzyme activity of approximately 50 % after 1 week. The second experiment was carried out with enzymes diluted in the buffer of their respective optimal pH. In the case of MaL1 the dilution was 1:10 and for SiLA the dilution was 1:100.

These dilutions were selected due to prior experiments where it was observed that these dilutions resulted in the optimal enzyme concentrations for the ABTS assays. Under these conditions, MaL1 showed in both experiments a similar residual enzyme activity after 1 week. SiLA showed a steep decrease with a loss of 50 % enzyme activity after 24 hours. For further applications, it will be necessary to determine the thermal stability at higher temperatures, for example at the temperature optimum.

In the next step, the utilization of other substrates by MaL1 and SiLA was tested using wavelength scans. Both enzymes showed a similar substrate range, hinting at a similar redox potential. For most substrates MaL1 showed higher activites indicating that SiLA had a lower redox potential than MaL1. A next step would be to perform the corresponding assays to determine the enzyme activities quantitatively for each substrate.

The main part of this thesis was to find new laccases for lignosulfonate polymerization. MaL1 showed promising results at neutral pH (6 to 8) and additionally at pH 3.4, indicating two pH optima. Because of purification issues, the protein concentration of MaL1 was rather low. It will be necessary to redo these experiments with higher enzyme concentrations. The data obtained from SiLA was not as promising because the oxygen consumption during lignosulfonate polymerization was rather low, which indicated that SiLA is not that suitable for the application with lignosulfonates. Further testing under different conditions and by applying higher enzyme concentrations, will be required.

For further characterizations, it will be important to determine the degree of polymerization by size exclusion chromatography. Further measurements will show if these enzymes are applicable for lignosulfonate polymerizations and associated applications.

SiLA revealed a stability in storage buffer and showed a higher enzyme activity compared to MaL1. These properties could be essential for a wide variety of applications. Mal1 showed a high stability in diluted and undiluted form. Additionally, this fungal laccase was able to polymerize lignosulfonate especially at neutral to alkaline conditions. This is particularly interesting for industrial applications because technical lignin, such as kraft lignin, is only soluble at alkaline pH.

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Figures

Tables

Equations

Appendix

Amino acid sequences of designed fungal and bacterial laccases

Sequence of CgL1

APPSAASQRDLLVPIEERQEAAVLPRQTSCHTPSNRACWTTGYNINTDYEVNSPDTGVVRPYTFTLTEEENWTGPD
GVVKNKVMLINSMSCRPTIFADWGDTIQVTVINNLDTNGTSIHWHGMHQKDTNLHDGANGVTECPIPPGGRRV
YRFKAQQYGTSWYHSHFSAQYGNGVVGTIQINGPASLPYDIDLGVFPLMDYYYASADELVHFTMNNGAPFSDNVL
FNGTAKHPTTGAGQWANVTLTPGKRHRLRIINTSTENHFQVSLVNHTMTVIASDMVPVNAMTVDSLFLAVGQRY
DVTIDANRTPGNYWFNVTFGGQAFCGGSLNPTPAAIFHYAGAPGGLPTNRGVPPTDHQCLDLPNLTPVVTRNVPV
SGFVKRPGNTLPVNIDLSGTPLFVWKVNGSAINVDWGKPVADYVMTQNTSYPTSDNIVQVDGANQWTYWLVEN
DPDGPFSLPHPMHLHGHDFLVLGRSPDVSPASQQRFVFDPTVDLPRLRGTNPVRRDVTMLPPRGWLLLAFRADN
PGAWLFHCHIAWHVSGGLSVTFLERPNELRQRITPADRADFNRVCEEWREYWPTNPFPKVDSGLRHRMVEESEW
MVKAWSHPQFEK*

Green: Strep-tag

Molecular weight (MW): 67492.76 Da

Sequence of ToLA

GIGPVADLTITNAAVSPDGFSRQAVVVNGGTPGPLITGNKGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQKGTNW
ADGPAFVNQCPISAGNSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPSADLYDVDNDDTVITLVD
WYHVAARLGPAFPLGADATLINGKGRSPSTTTADLAVISVTAGKRYRFRLVSLSCDPNYVFSIDGHNMTIIETDSINT
QPLVVDSIQIYAAQRYSFVLDANQAVDNYWIRANPNFGNVGFTDGINSAILRYDGAAAVEPTTTQTTSTEPLNEVN
LHPLVATAVPGSPVAGGVDLAINMAFNFNGTNFFINGASFVPPTVPVLLQIISGAQSAQDLLPSGSVYSLPSNADIEI
SFPATTAAPGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAANDNVTIRFRTDNPGPWFLHCHIDFH
LDAGFAVVFAEDIPDVASANPVPQAWSDLCPTYDALDPSDQWSHPQFEK*

Green: Strep-tag

MW: 54491.54 Da

Sequence of MaL1

APPSTPAQRDLVELREARQEGGKDLRPREPTCNTPSNRACWSDGFDINTDYEVSTPDTGVTQSYVFNLTEVDNW
MGPDGVVKEKVMLINGNIMGPNIVANWGDTVEVTVINNLVTNGTSIHWHGIHQKDTNLHDGANGVTECPIPPK
GGQRTYRWRARQYGTSWYHSHFSAQYGNGVVGTIQINGPASLPYDIDLGVFPITDYYYRAADDLVHFTQNNAPPF
SDNVLINGTAVNPNTGEGQYANVTLTPGKRHRLRILNTSTENHFQVSLVNHTMTVIAADMVPVNAMTVDSLFLAV
GQRYDVVIDASRAPDNYWFNVTFGGQAACGGSLNPHPAAIFHYAGAPGGLPTDEGTPPVDHQCLDTLDVRPVVP
RSVPVNSFVKRPDNTLPVALDLTGTPLFVWKVNGSDINVDWGKPIIDYILTGNTSYPVSDNIVQVDAVDQWTYWLI
ENDPEGPFSLPHPMHLHGHDFLVLGRSPDVPAASQQRFVFDPAVDLARLNGDNPPRRDTTMLPAGGWLLLAFRT
DNPGAWLFHCHIAWHVSGGLSVDFLERPADLRQRISQEDEDDFNRVCDEWRAYWPTNPYPKIDSGLKRRRWVEE
SEWLVRWSHPQFEK*

