**University of Natural Resources and Life Sciences**

**Department for Agrobiotechnology, IFA-Tulln**

**Institute for Environmental Biotechnology**

Master Thesis

# Characterization and modification of a laccase from *Aspergillus flavus* for application as antioxidant biosensor

*Author:*

**Sarah Fürst, BSc**

Master Program Biotechnology

*Supervisor:*

**Univ.Prof. Dipl.-Ing. Dr.techn. Georg M. Gübitz**

Institute for Environmental Biotechnology

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## Abstract

Oxidative stress related diseases like cardiovascular and neurodegenerative diseases (Alzheimer's disease, Parkinson) are increasing in number in the last years. Consequently, measurement of the antioxidant capacity of human plasma and blood is gaining importance in the clinics. Assays using peroxidases and $H_2O_2$ for oxidizing ABTS to the stable radical $ABTS^+$ are known. The radical is reduced by antioxidants present in the biological samples, which results in a decrease in absorbance of the solution, and antioxidant capacity can be evaluated.

Unlike peroxidases, laccases do not need $H_2O_2$ as a cofactor for oxidation reactions. The laccase 1 from *Aspergillus flavus* (*AfLacc1*) and its variant *AfLacc1:CBD* (*AfLacc1* fused with a cellulose-binding-domain) were heterologously expressed in *Pichia pastoris* and biochemically characterized. Optimal reaction conditions were determined to be pH 3.4 and 40°C for both enzymes with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as substrate. Furthermore, kinetic parameters were evaluated and showed $K_M$ values of 1.27 mM (*AfLacc1*) and 0.71 mM (*AfLacc1:CBD*) as well as $V_{max}$ values of 9.4 U/mg (*AfLacc1*) and 16 U/mg (*AfLacc1:CBD*).

Besides that, the heterologously expressed enzyme *AfLacc1:CBD* proved to be highly substrate specific for ABTS, and no interactions with human plasma antioxidants and amino acids occurred. Hence, *AfLacc1:CBD* is very promising for the application as antioxidant biosensor. Experiments to proof the concept were performed.

Linear correlation between the amount of oxidized ABTS and the absorbance at 420 nm as well as a between the amount of Trolox added to a solution of ABTS radical and the resulting decrease in absorbance were observed. Trolox was used as reference antioxidant to compare it to other human antioxidants.

Moreover, first experiments with human plasma samples were performed which showed no significant matrix effects interfering with the assay.

## Keywords

Laccase, *Aspergillus*, Oxidative Stress, Antioxidant capacity, Biosensor

## Kurzfassung

Oxidativer Stress wird häufig mit vielen sogenannten Zivilisationskrankheiten wie Herz-Kreislauf, oder neurodegenerativen Krankheiten (Parkinson, Alzheimer) in Verbindung gebracht. Das Ungleichgewicht zwischen reaktivem Sauerstoff (und Stickstoff) Radikalen und Antioxidanten kann zu Zell- und daraus folgenden Gewebeschäden führen, daher gehört das Testen des Antioxidantienstatus zur klinischen Routine. Assays, in denen das durch Oxidation mit Peroxidasen und $H_2O_2$ hergestellte Radikal $ABTS^+$, welches eine stark grüne Farbe aufweist, durch Antioxidantien reduziert und somit entfärbt wird, sind üblich.

Anders als Peroxidasen benötigen Laccasen, die ebenfalls zu Gruppe der Oxidoreduktasen gehören, keine Cofaktoren wie $H_2O_2$ für Oxidationsreaktionen. Die Laccase 1 aus *Aspergillus flavus* (*AfLacc1*) und seine Variante *AfLacc1:CBD* (*AfLacc1* wurde mit eine Cellulose Bindungsdomäne ergänzt) wurden heterolog *Pichia pastoris* exprimiert und biochemisch charakterisiert. Die exprimierte Laccase 1 von *Aspergillus flavus* (*AfLacc1*) weist mit einem pH Wert von 3.4 und einer optimalen Temperatur von 40 °C die für saure Laccasen typischen Reaktionsbedingungen auf. Ebenso konnte ein für die Kupferzentren von Laccasen typisches UV/VIS Spektrum aufgenommen werden. Im Unterschied zu dem Großteil bisher bekannter Laccasen, ist *AfLacc1* jedoch hoch substratspezifisch für ABTS. Bei keinem der getesteten typischen Substrate konnte eine Oxidation durch das Enzym gezeigt werden. Zusätzlich wurde das Enzym mit einer Cellulose-Bindugsdomäne ergänzt, um es spezifisch an Cellulose immobilisieren zu können und somit die Stabilität sowie die Wiederverwendbarkeit des Enzyms zu verbessern.

Es konnte in dieser Arbeit gezeigt werden, dass das Enzym keine Wechselwirkungen mit wichtigen körpereigenen Antioxidantien aufweist. Ein linearer Zusammenhang zwischen der Menge an oxidiertem ABTS und der daraus resultierenden Absorbtion bei 420 nm besteht, sowie zwischen der Abnahme der Absorbtion bei Zugabe definierter Mengen des Referenzantioxidants Trolox. Außerdem konnten erste Versuche mit humanen Plasma Proben zeigen, dass keine Matrixeffekte negativ auf den Assay einwirken, jedoch die Konzentration an Antioxidantien im Plasma zu hoch ist um sie unverdünnt zu verwenden. Dies stellt auf Grund des erhöhten Arbeitsaufwands einen klaren Nachteil gegenüber bereits verfügbaren Assays dar.



## Schlagworte

Laccase, Aspergillus, oxidativer Stress, Biosensor

# Table of Contents

# 1 Introduction

The following work can be thematically divided into two parts, that are linked to one another by the laccase *AfLacc1* and its variant *AfLacc1:CBD*. In the first part the laccases were engineered, heterologous expressed and characterized. Following that, variant *AfLacc1:CBD* was immobilized on a cellulosic support material. The characteristics and immobilization ability of the enzyme directly influenced the application as an antioxidant biosensor and further experiments. **Figure 1** shows a graphical abstract of the thesis. It shows the two distinguished parts, but also their connections.

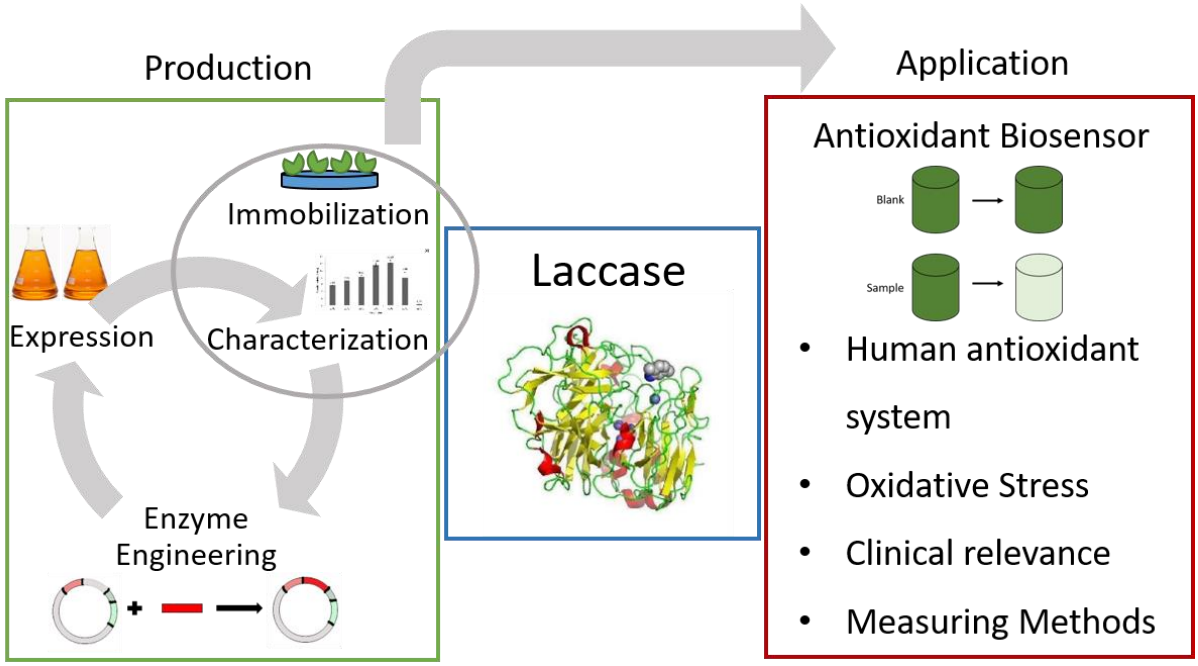



**Figure 1: Graphical abstract**: on the left side the production steps of the enzyme are shown, the characterization and immobilization results directly influence the application, which is shown by the grey arrow. On the right side important points to be considered for the application are listed.

For the theoretical part of this work the first topic addresses the reaction mechanism of laccases and heterologous expression and possibilities to engineer and immobilize them to improve their functions. The second part explains the application of the expressed laccase as an antioxidant biosensor by giving an overview about the importance of antioxidants for avoiding oxidative stress in the human body and already existing measuring methods of human serum antioxidants.

## 1.1 Laccases

Laccases (benzendiol:oxygen oxidoreductases, E.C. 1.10.3.2) belong to the multicopper oxidases (MCO) among which they represent the largest subgroup [1]. The first laccase was discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* [2] at the end of the 19$^{th}$ century. Only few years later laccases were also observed in fungi and got more attention after observation of their ability to enzymatically degrade wood in white-rot and wood-rotting fungi [3].

This class of enzymes is able to oxidize various substances such as phenols, aminophenols, polyphenols, polyamines, methoxy phenols, aromatic amines and thiols, and also some inorganic substrates [4]. Their broad substrate range makes laccases interesting for many biotechnological purposes in the textile, dye or printing industry as well as in the pulp and paper industry [5]. Furthermore, laccases are employed for medicinal purposes and as biosensors [6].

Not only fungi, but many other organisms like plants, prokaryotes and arthropods are known to produce laccases with varying functions in the different organisms [7]. In plants they are located in wood and cellular walls of herbaceous species, and play a role in lignin biosynthesis [8], [9]. Bacterial laccases are involved in morphogenesis as well as in the biosynthesis of the brown spore pigment and protection against UV light and hydrogen peroxide [10]. Fungal laccases take part in morphogenesis, plant pathogen/host interactions, stress defence and lignin degradation [11].

**Reaction mechanism**

Laccases catalyze the oxidation of a wide range of substrates by utilizing atmospheric oxygen as electron donor which is reduced to water while a phenol is oxidized to form a radical [3][12][13]. There are six groups of enzymes able to perform that type of oxygen reducing reaction, namely cytochrome-c oxidases, l-ascorbate oxidases, ceruloplasmins, bilirubin oxidases, phenoxazinone synthases and laccases [1].

A minimum of 4 copper atoms per active protein unit is necessary for the catalytic activity of laccases (see **Figure 2**). Three types of copper can be found in the active site of laccases. Type 1 is the paramagnetic “blue” copper and shows absorbance at 610 nm (ox.) which results from the intense electronic absorption that is caused by a covalent copper-cysteine bond. Additionally one methionine and two histidine molecules are coordinated to the T1 copper. The redox potential of an enzyme is dependent on the T1 copper [14]. Type 2 copper shows no absorption in the visible spectrum but shows paramagnetic properties in EPR (electro paramagnetic resonance) studies. It is coordinated by 2 histidines and can bind one water molecule. Type 3 copper is coordinated by 3 histidines. A hydroxyl-

bridge connects two of the histidines to form a binuclear center. This hydroxyl-bridge shows a weak UV (ultraviolet) absorbance at 330 nm [15][16]. Together, type 2 and type 3 copper form a so called trinuclear cluster (see **Figure 2**), which is characteristic not only for laccases but also for other multicopper-enzymes like ascorbate oxidases or ceruloplasmin. This similarity has been proven by detailed spectroscopic studies as well as X-ray crystallography [13]. The hydrogen bonds and salt bridges between the four copper atoms in the active site of a laccase define the stability of the enzyme [17].

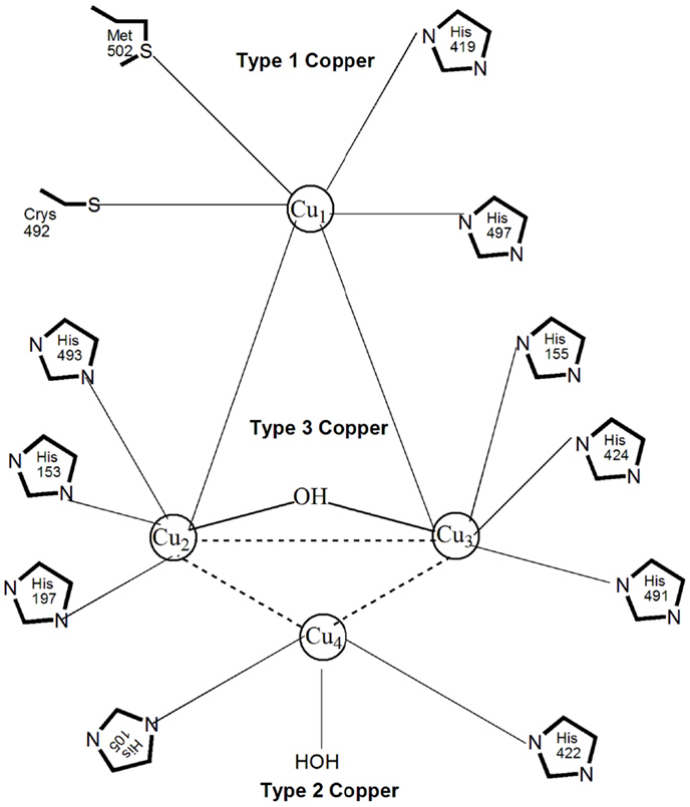



**Figure 2: Laccase Structure:** The copper coordination network of laccase [4]

These copper centers drive the electron transport from a reducing substrate to molecular oxygen without releasing any toxic peroxide intermediates [12]. **Figure 3** shows one catalytic cycle of substrate oxidation by laccase. It has been widely discussed and demonstrated that the T1 copper acts as the primary redox center accepting electrons from the substrates. Thereby the oxidized laccase undergoes four fast single electron transfers, oxidizing one substrate at a time, which means that four substrate molecules are needed to fully reduce a laccase. Molecular oxygen reacts via a fast 2-step-electron-transfer process that takes place at the fully reduced T2/T3 cluster, resulting in peroxide formation. The peroxide is tightly bound to the T2/T3 cluster, thereby release of peroxide is prevented. The fully oxidized laccase comprises the µ-oxo-bridged trinuclear T2/T3 site, and is thermodynamically stable which enables the laccase to perform further catalytic processes [7].

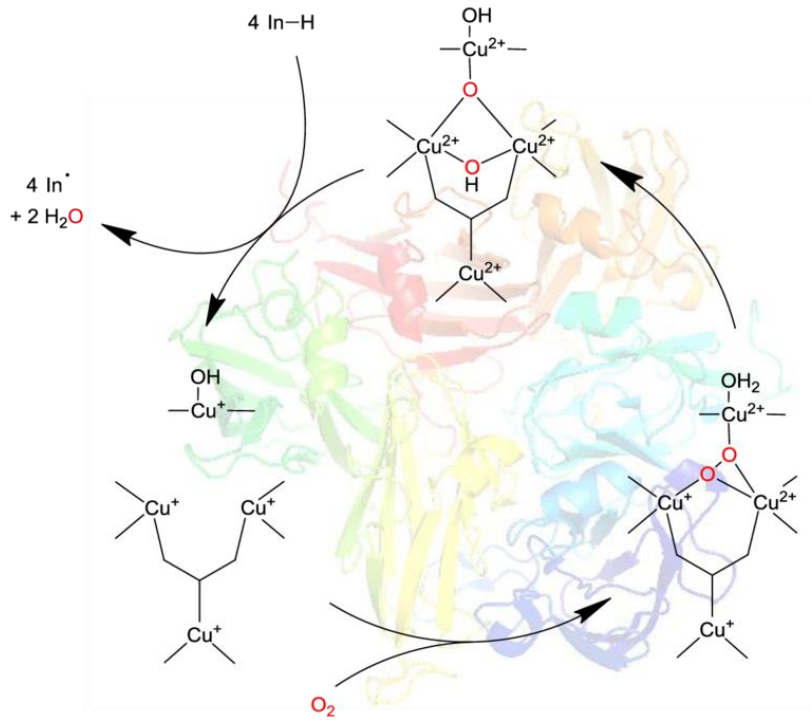



**Figure 3: Reaction mechanism of laccase:** Schematic mechanism for one catalytic cycle of radical formation and substrate oxidation by laccase [7]

### 1.1.1 Fungal laccases

Laccases play different roles in fungi: in morphogenesis, plant-pathogen/host interactions, stress defence and lignin degradation [18]. Typical laccases producers are wood-rotting basidiomycetes and ascomycetes which are capable to degrade lignin [19]. Among the division of ascomycetes, laccase production occurs mostly in white-rot and brown-rot fungi such as the *Aspergillus* species [20]. Laccases originating from brown-rot fungi are used for industrial and biotechnological applications, for example a laccase from *Aspergillus ochraceus* for the degradation of textile dyes [21], [22]. Fungal laccases are usually glycosylated. Therefore eukaryotic expression systems, which are able to perform these complex post-translational modifications, are needed. The high levels of expression of laccases in *Aspergillus niger* and *Aspergillus oryzae* was exploited for heterologous production of the enzymes [19]. However, the most common expression systems for fungal laccases are *Saccharomyces cerevisiae* and *Pichia pastoris* [23],[24]. Fungal laccases typically have pH optima at acidic pH values (pH 3-4), an optimal reaction temperature at around 50 °C [25], and typical activities (with ABTS as a substrate) are reported to range between 4 and 100 U mL-1 [26].

**_Aspergillus_ as source for new laccases**

*Aspergilli* belong to the Fungi kingdom and the division of Ascomycota and were first catalogued by Pier Antonio Micheli. A few hundred species are known to date. Approximately a third of *Aspergillus* species are capable of sexual reproduction but the asexual spore forming type is more common [27].

There are no publications about recombinant expression of laccases from *A. flavus* to date, but the role of *A. flavus* laccases in wood rotting and mycotoxin degradation has already been shown. Laccases from other *Aspergillus* species that were heterologously expressed are already described, for example a novel laccase from *A. niger* expressed in *Saccharomyces cerevisiae* or a laccase purified from *A. nidulans* [28]. *Aspergillus nidulans* was one of the first *Aspergillus* species used as research organism and also one of the first to have its genome sequenced. Until 2008 eight *Aspergillus* species have been sequenced, including *Aspergillus flavus* [29].

### 1.1.2 Laccase engineering

Due to the wide range of biotechnological applications of laccases, the demand for industrial-scale production of stable and active enzymes is increasing. To reach that goal with unmodified native laccases in wild-type fungal strains is unrealistic. Yields are too low and the demand for specialized enzymatic reactions cannot be satisfied [30]. Protein engineering can be the solution for efficient biocatalysis and high expression levels [31]. However, engineering of fungal laccases is challenging: While ascomycete fungi are more susceptible to genetic manipulations, the high-redox potential laccases interesting for industrial processes exist mainly in basidiomycete fungi [32][33].

**Fusion of binding domains**

Carbohydrate binding domains have been known for centuries, first they were discovered in (hemi) cellulases, but have recently been discovered in other enzyme classes, like chitinases [34]–[36]. Binding domains play an important role in the controlled adsorption and desorption of enzymes during reactions with polymers. Binding domains are linked to catalytic domains through spacers [37] and can improve the interaction between the enzyme and the polymer in two ways: Firstly, fusion of binding domains results in improved adsorption of active enzyme onto the surface of the polymer. Secondly they enable the hydrolysis of insoluble polymers by making targeted bonds more accessible to the catalytic domain through disruption of polymer structures. [38]–[41]. The improvement of polymer to enzyme interactions become noticeable through an increased enzyme activity.

### 1.1.3 Enzyme immobilization

Immobilization of enzymes to solid supports has received great attention over the last decades. Immobilized enzymes are understood as enzymes, physically attached to a specific solid support material. Thus they can be repeatedly and continuously used, while they maintain their catalytic activity [42]. The increased interest in immobilized enzymes comes from multiple advantages for applications in biotechnology, such as reusability, increase in enzyme stability, reaction control through easy separation of the enzyme from the reaction mixture, as well as the fast separation of the enzyme from a product [43][44].

A variety of methods to immobilize enzymes on solid supports is available, such as covalent bonding, entrapment or encapsulation, cross-linking and adsorption [45]. For this thesis the enzyme was fused with a cellulose-binding-domain (CBD) and immobilized on a cellulose support through adsorption.

Adsorption represents a straightforward immobilization method, based on electrostatic or hydrophobic interactions or Van der Waals forces [46]. The immobilization procedure is easy and fast and schematically shown in Figure 4. The enzyme is incubated with the solid support for a defined period of time under suitable reaction conditions, to ensure the stability of the enzyme. The non-adsorbed enzyme is washed away from the support with buffer, leaving only the support material with the attached enzyme. The drawbacks of this technique are the weak bonds that bind the enzyme, which can be broken by a change in temperature, pH or ionic strength [47]. Furthermore, non-specific binding of other substances or proteins can occur.

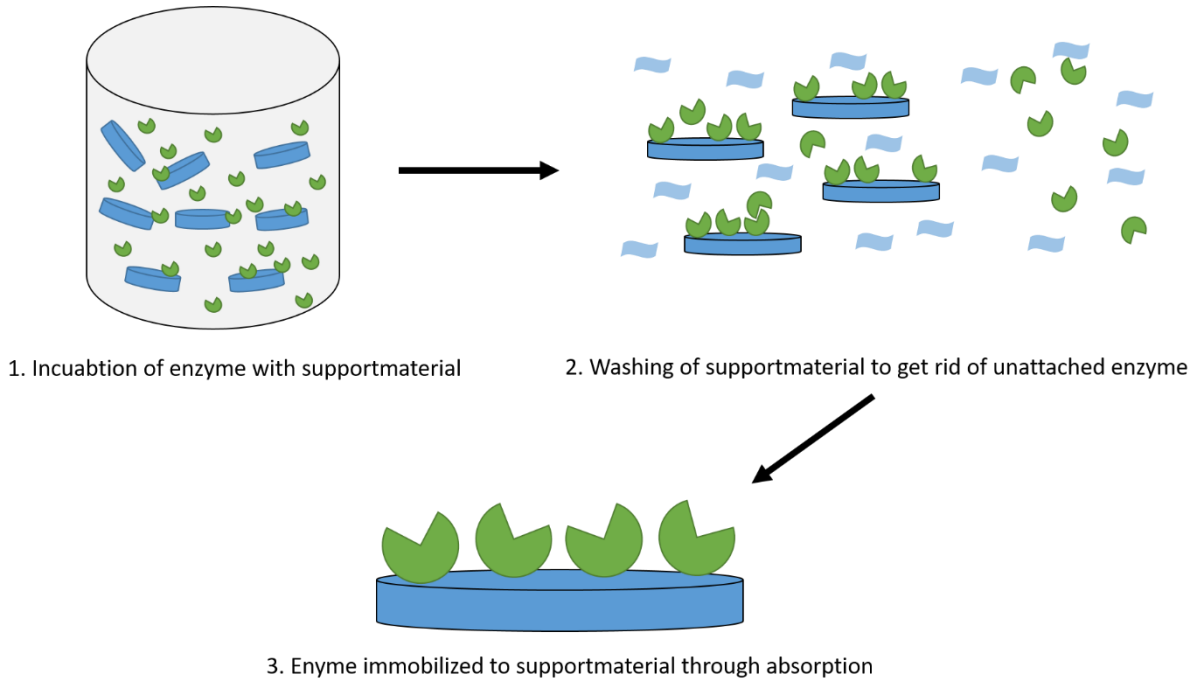



Figure 4: Enzyme immobilization: Procedure of enzyme immobilization through adsorption.

## 1.2 Recombinant enzyme expression in *Pichia pastoris*

The methylotrophic yeast *P. pastoris*, also called *Komagatella pastoris*, plays an important role in many biotechnological applications and in research for the heterologous production of proteins [48]. *P. pastoris* was first used for the production of single-cell proteins (SCP) as feedstock, because the synthesis from methane to methanol was cheap and effective in the late 1960s. When the price for methane increased in the following decade and simultaneously the price for soybeans (major feedstock source) decreased, the production of SCP using *P. pastoris* became economically irrelevant [49]. In the 1980s the first system for recombinant protein expression with *P. pastoris* was developed by Phillips Petroleum Company together with the Salk Institute Biotechnology/Industrial Associates, Inc.(SIBIA). With this new system very high levels of heterologous protein was produced. This success could be reached through the use of the alcohol oxidase promoter AOX1, which can be highly active while being tightly regulated [50]–[52].

*P. pastoris* is a single-celled organism and therefore easy to manipulate and cultivate. Other advantages lie in its capability to perform post-translational modifications. As eukaryotes yeasts can perform proteolytic processing, folding, disulfide bond formation and glycosylation. But yeast cultures are faster, easier to handle and less expensive than higher eukaryotic expression systems [53]. Heterologous protein expression in *P. pastoris* is a straightforward approach. After inserting the gene

of interest into plasmids they are linearized and transformed into the *P. pastoris* cells. The transformants integrate into the genome and under an inducible promoter the protein is produced and secreted to the fermentation broth. The most important steps of this procedure are shown in **Figure 5**.

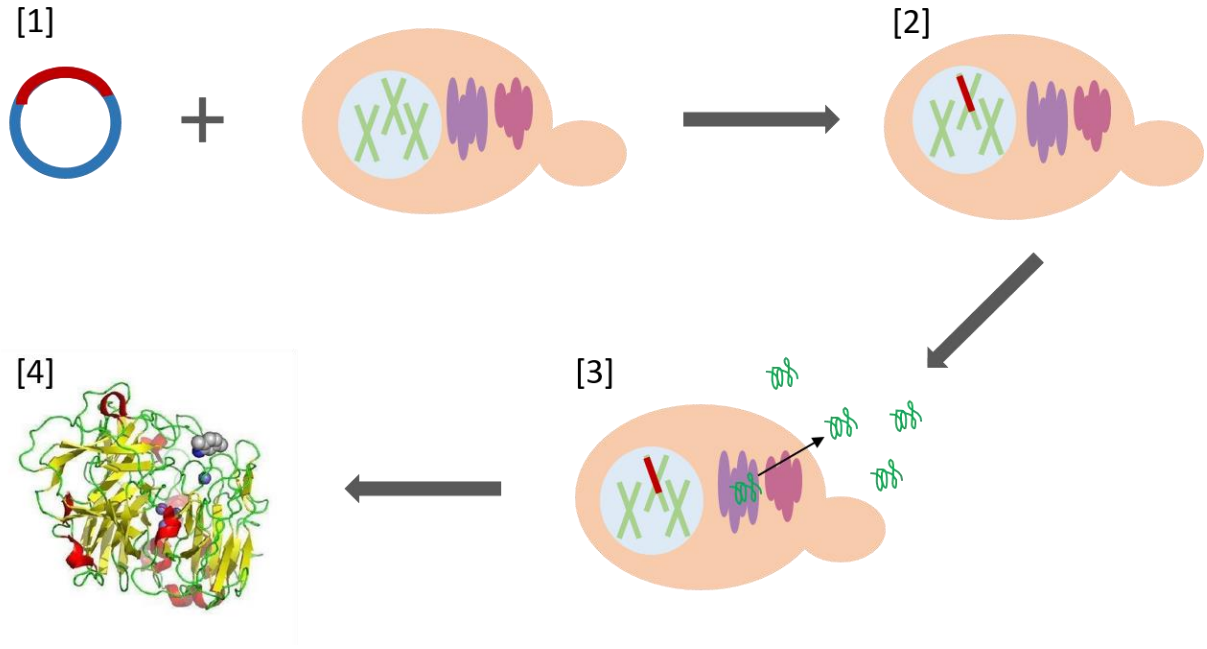



**Figure 5: Heterologous enzyme expression in *P. pastoris*:** (1) and (2) the gene is integrated into the genome, (3) the enzyme is produced and secreted and (4) the enzyme can be purified from the fermentation broth.