Green: Strep-tag

MW: 67868.71 Da

Sequence of SiLA

MAGDAKGVTARTAPAGGEVRHIKMYAEKLPDGQMGYGLEKGKASVPGPLIELNEGDTLHIEFTNTMDVRASLHV
HGLDYEISSDGTAMNKSDVEPGGTRTYTWRTHKPGRRADGTWRAGSAGYWHYHDHVVGTEHGTGGIRKGLYGP
VIVRRKGDVLPDATHTIVFNDMLINNRAPHTGPNFEATVGDRVEIVMITHGEYYHTFHMHGHRWADNRTGMLT
GPDDPSQVIDNKITGPADSFGFQIIAGEGVGAGAWMYHCHVQSHSDMGMVGLFLVKKPDGTIPGYDPHEHVHG
GGEPTADAPAHQHWSHPQFEK*

Green: Strep-tag

MW: 34241.16 Da

Sequence of SiLA SBD

MAGDAKGVTARTAPAGGEVRHIKMYAEKLPDGQMGYGLEKGKASVPGPLIELNEGDTLHIEFTNTMDVRASLHV
HGLDYEISSDGTAMNKSDVEPGGTRTYTWRTHKPGRRADGTWRAGSAGYWHYHDHVVGTEHGTGGIRKGLYGP
VIVRRKGDVLPDATHTIVFNDMLINNRAPHTGPNFEATVGDRVEIVMITHGEYYHTFHMHGHRWADNRTGMLT
GPDDPSQVIDNKITGPADSFGFQIIAGEGVGAGAWMYHCHVQSHSDMGMVGLFLVKKPDGTIPGYDPHEHVHG
GGEPTADAPAHQHPPGGNRGTTTTRRPATTTGSSPGPAFTCTATTASNYAHVQAGRAHDSGGIAYANGSNQSM
GLDNLFYTSTLAQTAAGYYIVGNCPWSHPQFEK*

Green: Strep-tag

Orange: Linker region of Cellobiohydrolase I from *Trichoderma. Reesei*

Blue: Substrate binding domain of polyhdydroxyalkanoate depolymerase from *Alcaligenes faecalis*

MW: 42796.43 Da

Sequence of SiLA CBD

MAGDAKGVTARTAPAGGEVRHIKMYAEKLPDGQMGYGLEKGKASVPGPLIELNEGDTLHIEFTNTMDVRASLHV
HGLDYEISSDGTAMNKSDVEPGGTRTYTWRTHKPGRRADGTWRAGSAGYWHYHDHVVGTEHGTGGIRKGLYGP
VIVRRKGDVLPDATHTIVFNDMLINNRAPHTGPNFEATVGDRVEIVMITHGEYYHTFHMHGHRWADNRTGMLT
GPDDPSQVIDNKITGPADSFGFQIIAGEGVGAGAWMYHCHVQSHSDMGMVGLFLVKKPDGTIPGYDPHEHVHG
GGEPTADAPAHQHPPGGNRGTTTTRRPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCLW
SHPQFEK*

Green: Strep-tag

Orange: Linker region of Cellobiohydrolase I from *Trichoderma. Reesei*

Blue: Carbohydrate binding domain of Cellobiohydrolase I from *Hypocrea jecorina*