For successful expression of recombinant proteins in *P. pastoris* four important points have to be considered: (1) a suitable promoter-terminator combination, (2) appropriate selection markers, (3) the vector system and (4) the host strain have to be chosen. The choice of expression vector in combination with the host strain is the most important consideration [54].

The tightly regulated inducible promotor AOX1 enables the separation of the growth and the enzyme production phase. Therefore, biomass production is done prior to protein production. In this way, proteins that are toxic to the *P. pastoris* cells can be produced, and the cells are not stressed by product formation during growth phase [54]. The AOX1 promoter was first described in 1987 and is still the most common promoter for protein production in *P. pastoris*. It is strongly repressed in the presence of glucose, glycerol or ethanol, which can be used as substrate for the growth phase of the organism. The promoter is de-repressed by the decrease of these carbon sources and fully induced by the addition of methanol [55].

Methanol is highly flammable and hazardous, which makes it useable in small scale experiments but undesirable for industrial scale protein production [56].

## 1.3 The human antioxidant system

### 1.3.1 Oxidative stress

Oxidative stress is defined as the imbalance between the ability of the body´s antioxidative protection system and its counterplayers, the reactive oxygen and nitrogen species (ROS/RNS)[57]. This means that oxidative stress is a result of either an increased ROS/RNS formation or a reduced capacity of endogenous systems to protect biomolecules from oxidative attack. Several pathologies are connected to the state of oxidative stress. For example in cardiovascular diseases and cancer, but also in aging the antioxidant status is known to play an important role [58].

ROS/RNS are products of cellular metabolism, and can either play a positive or a negative role in living organisms. At low concentrations ROS/RNS act in the defence mechanism against infectious agents and in numerous signalling pathways. They can induce a mitogenic response [59]. The negative effect of ROS/RNS occurs at high concentrations leading to an imbalance with enzymatic and non-enzymatic antioxidants as shown in **Figure 6**. The increased number of radicals in the body leads to damaging reactions on biomolecules like DNA. This can result in incorrect DNA replication which in turn can encourage the formation of tumour cells by preventing cells from apoptosis [60]–[62].

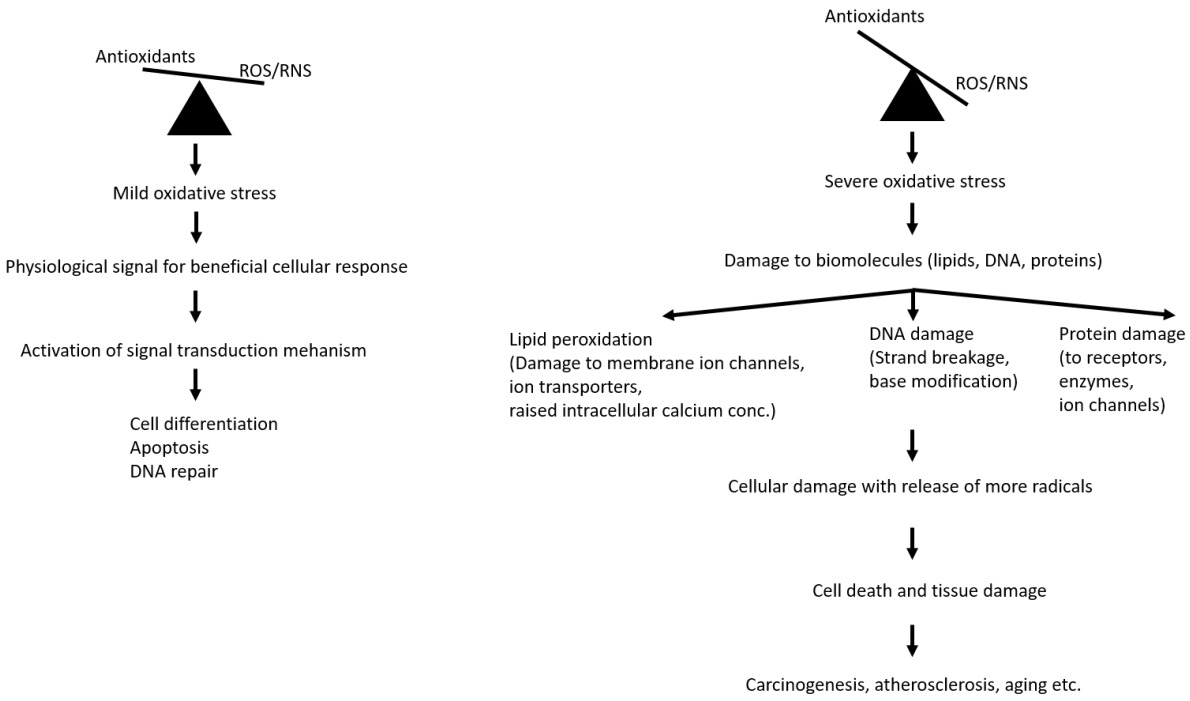



**Figure 6: Oxidative stress:** Scheme of responses and signals during oxidative stress and its possible effects on cells and tissue, adapted from [63], positive effects of mild oxidative stress at the left and possible effects of severe oxidative stress on the right side.

**Reactive oxygen species**

ROS is a group of reactive molecules comprising free radicals and non-free radical oxygenated molecules. Free radicals have one or more unpaired electrons in their atomic or molecular orbitals, which makes them very reactive. Non-free radicals are hydrogen peroxide ($H_2O_2$), superoxide ($O_2^{\cdot-}$), singlet oxygen (1/2 $O_2$) and the hydroxyl radical (OH) [64]. In living organisms free radicals originating from oxygen are the most important reactive species [65]. Free radicals are generated by aerobic processes like cellular respiration, microbial infections and phagocyte activation or during physical activity. Cigarette smoke, alcohol, ionizing and UV radiations, pesticides, ozone or similar toxins can increase ROS as well [66].

**Antioxidants**

Organisms developed a variety of defence mechanisms against oxidative stress. These involve: (1) preventative mechanisms, (2) repair mechanisms, (3) physical defences an (4) antioxidant defences. The antioxidant defence mechanism can include enzymatic antioxidant defence performed by superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). This first line of defence acts by quenching of $O_2^{\cdot-}$ and decomposition of $H_2O_2$ as well as sequestration of metal ions. The second line of defence is performed by non-enzymatic antioxidants which are able to scavenge radicals. These are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids and many others [67][68][63]. Vitamin C for example, can prevent damage to red blood cells by directly interacting with radicals like $O_2^{\cdot-}$ and HO in plasma [69].

### 1.3.2 Oxidative stress related diseases and aging

Aging can be defined as gradual loss of tissue- and organ functions. This functional loss can be related to accumulation of ROS/RNS, and the respective damages to macromolecules in the cells [70]. The exact mechanism of the relation between oxidative stress and aging is still not clear, but it seems that increased ROS/RNS levels lead to cellular senescence [71].

Furthermore, oxidative stress is related to many age-related conditions and diseases such as cardiovascular diseases, chronic obstructive pulmonary diseases, chronic kidney diseases, neurodegenerative diseases and cancer [70]. Three of these conditions are explained in detail in the following paragraphs**.**

Cardiovascular diseases (CVDs) are one of the main causes of mortality in old people and are often related to atherosclerosis. There are numerous studies showing that the tolerance for oxidative stress decreases with age due to a reduction in the intracellular enzymatic defence mechanism. This fact

results in cardiovascular alterations [72]. Oxidized low-density-lipoprotein (LDL)-cholesterol is suspected to correlate with a rise in free radical level. Consequently the risk of artherosclerosis and higher arterial stiffness independent of other CDV risk factors increases [73].

ROS/RNS can damage structural components in cells and can also lead to production of additional reactive species, which can in turn increase the damage. This can lead to chronic inflammation which can be directly related to carcinogenesis [74], [75]. In detail, transcription factors like NFκB, can be activated by ROS/RNS and inflammatory cytokines. These transcription factors induce the expression of genes that are involved in carcinogenesis.

Free radicals can cause apoptosis, necrosis and cell death through oxidative damage to macromolecules like lipids, DNA and proteins. These oxidative insults occur more often in older individuals because of higher accumulation of free radicals which leads to loss in physiological functions and physical characteristics [76], [77].

### 1.3.3 Methods for evaluation of antioxidant activity

Since several studies showed the importance of antioxidants in biological systems, a great interest for methods to quantify the amount of antioxidants in biological samples arose [78]. There are quantification methods for antioxidants in human blood and plasma samples, as well as in food and beverages, since nutrition is believed to influence the antioxidant homeostasis in the human body.

Antioxidant measurement systems have to include an oxidation initiator, a suitable substrate and a method to measure changes in the reaction mixture resulting from the amount of present antioxidants. There are several methods for such measurements which include (1) the measurement at an exact time point, (2) the measurement of a reaction rate, (3) lag phase measurement or (4) integrated rate measurement [79]. Oxidation initiators can be temperature, exposure to light, enzymes and many more [80][81]. Requirements for a suitable substrate are the ability to be oxidized as well as being reduced again, and the concomitant, measurable change in absorbance. The different methods can be further divided by their mechanism. Inhibition methods make use of the inhibition of oxidation of the target molecule in presence of the antioxidant, whereas there are also methods based on the direct measurement of radical scavenging activity in the sample, using already oxidized target molecules which are then reduced by the antioxidants [81].

For quantification of the amount of antioxidants present in a biological sample a reference substance with known concentrations is needed. For that purpose either a mixture of antioxidants is used, or very

commonly, the antioxidant Trolox, a water-soluble analog of vitamin E, is used. Results obtained using Trolox standard curves are referred to as Trolox equivalent antioxidant capacity (TEAC) [82].

Moreover the different measurement systems can be divided into *in vivo* and *in vitro* methods. Since for this thesis an *in vitro* approach is used, *in vivo* methods will only be mentioned shortly in the next sections and some of the most common *in vitro* methods will be described in more detail.

**DPPH method**

Through the delocalization of the spare electron on the molecule DPPH (2,2 diphenyl-1-picrylhydrazyl) a stable free radical is formed, namely DPPH° (oxidized), which has a violet colour with an absorption maximum at 520 nm. When the radical gets in contact with a hydrogen donor, either an antioxidant or another radical, the reduced form of the molecule, DPPH is formed, accompanied by a decrease or loss of the violet colour. This decrease in colour can be measured spectrophotometrically. As a standard antioxidant Trolox is used [83]–[85]. In **Figure 7** the principle of the DPPH method is explained graphically.

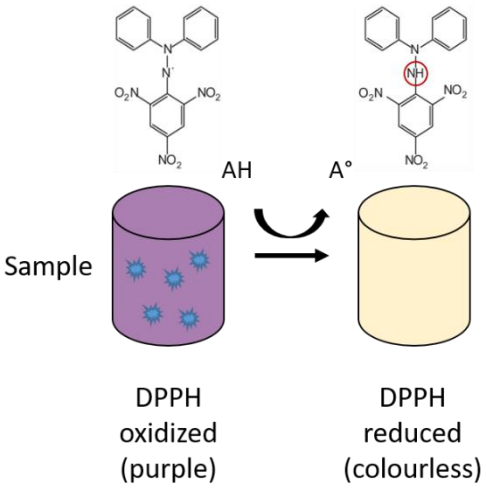



**Figure 7: DPPH Method:** Principle of the DPPH antioxidant scavenging method, adapted from [86].

**FRAP assay**

This FRAP (ferric reducing antioxidant power) method relies on the reduction of the complex ferric iron-TPTZ (2,4,6-tri(2-pyriolyl)-1,3,5-triazine) by antioxidants. The binding of Fe2+ to the ligand results in an intense navy blue colour (see **Figure 8**). The FRAP-antioxidant complex has an absorption maximum at 593nm, and therefore the formation of the complex can be measured spectrophotometrically and can be correlated to the amount of antioxidants present. As references Trolox or ascorbic acid are used [84], [87].

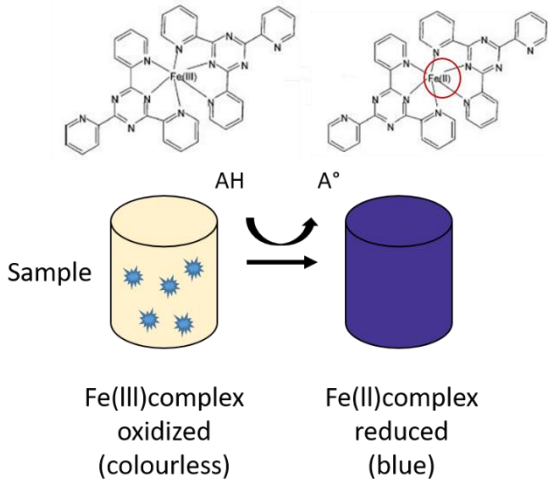



**Figure 8: FRAP Method:** Principle of the FRAP antioxidant capacity measurement method [88]

**ORAC assay**

For the ORAC (oxygen radical absorption capacity) assay a free radical, mostly peroxyl radical, is generated by 2,2-azobis-(2-amido-propane)dihydrochloride (AAPH) at 37°C. A fluorescent molecule, namely fluorescein or B-phycoerythrin (B-PE) is used as target molecule. The decrease in fluorescence of that molecule in presence of radical scavengers can be measured. The more antioxidants present in the sample the less decrease in absorbance can be measured (**Figure 9**). An excitation wavelength of 493 nm is used and emission is measured at 515 nm [84], [89].

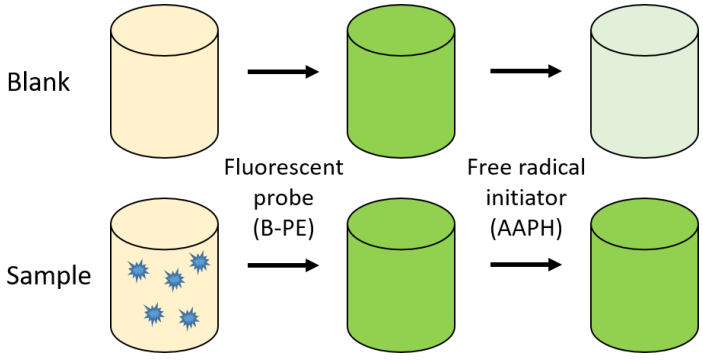



**Figure 9: ORAC Method:** Principle of the ORAC antioxidant capacity measurement method

**TRAP assay**

For the TRAP (total peroxyl radical trapping antioxidant parameter) assay, 2,2-diazobis-(2-amidino-propane)dihydrochloride (ABAP) is used as radical generator inducing a controlled peroxidation reaction which results in a decay of fluorescence of R-phycoerythrin (R-PE). If antioxidants are present in the reaction mixture the fluorescence is protected from decay [90].

**ABTS assay**

ABTS (2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid) forms the radical cation $ABTS^+$ when it is oxidized. This results in a colour change from light green to a very dark-blueish green with absorbance maxima at 420 nm and 743 nm. For the quantification of antioxidants with ABTS two methods are used. For the first method the ABTS is directly mixed with an oxidizing substance like potassium persulfate, manganese dioxide or an oxidizing enzyme, and the antioxidant sample. In the blank reaction no antioxidants are added. The reaction mixture including antioxidants will turn green slower and to a lower extent than the blank. As reference Trolox can be used and the TEAC (Trolox equivalent antioxidant capacity) can be evaluated (**Figure 10**) [84], [91]. Another approach is to oxidize the ABTS in a first step to the radical $ABTS^+$, and afterwards mixing it with either antioxidant, or the same amount buffer for the blank. From the difference in decrease of absorbance the TEAC can again be evaluated.

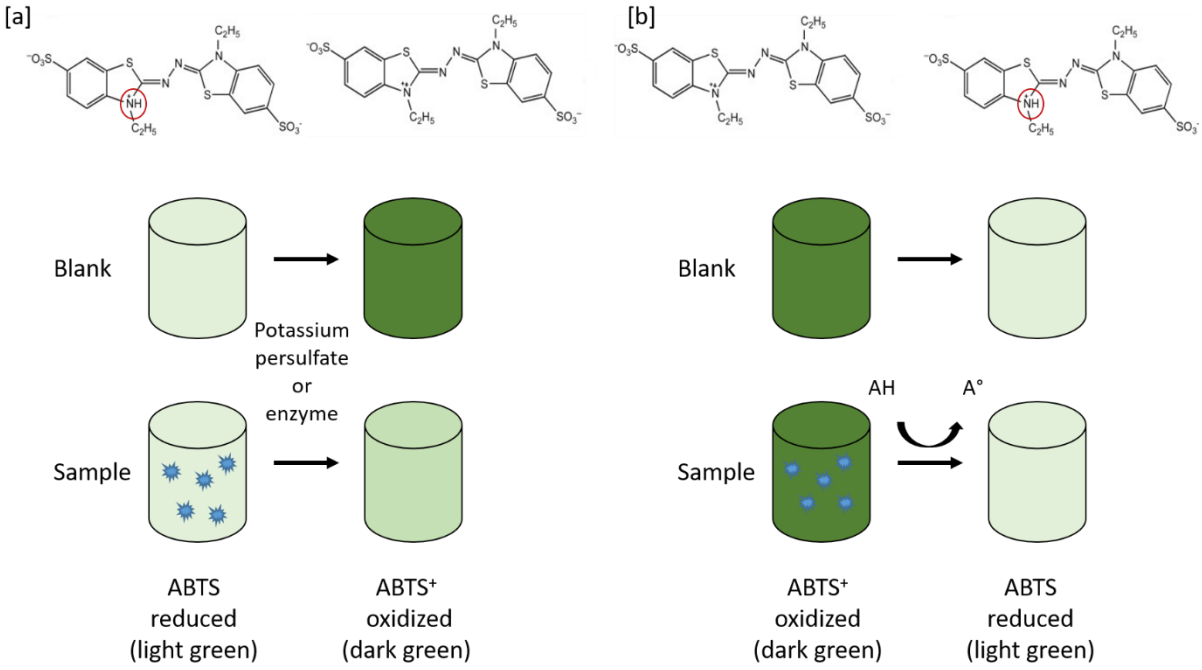



**Figure 10**: **ABTS Method:** Principle of the ABTS antioxidant assay [92]

# 2 Aim

The aim of this thesis was to heterologously express two newly designed laccases, namely *AfLacc1* and *AfLacc1:CBD*, to purify and characterize them while concomitantly design an antioxidant biosensor making use of the examined abilities of *AfLacc1* and its variant *AfLacc1:CBD*.

Antioxidant status in human blood and plasma is used as an indicator for the general health of patients nowadays, since oxidative stress is often related to various diseases. Many assays and medicinal test kits have been employed over the last decades and are in clinical use on a daily basis. Assays using enzymes to oxidize substances like syringaldazine or ABTS that produce stable radicals as oxidized forms are quite common. These assays use the ability of antioxidants in blood or plasma samples to reduce these radicals, resulting in a colour change that can be measured photometrically. The change in absorbance can be related to the amount of antioxidants present in the samples using a reference antioxidant like Trolox. The stable ABTS radical for antioxidant capacity measuring methods is well studied and common. The radical is either built by oxidation using chemicals or enzymes.

To date peroxidases (**Figure 11**) [93], potassium persulfate [84] or manganese dioxide [91] are used for the generation of the radical.

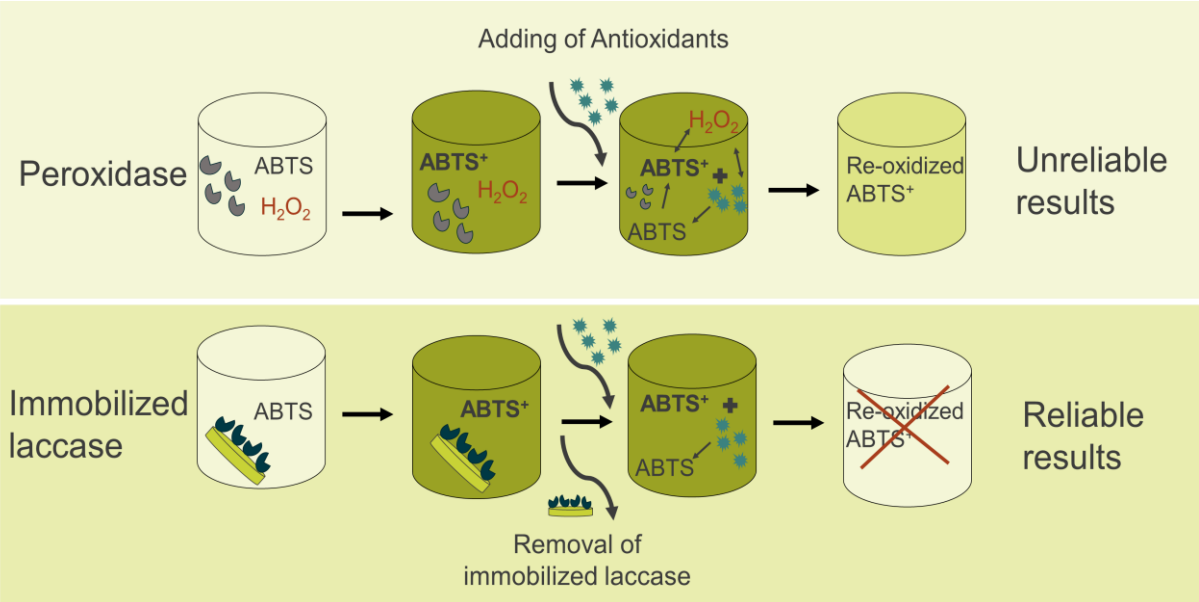



**Figure 11: Design of an antioxidant assay**: The common approach using Peroxidases and $H_2O_2$ to oxidize ABTS compared to the method using laccase, where no $H_2O_2$ is needed and no side reactions occur in the reaction mixture.

Peroxidases need $H_2O_2$ as a cofactor in the reaction mixture. This may produce re-oxidized $ABTS^+$ in the reaction mixture after addition of the antioxidants as a result of the oxidation ability of $H_2O_2$ still

present in the solution. Consequently the decrease in absorbance is falsified and does not fully correlate with the amount of present antioxidants in the sample. This makes assays using peroxidases inaccurate and hardly reproducible [93].

The high substrate specificity of the laccases *AfLacc1* and *AfLacc1:CBD* makes them very promising for the use in an antioxidant biosensor. Laccases do not need $H_2O_2$ as cofactor for the oxidation reaction, therefore no further oxidation of ABTS during the antioxidant assay can occur after removing the enzyme from the solution. For that purpose the enzyme *AfLacc1:CBD* was immobilized to a nanocellulose gel, creating an easy way to remove it from the assay mixture. All the stated properties of the expressed enzymes make them very promising for the use in an antioxidant biosensor.

# 3 Materials and Methods

## 3.1 Materials

### 3.1.1 Chemicals

All bulk chemicals were of analytical grade and purchased by Sigma-Aldrich (Germany) if not stated otherwise. Substrate for enzyme activity measurements 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS) and p-nitrophenol acetate (pNPA) and hemoglobin were purchased from Sigma-Aldrich (Germany). For protein concentration determination, a Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, USA). For SDS-PAGE pre-cast tris-glycine gels with 4-15% polyacrylamide, 10x tris-glycine-SDS (TGS) buffer and 4x Laemmli sample buffer were purchased from Bio-Rad Laboratories (Hercules, USA). Laccase substrates and antioxidants for screening experiments were obtained from Sigma-Aldrich (US).

#### 3.1.1.1 Media

**Table 1: Media:** List of used media with ingredients in [%]

| Media | Ingredient | Amount [%] |
|---|---|---|
| **YP** | Yeast Extract | 1.0 |
| | Peptone | 2.0 |
| **10xYNB** | Yeast Nitrogen Base | 13.4 |
| **10xD** | D-Glucose-Monohydrate | 20.0 |
| **500xB** | Biotin | 0.02 |
| **YPD** | 10xD | 10.0 |
| **BMD 1%** | 1M Potassium Phosphate Buffer pH6 | 20.0 |
| | 10xYNB | 10.0 |
| | 10xD | 5.0 |
| | 500xBiotin | 0.2 |
| | MQ water | 64.8 |
| **BMM 10 (Induction medium)** | 1M Potassium Phosphate Buffer pH6 | 20.0 |
| | 10xYNB | 10.0 |
| | Methanol (absolute) | 5.0 |
| | 500xBiotin | 0.2 |
| | MQ water | 64.8 |

### 3.1.2 Instruments

Table 2: Instruments: List of used instruments

| Instrument | Name | Company |
|---|---|---|
| Plate reader | Infinite 200 Pro | Tecan, Switzerland |
| Spectrophotometer | U-2900 | Hitachi |
| Water bath | | Julabo |
| Thermomixer | Thermomixer Comfort | Eppendorf, Germany |
| Centrifuge | 5427 R | Eppendorf, Germany |
| Autoclave | | |
| Incubator | Innova 44 | New Brunswick |
| Imaging | Chemidoc MP | BioRad |

### 3.1.3 Enzymes

*Myceliophthora thermophila* laccase (*MtL*) with the reference number 51003 was supplied by Novozymes(Land). *AfLacc1* and *AfLacc1:CBD* (cellulose binding domain) laccase were expressed and purified as described in 3.2. For the characterization of the laccases ÄKTA concentrated and purified aliquots of both enzymes were kindly provided by Sabine Zitzenbacher. For further experiments fermentation supernatant containing*AfLacc1* or *AfLacc1:CBD,* desalted using PD10 columns, were used.

## 3.2 Enzyme production

### 3.2.1 Expression of *AfLacc1* laccase in *Pichia pastoris*

Enzymes were recombinantly expressed in *P. pastoris* KM71H cells. *P. pastoris* cells transformed with pPICZαB vector (ThermoFisher Scientific (USA), containing the gene of interest, were kindly provided by co-workers. Amplification of the plasmid was performed with *Escherichia coli* BL21-Gold(DE3) (Agilent Technologies, USA) and isolated with a Promega Midiprep kit (USA). The vector was linearized with SacI (New England Biolabs (USA)) and transformed into *P. pastoris* via electroporation (MikroPulserTM, Bio-Rad) according to manufacturer's protocol.

For the pre-culture, transformed *P. pastoris* was cultivated in yeast extract peptone dextrose (YPD) medium supplemented with 100 µg $mL^{-1}$ Zeocin, at 28°C and 150 rpm, overnight. This culture was used to inoculate 100 mL buffered minimal dextrose (BMD) medium to an $OD_{600}$ of 0.1 in 1 L baffled flasks.

The cultures were incubated at 28°C and 150 rpm for 60 h. Expression was induced by the addition of 10 mL BMM 10 medium. Induction was continued by adding 500 µL methanol (absolute) 6 h after the first induction and then twice a day over a period of five days. Additionally, 0.1 mM $CuSO_4$ were added with the first induction for the proper folding of laccase.

The fermentation broth was centrifuged at 3428 rcf for 20 min, the pellet was discarded and the supernatant, containing the protein, was aliquoted and stored at -20°C or used directly.