MW: 40274.76 Da

Colony PCR agarose gels

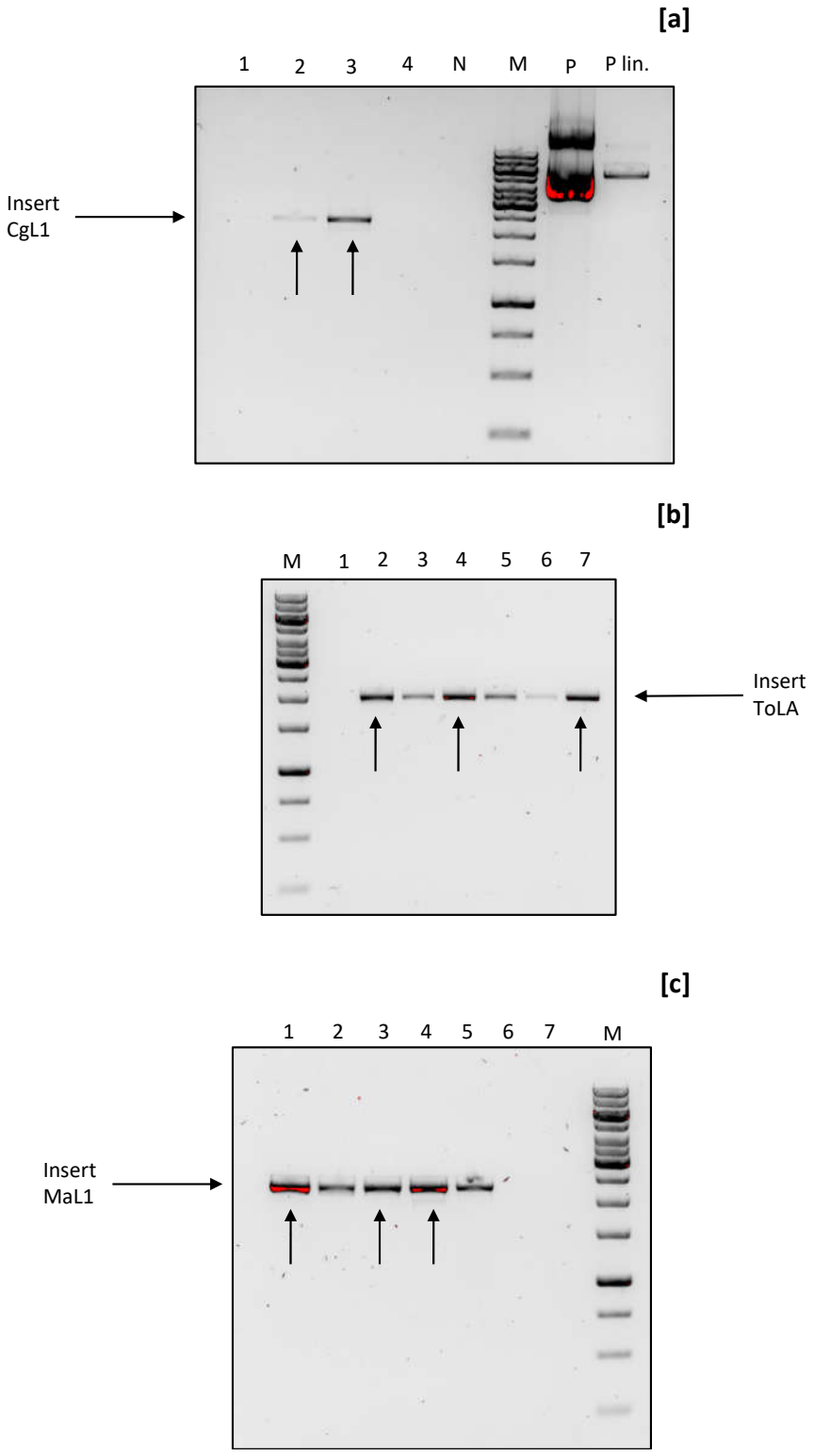


Figure 18: Colony PCR of transformed clones. [a] CgL1; [b] ToLA; [c] MaL1; Arrows indicate the P. pastoris clones used for expression

Expression and purification of laccases

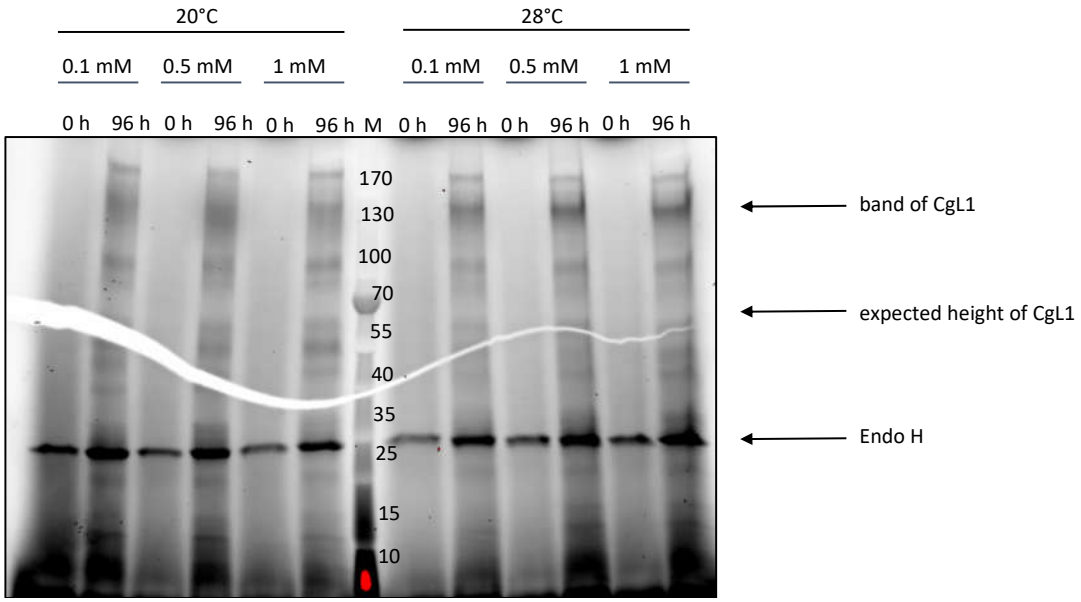


Figure 19: CgL1 expression at 20 °C and 28 °C for 96 hours with varying copper concentrations (0.1 mM, 0.5 mM and 1 mM). SDS-PAGE of deglycosylated CgL1 time points collected at the beginning and after 96 hours of induction. Lane M shows the peqGold protein marker IV in kDa.

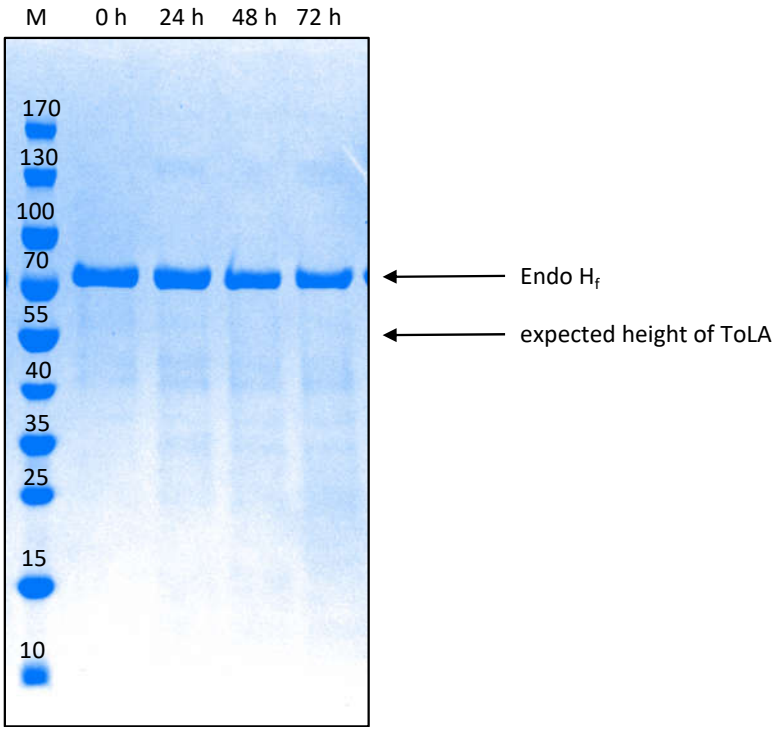


Figure 20: ToLA expression at 28 °C. SDS-PAGE of deglycosylated ToLA time points of expression at 20 °C. Lane M shows the peqGold protein marker IV in kDa.