### 3.2.2 Enzyme purification

In order to clear the fermentation supernatant of macromolecular media components, it was vacuum-filtrated with a pore size of 0.45 and 0.2 µm. Secondly, the enzyme was purified by buffer exchange to citrate(10mM)-phosphate(20mM) buffer pH 3.4 with PD10 desalting columns (GE Healthcare, UK). The columns were equilibrated with 25 mL of buffer letting it enter the bed completely and discarding the flow-through. To each column 2.5 mL supernatant were added, again letting the sample enter the packed bed completely and discarding the flow-through. For sample elution a tube was placed under the column and the sample was eluted using 3.5 mL buffer. The desalted protein solution was directly used for further experiments or stored at -20°C.

To reach higher protein concentrations the desalted enzyme solution was further concentrated using ultrafiltration with Vivaspin or freeze drying. For freeze drying, 40 mL of filtrated fermentation supernatant were frozen at -80°C and lyophilized. The dried enzyme was dissolved in only 5.5 mL buffer, in that way a concentration of nearly 8-fold could be reached.

The protein concentrations and activities of the untreated supernatant, as well as, from aliquots of all further concentration and purification steps were measured using the Bradford method and ABTS assay (3.3.1), respectively.

### 3.2.3 Deglycosylation of laccase and acetone precipitation

Fungal laccases are glycoproteins with an varying extent of glycosylation typically between 10 and 25%, but laccases with a glycosylation degree higher than 30% have been found [3].

Due to that and since proteins expressed in *Pichia pastoris* are normally not homogenously glycosylated, it is necessary to deglycosylate those proteins before running them on an SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or western blot. The native, glycosylated form has varying glycosylation patterns which results in different sizes and therefore they cannot be clearly identified. Additionally it is to mention that the expressed enzymes *AfLacc1* and *AfLacc1:CBD*

are not naturally produced in *P. pastoris* and therefore the glycosylation pattern is even more inhomogeneous.

For deglycosylation, 61.2 µL of fermentation supernatant were mixed with 6.8 µL 10x denaturation buffer and heated to 100°C for 10 minutes and instantly cooled on ice. After that 6.8 µL G5 reaction buffer and 1 µL Endo H were added. The samples were incubated at 37°C and 150 rpm for 2.5 h.

Proteins were precipitated by the addition of 227 µL ice cold acetone to deglycosylation samples and incubation for 3 h. Afterwards, the samples were centrifuged and the supernatant discarded. The reaction tubes were left open overnight to evaporate all the acetone. The pellets were used for the SDS-PAGE.

### 3.2.4 SDS-PAGE

SDS-PAGE was performed to analyse size and purity of the expressed enzyme.

In a first step 15 µL 2x Laemmli buffer, containing 2-mercaptoethanol, were added to each sample and heated to 99°C for 10 minutes. The samples were analyzed directly [94] by loading 15 µL sample onto the gel. As reference for molecular weight, 5 µL of pre-stained protein marker (peqGold marker IV) were also loaded onto the gel, and subsequently let run for 30-45 min at 170 mV in tris-glycine running buffer. For visualization of the protein bands, the gels were incubated with Coomassie blue staining solution (0.1% v $v^{-1}$ Coomassie R250, 10% v $v^{-1}$ acetic acid, 45% v $v^{-1}$ methanol) for 30 min. Afterwards a destaining step was performed by addition of destaining solution (10% v $v^{-1}$ acetic acid, 40% v $v^{-1}$ methanol), incubation for 40 min and subsequent discarding of the solution. This was repeated 2-3 times, the last destaining step was done overnight.

## 3.3 Enzyme Characterization

For all enzyme characterization experiments, enzymes were purified with affinity chromatography and concentrated , enzymes were kindly provided by Sabine Zitzenbacher.

### 3.3.1 Laccase activity assay

The activity of *AfLacc1* and *AflAcc1:CBD* was determined spectrophotometrically by monitoring the oxidation of ABTS as described by Kudanga et al [95]. ABTS oxidation occurs in two steps, for the activity assay the formation of the first oxidation product $ABTS^+$ is measured (see **Figure 12**). Modifications regarding the volumes were made, 170 µL of enzyme solution appropriately diluted in 100 mM citrate buffer (pH 3.4), and 50 µL of 10 mM ABTS (in distilled water) were mixed. The formation of the $ABTS^+$

radical can be monitored spectrophotometrically by the increase in absorbance at 420 nm and the dark green colour that appears.

The change in absorbance at 420 nm was immediately determined in 20 cycles with an interval time of 10 s using a plate reader (Tecan infinite M200 pro). Enzyme activity is expressed in Units, one Unit is defined as the conversion of 1 µmol ABTS per minute. This can either be stated as Unit per mL of enzyme solution or per mg to include the amount of enzyme. For the negative controls the same procedure was used, but the enzyme solution was substituted by buffer. Enzyme activity was calculated using **Equation 1**, which is an adaptation of the Lambert-Beer law (see Equation 2).

$$V_0 = \frac{\Delta abs}{min} \times \frac{Vtot}{Vsample * \varepsilon * d} \times f$$

**Equation 1:** Calculation of volumetric enzyme activity

$V_0$ = volumetric enzyme activity [U/mL]

Δ abs/min = change of absorbance per minutes

$V_{tot}$ = total volume of reaction mixture [mL]

$V_{sample}$ = sample volume in reaction mixture

ε = molar extinction coefficient of ABTS at 420nm at certain pH

d = optical path length [cm]

f = dilution factor

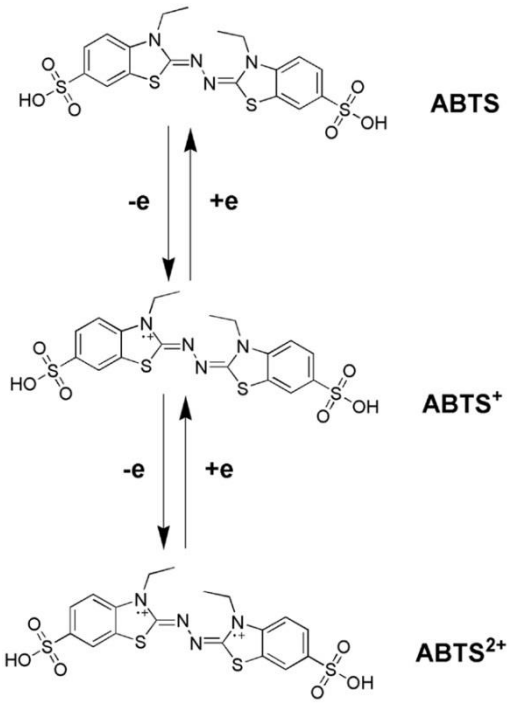



**Figure 12: Oxidation of ABTS:** Chemical structures of the oxidation products of ABTS in presence of laccase[4]

#### 3.3.1.1 Determination of the extinction coefficient of ABTS

The molar extinction coefficient of substrates is pH- as well as buffer dependent. Therefore, the extinction coefficients for ABTS at defined pH values and buffers for this thesis were determined prior to laccase activity measurement using the Lambert-Beer law (Equation 2).

$$E_{420} = \varepsilon_{ABTS} * c_{ABTS} * d * f \rightarrow \varepsilon_{ABTS} = \frac{E_{420}}{c_{ABTS} * d} * f$$

Equation 2: Lambert-Beer law

$E_{420}$ = extinction of ABTS at 420nm

$\varepsilon_{ABTS}$ = molar extinction coefficient of ABTS

$c_{ABTS}$ = concentration of ABTS [mM]

d = optical path length

f = dilution factor

Measurements were performed in the range from pH 3 to pH 8 using 100 mM citrate buffer (pH 3), 100 mM sodium-acetate buffer (pH 4-5) and 100 mM sodium-phosphate buffer (pH 6-8), as well as at

pH 3.4 in citrate-phosphate buffer. To determine the molar extinction coefficient of ABTS, $ABTS^{+}$, the first oxidation product is needed. Therefore, ABTS stock solution with a concentration of 5 mM in the various buffers was prepared and varying amounts of that solution were mixed, with enzyme solution (see **Table 3**). The absorbance change at 420 nm was measured immediately after the addition of enzyme, using a spectrophotometer, until maximal absorbance was reached. To convert ABTS into its completely oxidized state the commercially available laccase *MtL* (Novozymes) was used and diluted 1:1000 in the various buffers.

The absorbances were plotted against the ABTS concentrations in the reaction mixtures. The slopes represent the decadic molar extinction coefficient of ABTS at 420 nm at the respective pH value.

**Table 3: Extinction coefficient:** Volumes of enzyme solution and ABTS stock solution, and resulting ABTS concentrations in the reaction mixture for the determination of the molar extinction coefficient of ABTS at 420nm.

| Enzyme solution (*MtL*) [µL] | ABTS stock solution (0.5 mM) [µL] | Total Volume [µL] | Resulting ABTS concentration in reaction mixture [mM] |
|---|---|---|---|
| 990 | 0 | 990 | 0 |
| 990 | 10 | 1000 | 0.005 |
| 990 | 20 | 1010 | 0.010 |
| 990 | 45 | 1035 | 0.022 |
| 990 | 70 | 1060 | 0.033 |
| 990 | 95 | 1085 | 0.044 |
| 990 | 120 | 1110 | 0.054 |
| 990 | 145 | 1135 | 0.064 |
| 990 | 170 | 1160 | 0.073 |
| 990 | 195 | 1185 | 0.082 |
| 990 | 220 | 1210 | 0.091 |

### 3.3.2 Protein concentration

For the determination of protein concentration the method described by Bradford [96] was used. This photometric assay was performed in microtiter plates. A standard calibration curve using bovine serum albumin was measured in the concentration range from 0.0125 mg/mL to 0.5 mg/mL. The absorbance at 595 nm was plotted against the protein concentration. The equation calculated for the standard curve was then used for protein concentration calculation (Equation 3) of the samples. For the assay 10 µL of sample solution (or BSA standard) were pipetted in to a well, 200 µL of Bradford reagent 1:5 diluted with MQ water were added and the plate was incubated for 10 minutes at room temperature

and 300 rpm. The absorbance was measured at 595 nm. For the blanks the same procedure was performed, except buffer was used instead of protein solution.

$$C = \frac{A595Sample - A595Blank}{k} * f$$

Equation 3: Calculation of protein concentration

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

$A_{595\text{-}Sample}$ = measured absorbance of the sample at 595 nm

A595 Blank = measured absorbance of the blank at 595 nm

k = slope of the calibration curve

f = dilution factor of the sample

### 3.3.3 Absorbance spectra of *AfLacc1* and the commercial laccase *MtL*

Blue copper proteins like laccases have a characteristic absorption band around 610 nm, caused by the type 1 paramagnetic blue copper atom in the active protein [97][12].

*AfLacc1* and *MtL*, as a reference, were diluted in MQ water and absorbance scans from 230-900 nm were measured using a spectrophotometer.

### 3.3.4 Laccase pH optimum

To establish a pH profile for *AfLacc1*, enzyme activities were measured at different pH values. Citrate(10 mM)-phosphate(20 mM)-buffer was used in the range from pH 3-5 and 100 mM sodium-phosphate buffer was used between pH 6 and pH 8. Enzyme activity measurement was done using the ABTS-assay as described above.

### 3.3.5 Laccase temperature optimum

To establish a temperature profile for *AfLacc1* at its optimal pH level (pH 3.4), enzyme activities were measured after heating the enzyme solution to different temperatures (20, 25, 30, 40, 50, 60°C) using a water bath. Enzyme activities were measured with the ABTS-assay as described above with some modifications according to the volume, which was upscaled to 250 µL 10 mM ABTS and 850 µL enzyme solution. For the measurement a spectrophotometer and 1.5 mL cuvettes were used.

### 3.3.6 Storage and Thermostability

Furthermore, storage- and thermostability were investigated. Thermostability was determined at the optimal temperature, as examined before, and room temperature, which is often chosen as reaction

condition, due to easy handling. Enzymes are usually stored in the fridge (4°C) or frozen (-20°C) to preserve their activity. Therefore, these two temperatures were chosen for the determination of the storage stability.

Aliquots of *AfLacc1* diluted 1:10 in citrate-phosphate-buffer were incubated at room temperature, 40°C, 4°C and -20°C. Furthermore, undiluted desalted fermentation supernatant containing *AfLacc1*, was incubated at the same temperatures. At every time point 100 µL of the enzyme dilutions were removed to be further diluted (1:10) in citrate(10 mM)-phosphate(20 mM)-buffer, resulting in a 1:100 dilution of the concentrated aliquots. Activity was measured and calculated as described above. Time points were taken after 10 min, 1 h, 3 h, 6 h. 24 h, 48 h, 72 h, 96 h as well as 1, 2 and 4 weeks.

### 3.3.7 Kinetic parameters

To determine kinetic values, laccase activity was measured at a range of ABTS concentrations from 0.5 mM to 20 mM. The activity measurement was conducted as described above. The laccase was diluted in 100 mM citrate buffer at pH 3.4 to a final concentration of 1.09 mg/mL. Kinetic values, $V_{max}$ and $K_M$, were calculated using Equation 4 and **Equation 5** obtained from Michaelis-Menten kinetics and Lineweaver-Burk diagrams.

$$V = \frac{V_{max} * [S]}{K_m + [S]}$$

Equation 4: Michaelis Menten equation

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_M}{V_{max}}$$

**Equation 5:** Rearrangement of the Michaeli-Menten equation to determine kinetic parameters using the Hanes-Woolfs plot

V = volumetric enzyme activity [U/mL]

$V_{max}$ = maximum reaction velocity [U/mL]

[S] = substrate concentration [mM]

$K_m$ = Michaelis constant

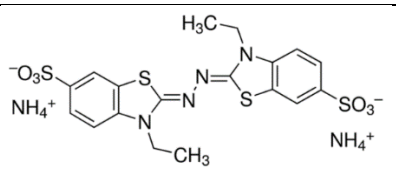
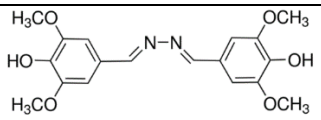
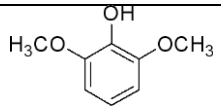
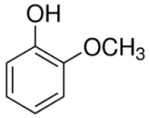
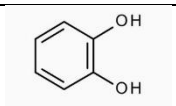
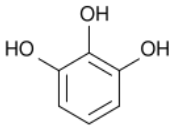
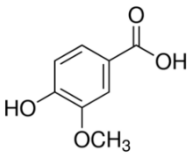
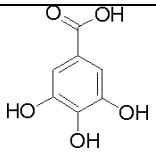
### 3.3.8 Screening for typical laccase substrates

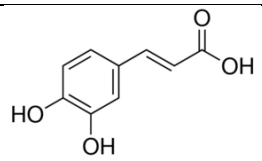
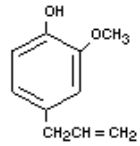
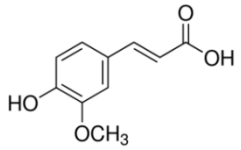
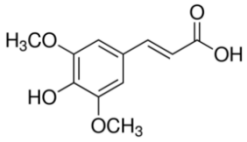
Laccases are known to oxidize a variety of substrates and the substrate range varies from one laccase to another [98]. This makes the substrate specificity an important factor for characterizing laccases. Phenols like guaiacol, catechol or 2,6-dimethoxyphenol are typical substrates for laccases, ABTS and

syringaldazine can be oxidized by the majority of laccases with high reaction rates and are therefore often used as laccase substrates for activity determination [99][100][101].

The substrates (**Table 4**) were dissolved in buffer, ethanol or methanol and diluted to a concentration of 200 µM in 100 mM citrate buffer pH 3.4. For the absorbance scans unoxidized substrate was compared to substrate incubated with *AfLacc1* as well as to substrate incubated with *MtL*. As the laccase *MtL* is known to oxidize most of the tested substrates, these absorbance scans were used as reference. Therefore 210 µL of pure substrate and 200 µL of substrate mixed with 10 µL *AfLacc1* or *MtL* were put in 96-well plates and absorbance scans from 230-900nm were measured directly after mixing and after 30 minutes of incubation. Differences in spectra of the substrate and the spectra of the substrate incubated with enzyme suggest interactions of enzyme and substrate.

**Table 4: Laccase substrates:** Chemical structures of the substrates used for the screening of interactions with *AfLacc1*

| No. | Monomer | Chemical structure |
|---|---|---|
| 1 | ABTS |  |
| 2 | Syringaldazine |  [102] |
| 3 | 2,6 Dimethoxyphenol |  |
| 4 | Guaiacol |  |
| 5 | Catechol |  |
| 6 | Pyrogallol |  |
| 7 | Vanillic acid |  |
| 8 | Gallic acid |  |

| | | |
|---|---|---|
| 9 | Caffeic acid |  |
| 10 | Eugenol |  |
| 11 | trans-Ferulic acid |  |
| 12 | Sinapic acid |  |

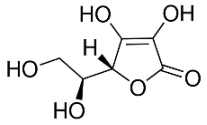
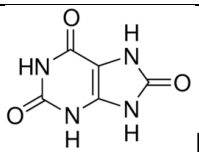
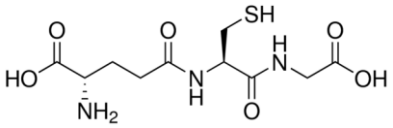
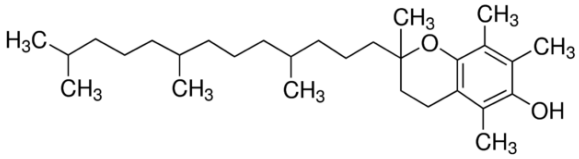
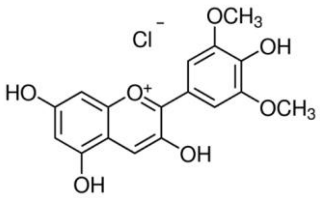
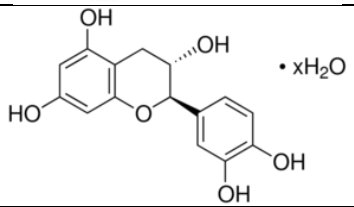
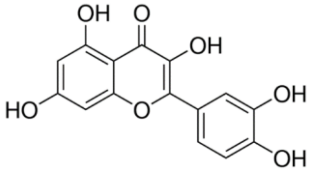
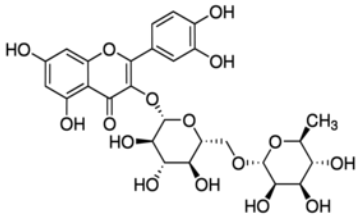
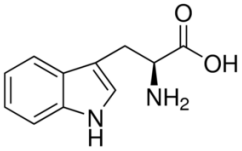
## 3.4 Development of an antioxidant activity testing kit

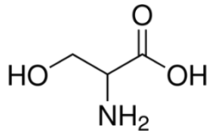
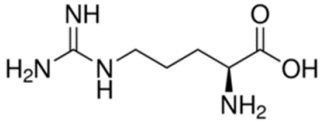
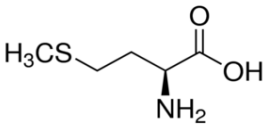
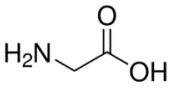
### 3.4.1 Screening for interactions of *AfLacc1* with human antioxidants and amino acids

To exclude interactions between *AfLacc1* and antioxidants present in the human body the most important ones were screened for interactions (see **Table 5**). Additionally, some amino acids were screened for interactions with the enzymes, since amino acids are present in human blood and plasma and could interfere with the assay.

The antioxidants and amino acids were solved in buffer, ethanol or methanol and diluted to a concentration of 1-200 µM in 100 mM citrate buffer pH 3.4. A solution of 210 µL pure antioxidant or amino acid and 200 µL of antioxidant or amino acid incubated with 10 µL *AfLacc1* or *MtL* were put in 96-well plates and absorbance scans from 230-900 nm were measured directly after mixing and again after 30 minutes incubation. Differences in spectra of the substrate to the substrate incubated with enzyme suggest interaction of enzyme and antioxidant or amino acid.

**Table 5: Antioxidants and amino acids:** Chemical structures of screened antioxidants and amino acids.

| Antioxidants | Human Serum Albumin | No Structure |
|---|---|---|
| | L-Ascorbic acid |  [103] |
| | Uric acid |  [104] |
| | Glutathione |  [105] |
| | alpha-Tocopherol |  [106] |
| | Malvidin Chloride |  |
| | Catechin hydrate |  |
| | Quercetin |  |
| | Rutin |  |
| Amino Acids | L-Tryptophan |  |

| | | |
|---|---|---|
| | L-Serine |  |
| | L-Arginine |  |
| | L-Methionine |  |
| | Glycine |  |

### 3.4.2 Immobilization of *AfLacc1:CBD* on Nanocellulose gel

ABTS could be further oxidized or re-oxidized in the reaction mixture after the addition of antioxidants. This would interfere with the antioxidant assay. Therefore laccase had to be removed from the solution. Immobilization on a nanocellulose gel (Tencel gel 2% G-62, Lenzing) created an easy way to remove the laccase.

Furthermore, immobilization of enzymes often leads to higher stability and has the advantage of reusability of the enzyme.

At first 1 g of nanocellulose gel was mixed with 15 mL *AfLacc1:CBD*, and mixed on a wheel at 4°C for 24 h. As a negative control 1 g nanocellulose mixed with buffer was treated the same way. To monitor the decrease in enzyme activity due to the 24 h incubation time an aliquot of the enzyme, was also treated in the same way and the activity before and after the 24 h incubation was measured.

After incubation the solution was aliquoted to 1 mL each, and centrifuged at 4°C and 2810 rcf for 5 minutes to separate the nanocellulose with the attached enzyme from the remaining free enzyme in the solution. The pellets were washed 3 times by adding 1 mL citrate-phosphate buffer pH 3.4 and centrifuging at 4°C and 2810 rcf for 5 minutes. The washing steps were performed to remove all enzyme that was not attached to the nanocellulose. Laccase activity was measured before and after immobilization, as well as in all washing supernatants, using the ABTS assay procedure. Furthermore, the protein concentration of the washing supernatants was measured using the Bradford method, but owing to too low concentrations, no results could be obtained.

The nanocellulose pellets were then used to completely oxidize ABTS, by mixing pellets with buffer and ABTS. The reaction mixtures were incubated at 40 °C for 4 h, and centrifuged at 2810 rcf for 5 minutes to separate the nanocellulose and enzyme from the oxidized ABTS solution. The supernatant,

containing oxidized ABTS, was used for further experiments. The pellets were washed 2 times with buffer as prior described and were reused 3-4 times.

**Activity measurement and reusability of nanocellulose pellets**

To measure the enzymatic activity of the enzyme immobilized to nanocellulose, the ABTS assay as described before could not be used. Instead the pellets remaining from 1mL immobilization mixture were dissolved in 850 µL buffer and 250 µL of 10 mM ABTS were added. After 5 minutes the gel was precipitated by centrifugation for 3 minutes and the absorbance of the supernatant was measured. This method gives only qualitative data, as the increase of absorbance over time could not be measured, only the colour change could be observed. To explore the reusability of the immobilized enzyme the gel pellets were washed 2 times with 850 µL buffer, as described in the paragraph above, and the same procedure was repeated.

### 3.4.3 $ABTS^+$ quenching by antioxidants over time

To show the quenching reaction of ABTS radical by different antioxidants, absorbance measurements after 0, 3, 5, 8, 10, and 20 minutes after adding 10 µL of 0.2 mM antioxidant to 200 µL oxidized ABTS were performed. Absorbance was measured at 420 nm.

### 3.4.4 Trolox standard curve

Oxidized ABTS was diluted to give an absorbance of 0.87 ± 0.02 at 734 nm and 1.97 ± 0.03 at 420 nm for 200 µL when measured on the plate reader in a 96-well-plate.

Trolox was dissolved to a concentration of 10 mM in 96 % ethanol and further diluted in 20 % ethanol to concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM.

Firstly 200 µL of ABTS solution were put in 96-well-plates and the absorbance at 420 nm and 734 nm was measured. Secondly 10 µL of the different Trolox standards were added and incubated for 3 minutes, afterwards the absorbance at 420 nm and 734 nm was measured, all was done in triplicates.

The ABTS quenching was calculated and expressed in % of decrease in absorbance. Standard curves showing the Trolox concentration in the reaction mixture against the decrease in absorbance were created.

### 3.4.5 Spiking of human plasma samples with Trolox

Experiments with human plasma samples were performed at the Medical University of Graz, under the kind supervision of Dr. Willibald Wonisch.

The purpose of this experiment was to test if the assay could be used for human plasma samples, to exclude matrix effects, and to determine antioxidant concentration ranges relevant for medical applications.

A Trolox standard curve was measured as described in chapter 3.4.4. Human plasma samples were diluted 1:100 in buffer and spiked with various amounts of Trolox. For the absorbance measurement firstly 200 µL of ABTS were put into 96-well plates and the timepoint zero read was done. After that 10 µL spiked samples were added and the absorbance was measured again after 3 minutes. As reference, diluted plasma samples without supplementary Trolox were measured to see if the added Trolox in the plasma changes the decrease in absorbance as expected from the Trolox standard curve.

# 4 Results and Discussion

To reach the aim of this thesis the enzymes *AfLacc1* and *AfLacc1:CBD* were heterologously expressed in *P. pastoris.* The following biochemical characterisation showed the optimal reaction conditions for the oxidation of ABTS to form a stable radical. Furthermore, AfLacc1 was discovered to have a high substrate specificity. This high substrate specificity is beneficial to design an antioxidant biosensor relying on the reduction of $ABTS^{+}$. Enzyme immobilization created an easy and fast method to remove the enzyme from the assay solution. Furthermore enzyme immobilization represented a way to reuse the enzyme and improve its stability.

## 4.1 Enzyme production

The enzymes *AfLacc1* and *AfLacc1:CBD* were expressed in *Pichia pastoris* according to an already established method as described in 3.2.1. As an expression control and to determine the size of the enzymes SDS-PAGE was performed. To estimate the degree of glycosylation and the size of *AfLacc1* the enzyme was loaded onto the gel deglycosylated as well as untreated (**Figure 13**). Protein marker IV (peqGold) was used to determine the size of the deglycosylated enzyme. *AfLacc1* had a size of around 60 kDa. As expected the untreated enzyme formed a broad and unclear band at a size of about 120 kDa due to the glycosylation of the enzyme and the resulting bigger size [3]. On the contrary the deglycosylated sample showed many bands, which are suspected to be cleavage products. The band at 60 kDa showed the enzyme *AfLacc1* and the clear band at approximately 30 kDa results from the endoglucanase EndoH in the sample.

The SDS-PAGE clearly shows that *AfLacc1* is a glycosylated enzyme and that the deglycosylation step before determining the size is necessary.

The size of *AfLacc1* is characteristic for fungal laccases which are approximately 60-70 kDa, but there is heterogeneity in the properties especially according molecular weight in ascomycetes.

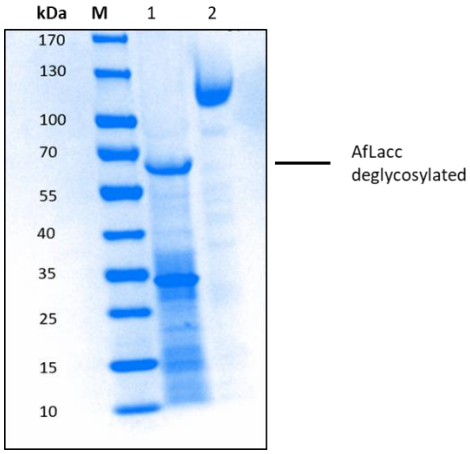



**Figure 13: Expression control:** SDS-PAGE gel of the deglycosylated (lane 1) and the untreated (lane 2) enzyme *AfLacc1*. In lane M the peqGold protein marker was run (protein sizes stated on the right side).