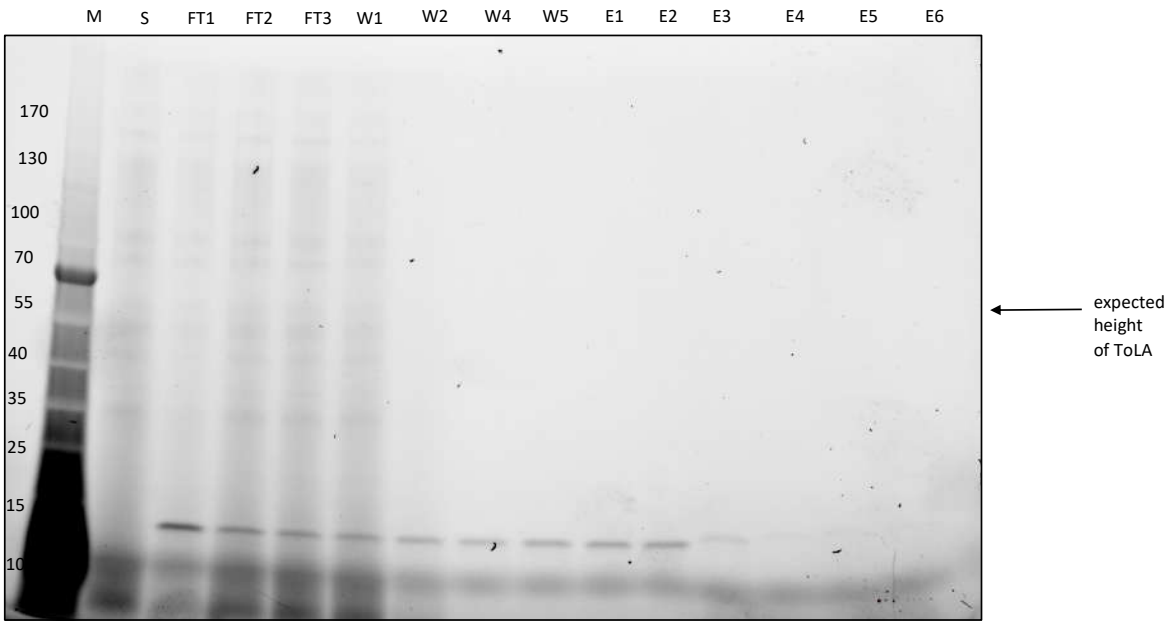


Figure 21: SDS-PAGE of ToLA purification fractions. Lane S shows the supernatant, whereas lane FT1 – FT3 show the flow-through fractions. Lanes W1-W5 show wash fraction and lane E1-E6 show the elution fractions of the purification. Lane M shows the peqGold protein marker IV in kDa.

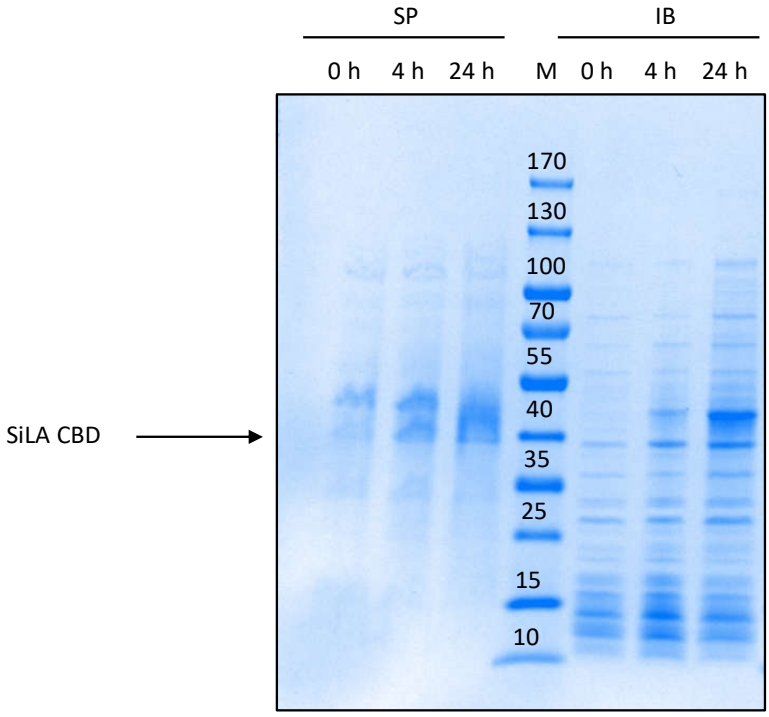


Figure 22: SDS-PAGE of expression timepoints of SiLA CBD at 25 °C. The expression timepoints were taken during the expression at 25 °C. The gel consists of two parts. SP refers to the soluble protein, which was gained through cell lysis and IB refers to inclusion bodies. The lanes indicate the timepoints in hours. Lane M shows the peqGold protein marker IV in kDa.