Activities of *AfLacc1* after various purification and concentration steps were determined by ABTS assay (**Figure 14**). The goal of performing various purification steps was to find a fast and easy method that results in an active enzyme solution. Lyophilization of the enzyme resulted in a total loss of activity. Ultrafiltration for purification of the enzyme did not have the desired effect. The volumetric activity could be increased, but the specific activity decreased to only one third compared to the specific activity of the desalted *AfLacc1.* The loss in the specific activity indicates that ultrafiltration may be stressful and harming to the enzyme. Furthermore, and the procedure is more work intensive than desalting with PD10 columns. Consequently the desalted fermentation supernatant was used for all further experiments.

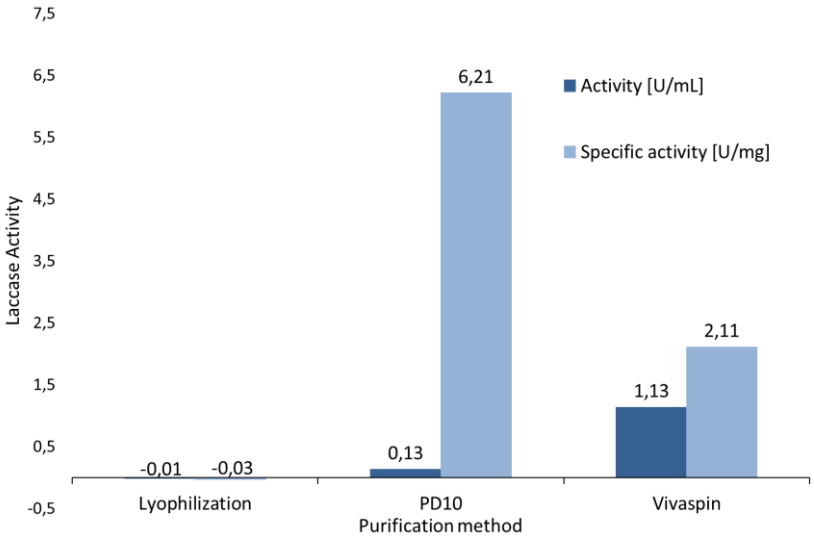



**Figure 14: Enzyme purification:** Volumetric activities and specific activities of *AfLacc1* after different purification and concentration methods.

## 4.2 Enzyme Characterisation

### 4.2.1 Absorbance spectra

To characterize *AfLacc1*, its absorption spectrum was compared to that of *MtL*. The characteristic peak at 610 nm is caused by the presence of the type 1 copper, which is known as paramagnetic blue copper (**Figure 15**). The blue colour resulted from the intense electronic absorption, as a result of the covalent copper-cysteine bond. A second characteristic peak at 330 nm, resulting from the type 3 copper, was expected [12].

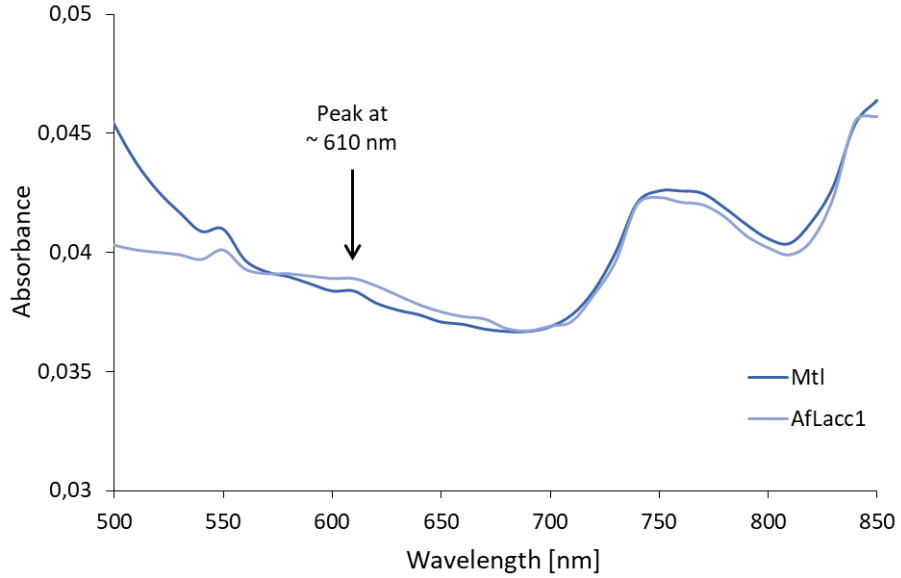



**Figure 15: UV/VIS absorbance spectra:** The spectra of *MtL* and *AfLacc1*, the black arrow shows the characteristic peak at around 620 nm

No peak at 330 nm could be observed (data not shown), which could be due to the fact that both enzymes were not completely purified. Neither of the solutions showed any visible blue colour. Moreover, peaks at a wavelength range around 750 nm, untypical for laccases could be observed (**Figure 15**). These untypical peaks could result from media components in the *AfLacc1* solution or undefined ingredients in the *MtL* solution, which has a brownish colour.

### 4.2.2 Optimal conditions and stability assay

For antioxidant biosensors relying on the reduction of prior formed $ABTS^{+}$, high concentrations of that radical are needed. Therefore optimal reaction conditions regarding the pH of the solution as well as the temperature at which the oxidation of ABTS is the most effective were obtained. The

determination of optimal reaction conditions as well as the kinetic parameters are also typically used methods to characterize and compare enzymes.

Prior to activity measurement with the ABTS assay, the molar decadic extinction coefficients of the substrate was determined, to be used for the calculation of the activity. Extinction coefficients are dependent on temperature, pH and other environmental influences. Since most of the experiments were performed at room temperature the extinction coefficients were only determined at that temperature.

**Table 6: Extinction coefficients of ABTS:** Determined molar decadic extinction coefficients of ABTS at various pH values, citrate(10mM)phosphate(20mM) buffer used for pH 3-6, sodium phosphate buffer (100mM) used for pH 7-8.

| pH | ε [L $mol^{-1}$ $cm^{-1}$] |
|---|---|
| 3 | 34.68 |
| 3.4 | 33.97 |
| 4 | 33.12 |
| 5 | 31.33 |
| 6 | 21.10 |
| 7 | 12.07 |
| 8 | 5.25 |

As expected the high pH dependence of the extinction coefficient can be clearly seen (**Table 6**). For all further activity measurements these coefficients were used for the calculation.

The pH optima for *AfLacc1* and *AfLacc1:CBD* on ABTS were 3.4 and 3.6, respectively (**Figure 16**). For calculation of the activities the molar decadic extinction coefficients that were determined prior to the other experiments were used (**Table 6**). Both enzymes showed optimal pH values in the acidic region, at pH 5 hardly any activity can be measured anymore. At pH 2, the enzymes were inactive. For that reason the pH range between 3 and 4 was explored in more detail.

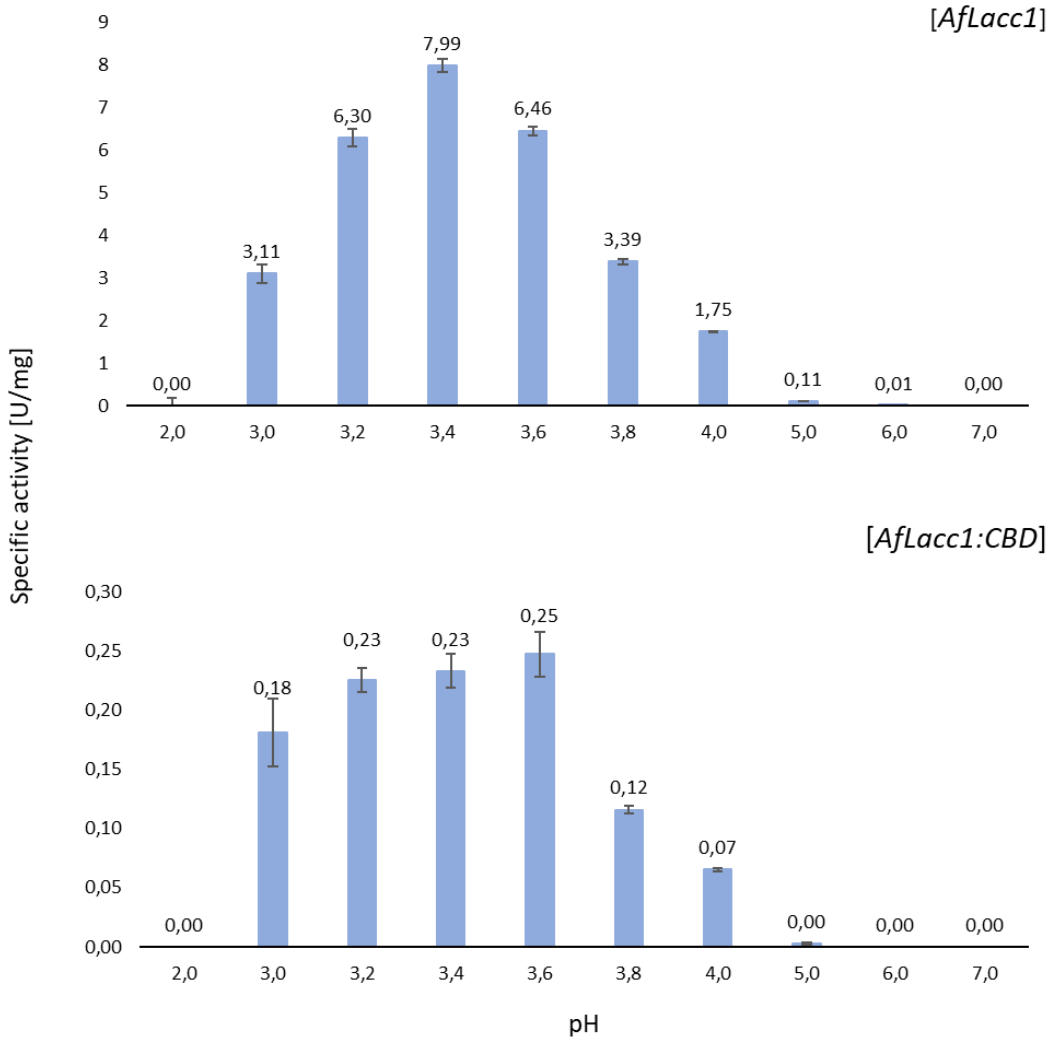



**Figure 16: pH Optimum:** Enzymatic activity of *AfLacc1* and *AfLacc1:CBD* with ABTS as substrate at different pH values.

The values correspond well with data found in literature stating that fungal laccases characteristically have an acidic isoelectric point around pH 4.0 [3]. This also fits to the fact that the optimal pH value for the oxidation of ABTS is typically lower than 4.0, in contrast to most phenolic compounds, like DMP, guaiacaol and syringaldazine, where higher optimal pH values between 4.0 and 7.0 can be observed [107][108].

The low activities of *AfLacc1:CBD* measured at the pH optimum assay (**Figure 16**), compared to the $K_M$ values (

**Table 7**) and the activities shown in the temperature optimum assay (**Figure 17**), are due to varying enzyme expression batches, and differences in protein concentrations.

The aim of this thesis was to design an assay for measuring the antioxidant activity of plasma samples using laccase-oxidized ABTS. Therefore, the enzyme had to be either inactivated or excluded from the

reaction of the antioxidants with the $ABTS^+$ radical. The inactivity of *AfLacc1* and *AfLacc1:CBD* to oxidize ABTS at pH 7 could be one approach, by oxidizing the ABTS at acidic pH and then changing the pH to pH 7 for the antioxidant activity measurement.

Additionally to the optimal pH, the reaction temperature has a great effect on enzyme activity. Therefore the optimal temperature was determined. The temperature optima determination gave similar results for both enzymes. Optimal reaction conditions were reached at 40°C, where the highest activity for both enzymes was measured (**Figure 17**). From 20°C to 40°C the specific activities of *AfLacc1* as well as *AfLacc1:CBD* were increasing. At 50°C the activities were decreasing and at higher temperatures no activities were measured anymore. This rather low optimal temperature is unusual for laccases, which characteristically have temperature optima between 50 and 70 °C, like other extracellular ligninolytic enzymes. There are, however, few enzymes with temperature optima lower than 35 °C [3].

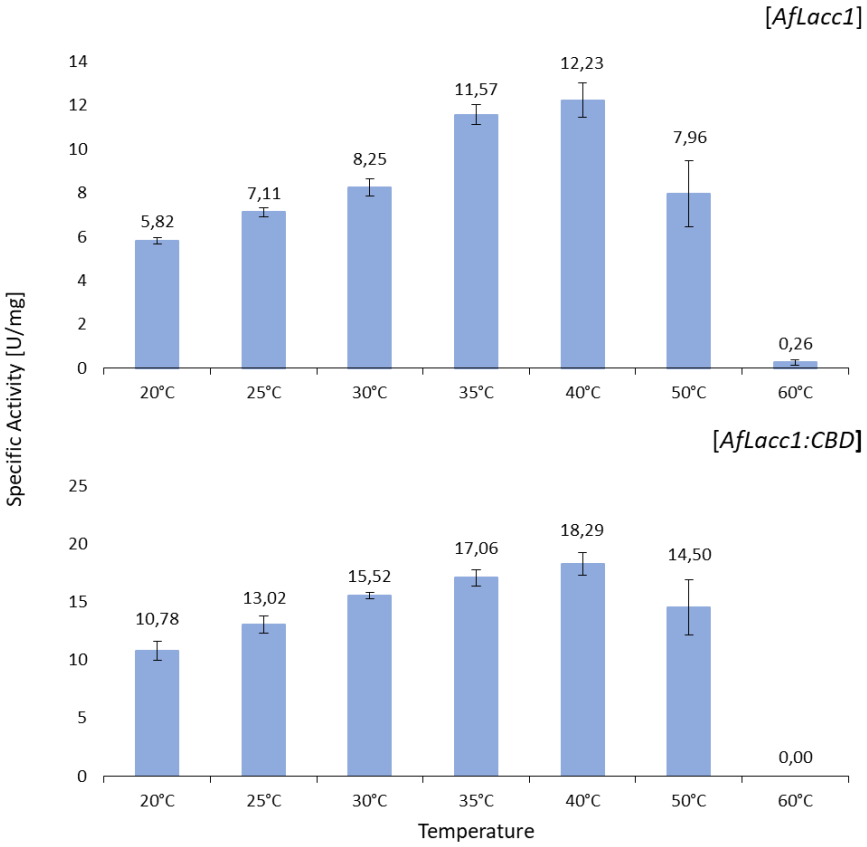



**Figure 17: Temperature optimum**: Enzymatic activity of *AfLacc1* and *AfLacc1:CBD* with ABTS as substrate at different temperatures.