Enzyme characterization

Table 16: Raw data of pH optimum determination

	enzyme activity [U/mg]		STD DEV [U/mg]	
pH	**MaL1**	**SiLA**	**Mal1**	**SiLA**
3	1.958259	2.465616	0.04799	0.114923
4	2.06372	5.862703	0.061831	0.371973
5	2.012598	6.976043	0.051439	0.269926
6	1.37919	1.937477	0.03099	0.020707
7	0.73662	0.588358	0.024887	0.044022
8	0.405008	0.20869	0.07215	0.007363

Table 17: Raw data of temperature optimum determination

	enzyme activity [U/mg]		STD DEV [U/mg]	
T [°C]	**MaL1 [pH 4]**	**SiLA [pH 5]**	**Mal1**	**SiLA**
20	1.3536191	1.780677	0.041249	0.402885
30	1.8745071	2.9004841	0.10545	0.084125
40	2.7023552	3.7554189	0.042777	0.120435
50	3.4350328	4.6234661	0.164881	0.177033
60	2.6591518	6.0632182	0.157748	0.27303
70	1.140194	7.6498302	0.377532	0.178426

Raw data of enzyme stability

Table 18: Raw data of enzyme stability in storage buffer at room temperature

MaL1				SiLA			
time [h]	**U/mg**	**STD DEV**	**%**	**U/mg**	**STD DEV**	**%**	**time [h]**
0.166667	1.028181	0.034267	100	4.296411	0.085746	100	0.166667
0.5	1.059387	0.032642	103.0351	4.412009	0.174223	102.6906	1
1				4.385036	0.308365	102.0628	2
2	1.109284	0.013619	107.8881	4.528571	0.266864	105.4036	4
6	1.159424	0.027773	112.7646	4.07292	0.163923	94.79821	6
24	0.871223	0.014728	84.73438	3.552727	0.115031	82.69058	24
48	0.783602	0.01935	76.21245	2.969918	0.089604	69.12556	48
72	0.700885	0.019316	68.16749	3.264694	0.13482	75.98655	72
				2.654912	0.08772	61.79372	96
144	0.558202	0.013881	54.29022				144
				2.021047	0.020503	47.04036	168

Table 19: Raw data of enzyme stability diluted in their respective optimal buffer at room temperature

MaL1 1:10 (pH 4)				SiLA 1:100 (pH 5)			
time [h]	**U/mg**	**STD DEV**	**%**	**U/mg**	**STD DEV**	**%**	**time [h]**
0.166667				4.219345	0.208938	100	0.166667
0.5	0.697795	0.017811	100	4.046911	0.071805	95.91324	1
1				4.09026	0.115791	96.94064	2
2	0.465171	0.006469	66.66299	3.683739	0.192401	87.30594	4
6	0.524261	0.075836	75.13101	3.317677	0.151165	78.63014	6
24	0.305191	0.00657	43.73642	1.7754	0.051724	42.07763	24
48	0.275761	0.003423	39.51886	0.957541	0.046269	22.69406	48
72	0.293991	0.007562	42.13147	0.565469	0.025902	13.40183	72
				0.324639	0.021691	7.694064	96
144	0.264436	0.007819	37.89598				144
				0.110782	0.019242	2.625571	168

Enzyme kinetics

Michaelis-Menten Plots

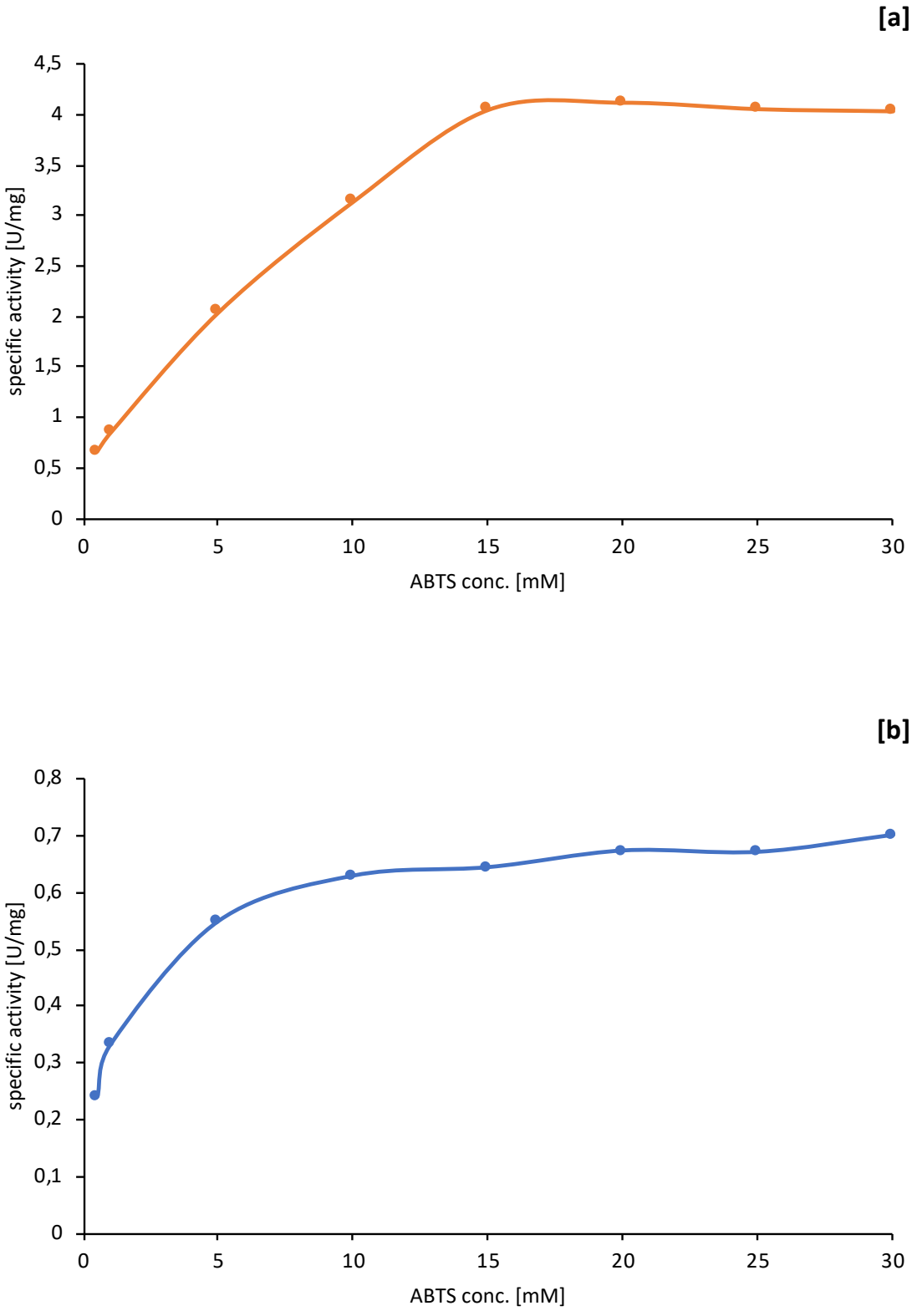


Figure 23: Hanes-Woolf plots of [a] SiLA and [b] MaL1. Conducted with ABTS concentrations ranging from 0.5 mM to 30 mM

Raw data enzyme kinetics

Table 20: Raw data of enzyme kinetics of SiLA

ABTS conc. [mM]	**actual ABTS concentration**	**specific enzyme activity [U/mg]**			**mean value [U/mg]**
0.5	0.113636	0.702261	0.618452	0.687811	0.669508
1	0.227273	0.817859	0.814969	0.930568	0.854466
5	1.136364	2.187702	1.970954	1.985404	2.048020
10	2.272727	3.785851	2.858173	2.791704	3.145242
15	4.467882	4.242465	3.863880	4.438982	4.053172
20	4.545455	4.777108	3.884109	3.710712	4.123976
25	5.681818	4.089297	4.031498	3.320567	4.060397
30	6.818182	3.748281	3.589333	4.777108	4.038241

Table 21: Raw data of enzyme kinetics of MaL1

ABTS conc. [mM]	actual ABTS concentration	specific enzyme activity [U/mg]			mean value [U/mg]
0.5	0.113636	0.257089	0.233792	0.232565	0.241149
1	0.227273	0.333521	0.332295	0.333521	0.333112
5	1.136364	0.52971	0.576304	0.54238	0.549465
10	2.272727	0.624125	0.633526	0.716089	0.628826
15	3.409091	0.647014	0.640066	0.621673	0.64354
20	4.545455	0.673581	0.671946	0.615542	0.672764
25	5.681818	0.666633	0.681756	0.663363	0.670584
30	6.818182	0.712002	0.68053	0.708323	0.700285

Table 22: Raw data of Hanes-Woolf plot of SiLA

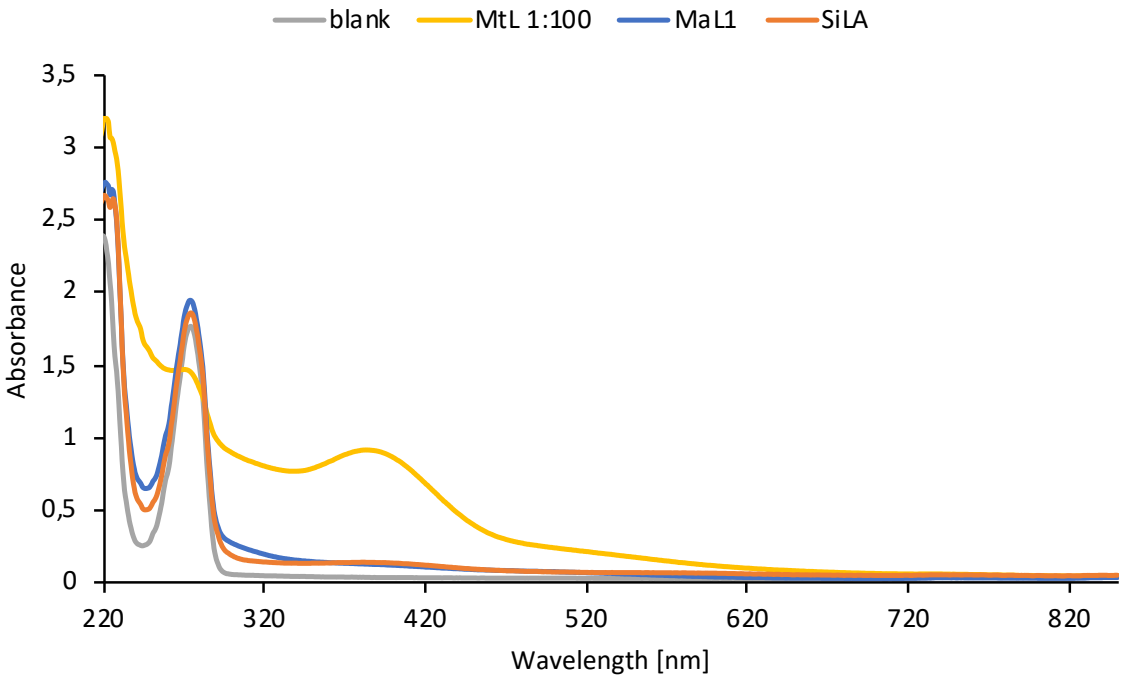
ABTS stock [mM]	[S] [mM]	[v] [µmol/min]	[S]/v
0.5	0.113636	0.669508	0.169731
1	0.227273	0.854466	0.265982
5	1.136364	2.048020	0.554860
10	2.272727	3.145242	0.722592
15	4.467882	4.053172	1.102317
20	4.545455	4.123976	1.102202
25	5.681818	4.060397	1.399326
30	6.818182	4.038241	1.688404

Table 23: Raw data of Hanes-Woolf plot of MaL1

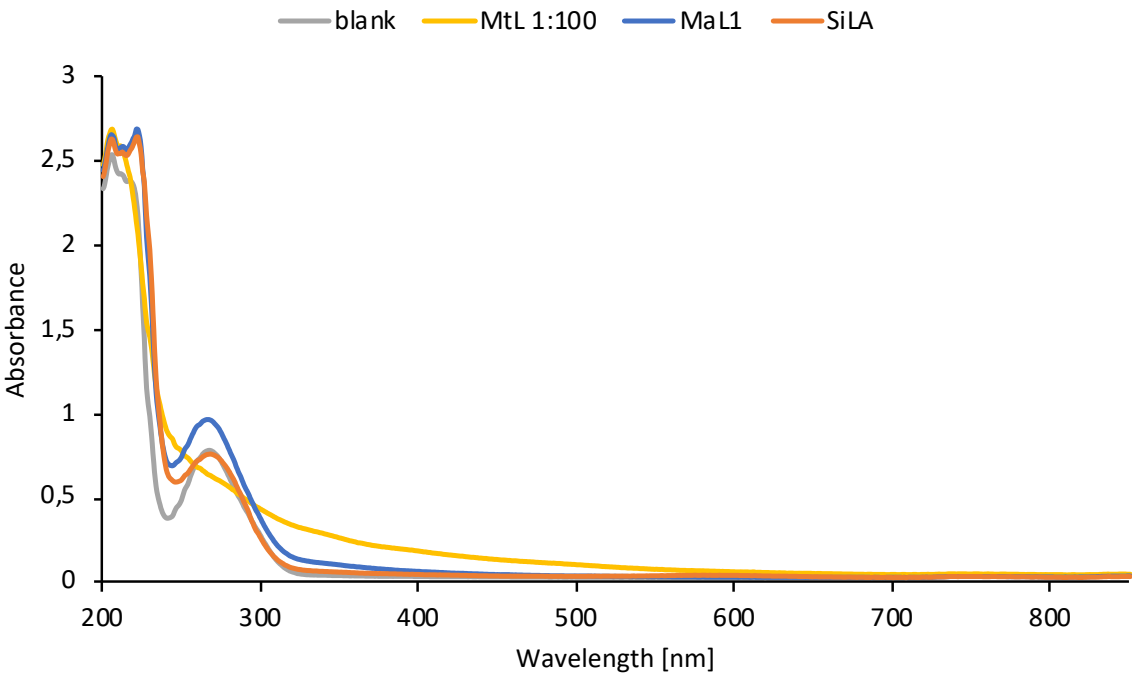
ABTS stock [mM]	[S] [mM]	[v] [µmol/min]	[S]/v
0.5	0.113636	0.241149	0.471229
1	0.227273	0.333112	0.682271
5	1.136364	0.549465	2.068128
10	2.272727	0.628826	3.61424
15	3.409091	0.64354	5.297403
20	4.545455	0.672764	6.756388
25	5.681818	0.670584	8.472939
30	6.818182	0.700285	9.736297

Substrate screening

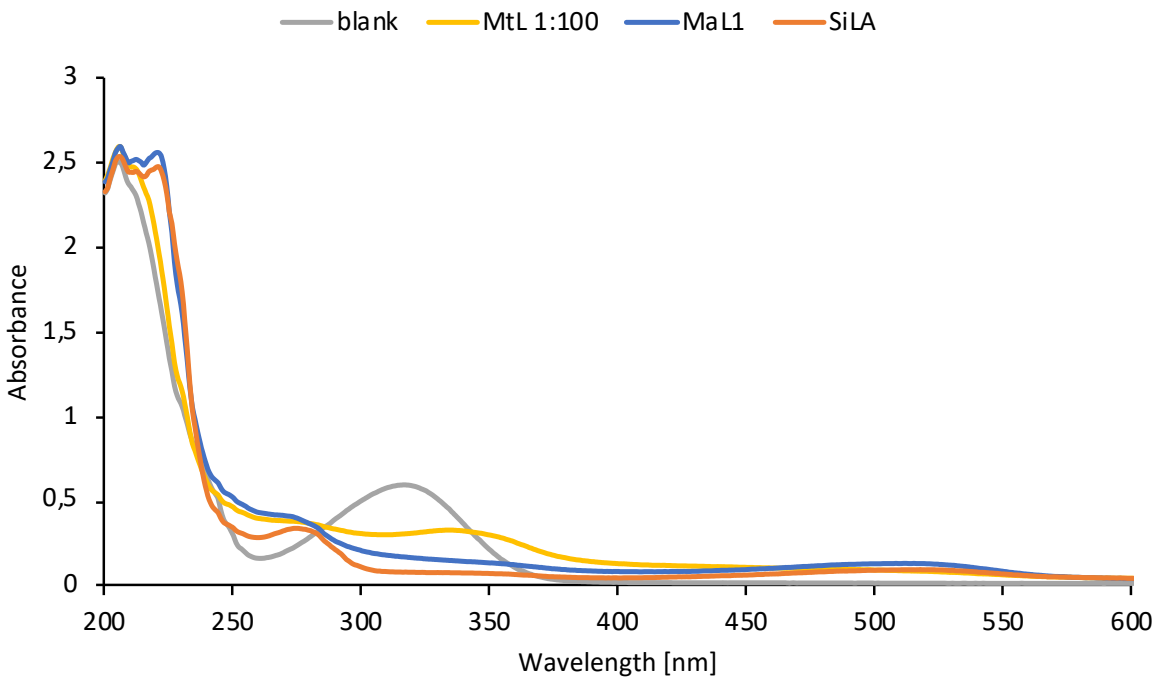
[a]



[b]



[c]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SiLA
Absorbance
Wavelength [nm]
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400
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500
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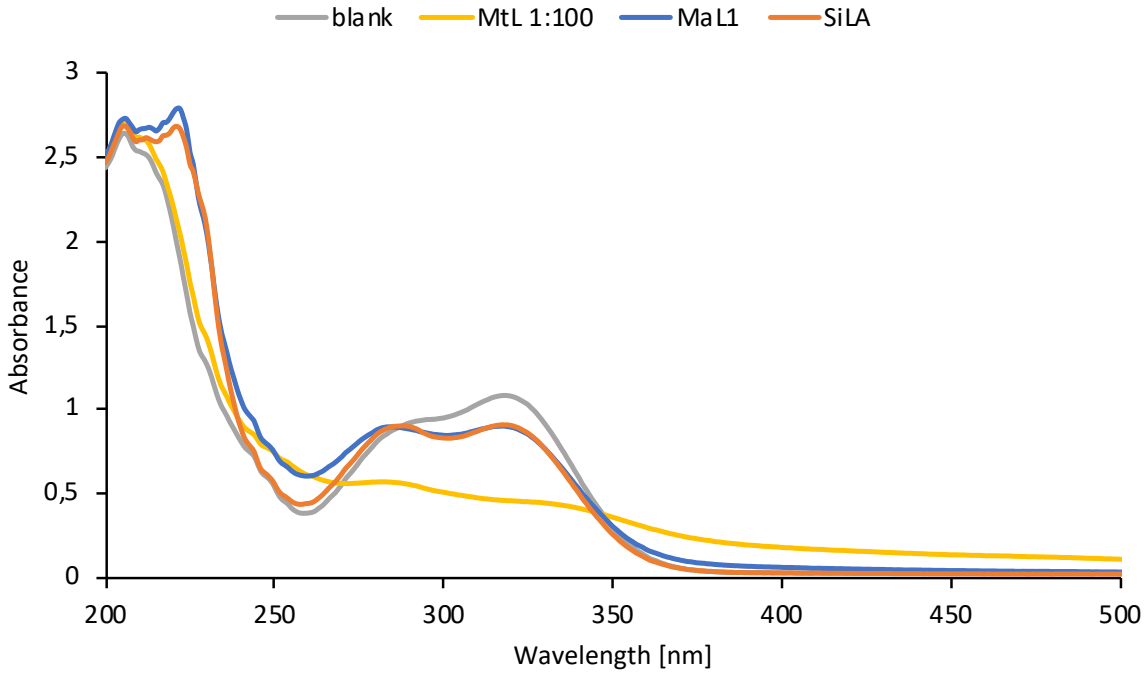
600

[d]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MaL1
SiLA
Absorbance
Wavelength [nm]
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[e]

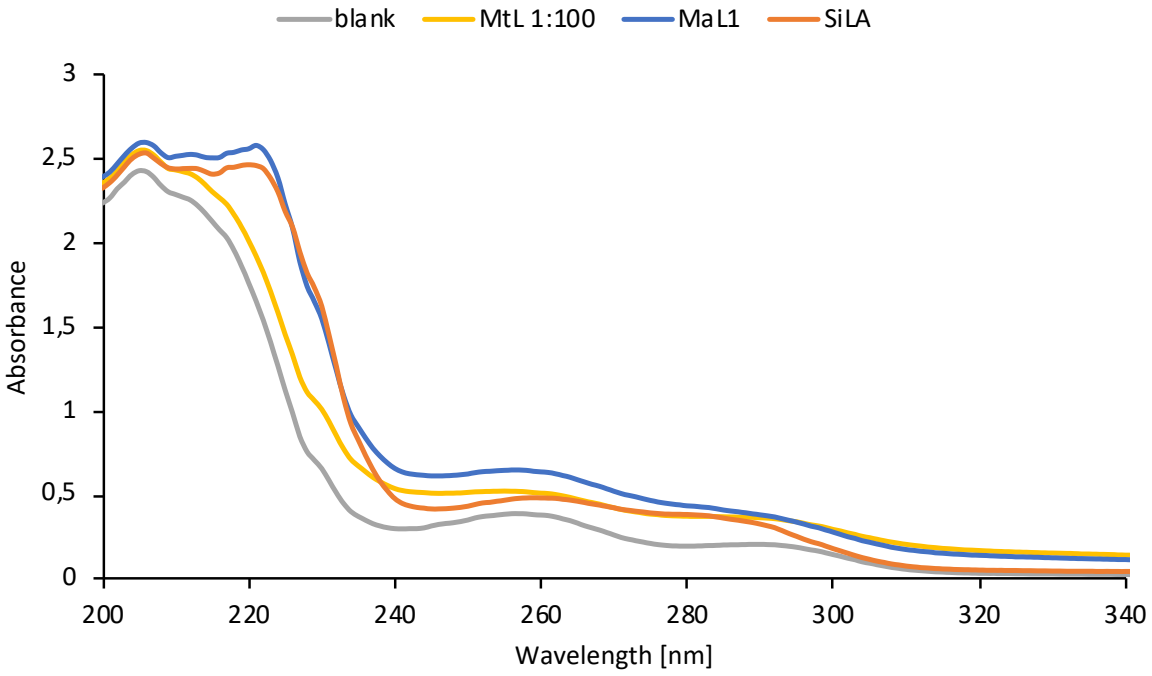

Figure 24: Oxidation of [a] Catechol, [b] Gallic acid, [c] Sinapic acid [d] Ferulic acid and [e] Vanillic acid with MtL diluted 1:100, MaL1 and SiLA