Likewise to the optimal temperature, the temperature stability of fungal laccases varies a lot. A*fLacc1* showed the highest stability at 40 °C, with 80 % remaining activity after 120 h (**Figure 18**). High enzyme stability at high temperatures are usual for fungal laccases.

For this work ABTS needed to be oxidized to the first oxidation product $ABTS^{+}$, which was performed for several hours at 40°C. Therefore, the high stability at the optimal reaction temperature was a great advantage.

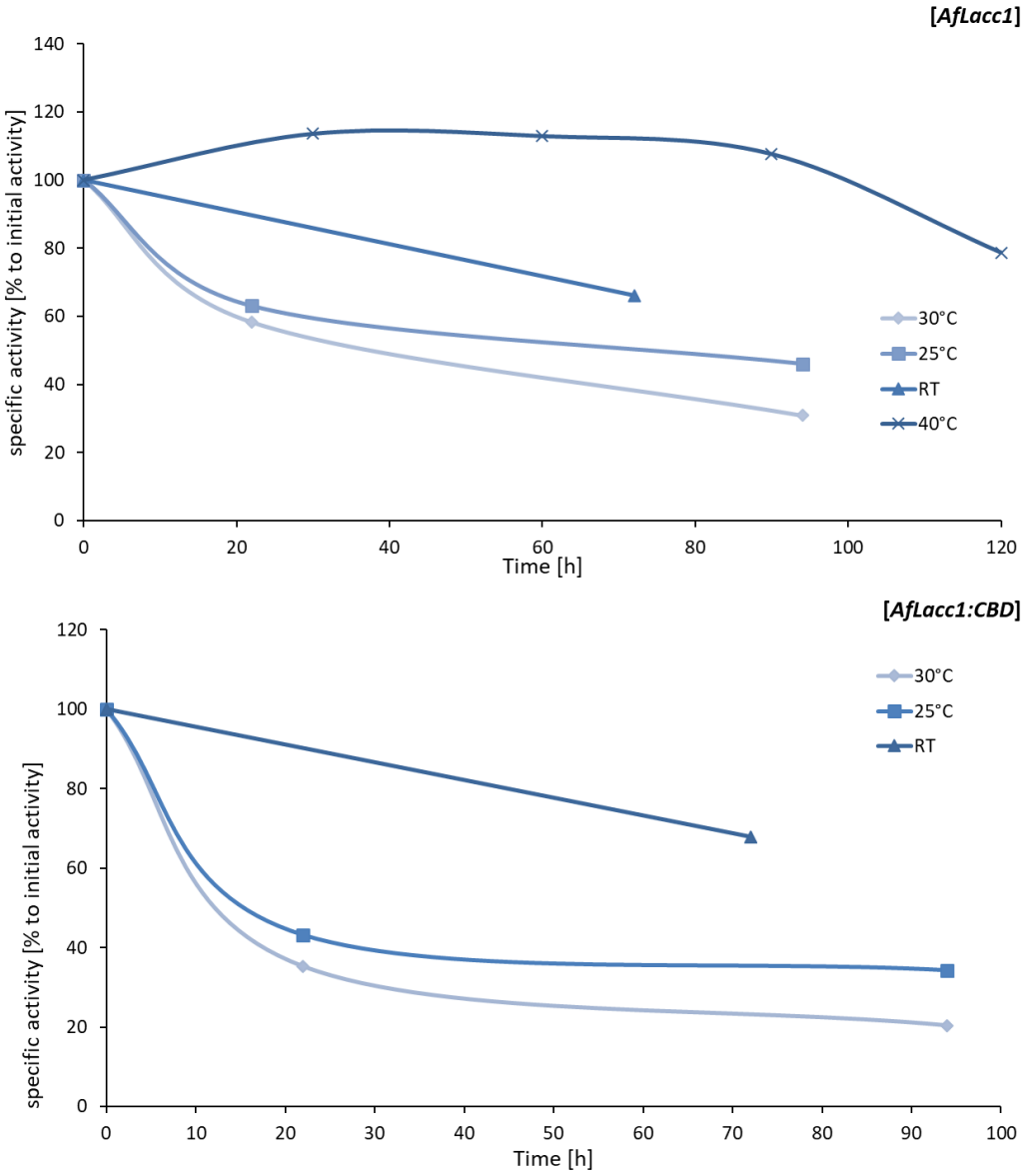



**Figure 18: Temperature stability determination:** Enzymatic activity of *AfLacc1* and *AfLacc1:CBD* with ABTS as substrate measured after storage at different temperatures over a time of 96/120 h. The decrease in activity is shown relative to initial enzyme activity.

### 4.2.3 Enzyme activities and kinetics

Additionally to the optimal reaction conditions for the oxidation of ABTS the enzyme kinetics were determined, since kinetic parameters are used to compare enzyme performances. For creating the Michaelis Menten kinetics for *AfLacc1* as well as *AfLacc1:CBD* preliminary results from pH and temperature optima measurements were used as reaction conditions. In **Figure 19** the graphs of the Michaelis-Menten kinetics of *AfLacc1* and *AfLacc1:CBD* are shown. A graphical determination of the kinetic parameters was made (dotted lines), secondly the values were calculated using the Hanes-Woolf plot (**Figure 20**) and the corresponding **Equation 5** (see 3.3.7).

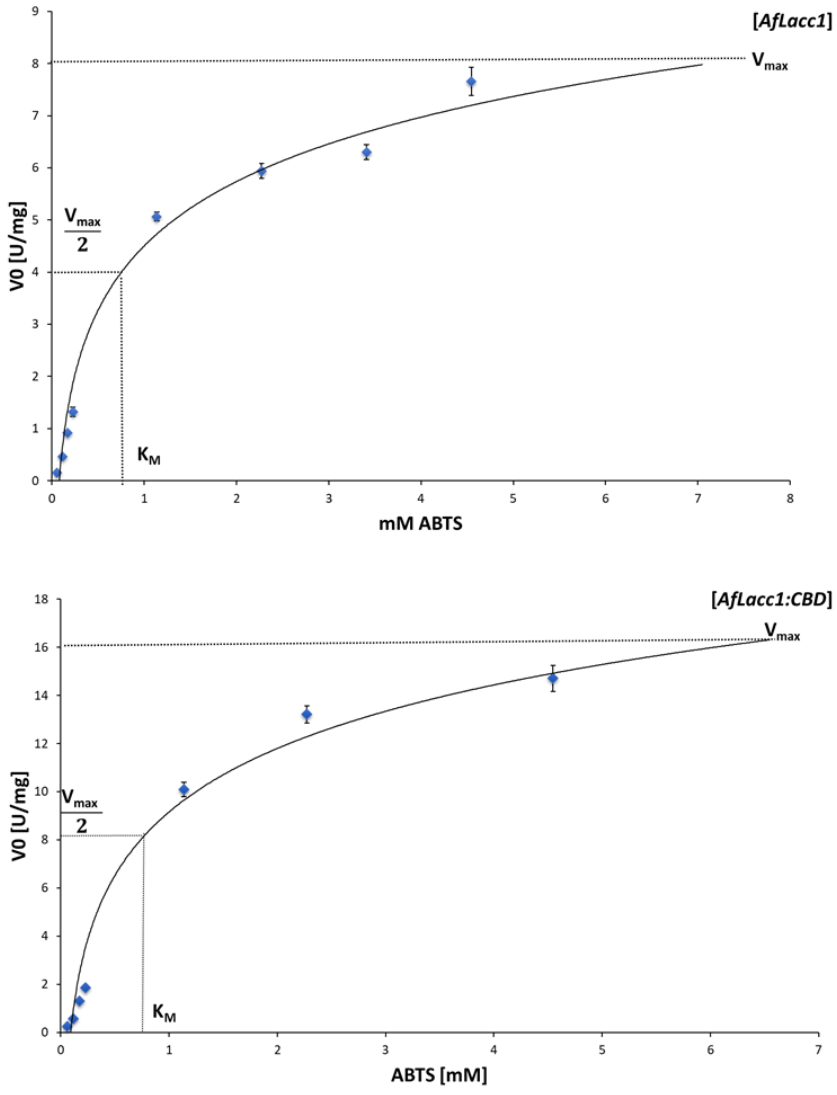



**Figure 19: Michaelis Menten kinetics**: Graph of the obtained Michaelis Menten curve of *AfLacc1* and *AfLacc1:CBD* with ABTS used as substrate, the dotted lines show the graphical determination of $K_M$ and $V_{max}$.

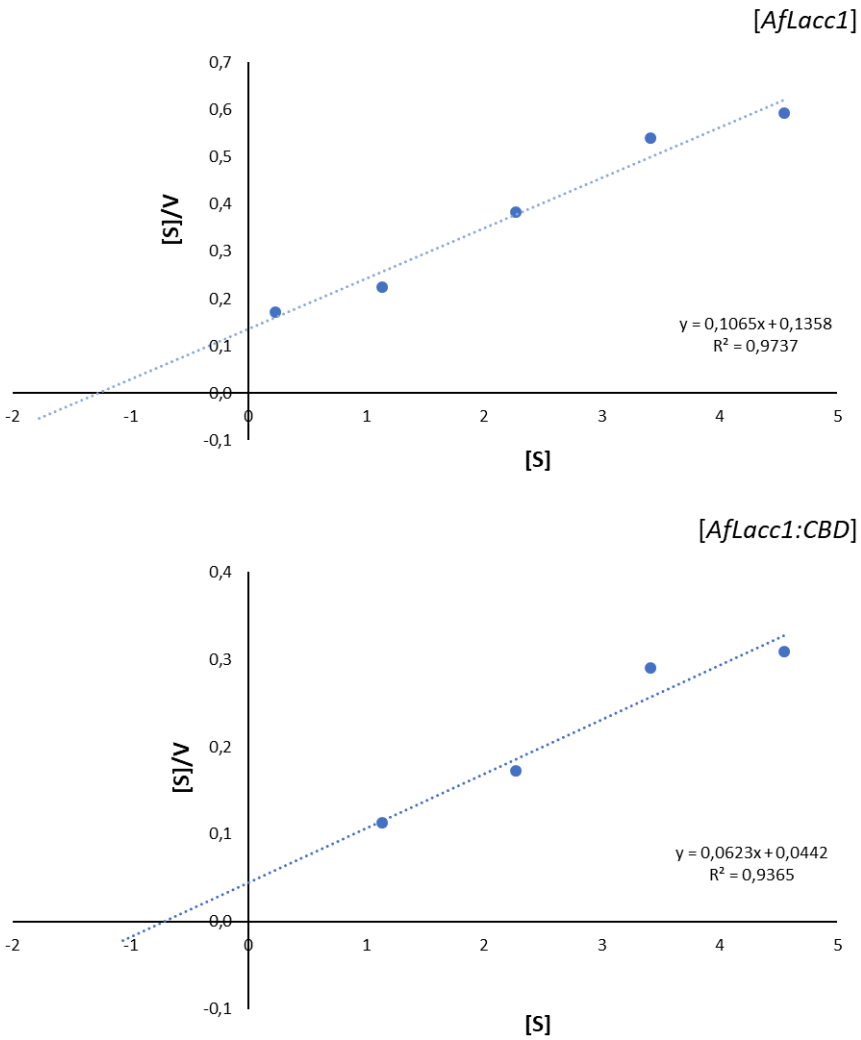



**Figure 20: Hanes-Woolf plot:** plotting of the Michaelis-Menten kinetic of AfLacc1 and *AfLacc1*:CBD according to Hanes Woolf.

Table **7**). For *AfLacc1* the $K_M$ value was expected to be around 0.8 mM and $V_{max}$ at 8 U/mg, the calculated values were slightly higher. For *AfLacc1:CBD,* in contrast, the expected values of $K_M$ (0.8 mM) and $V_{max}$ (16 U/mg) did not differ from the calculated ones.

Moreover the calculated $V_{max}$ values correspond well to the activities determined at the optimal reaction conditions, however for the pH optima of *AfLacc1:CBD* the measured activities were lower due to different enzyme batches.

**Table 7: Enzyme kinetics:** Calculated kinetic parameters of *AfLacc1* and *AfLacc1:CBD* by solving **Equation 5** of the Hanes-Woolf plot.

| Enzyme | Substrate | $K_M$ [mM] | $V_{max}$ [U $mg^{-1}$] |
|---|---|---|---|
| *AfLacc1* | ABTS | 1.27 | 9.39 |
| *AfLacc1:CBD* | ABTS | 0.71 | 16.05 |

The determined kinetic parameters indicate a higher activity for *AfLacc1:CBD* compared to *AfLacc1*. This difference can also be seen in the activity measurements at the optimal reaction conditions. For this reason and the possibility to immobilize *AfLacc1:CBD* to a nanocellulose gel, this enzyme was used for experiments for the antioxidant biosensor.

### 4.2.4 Screening for laccase substrates

One cornerstone of this thesis was the high substrate specificity of *AfLacc1* and *AfLacc1:CBD* to ABTS, which is very untypical for laccases. Laccases usually have a very broad range of oxidizable substrates. The most important group of substrates are phenols due to their low oxidation potentials [109], and as a phenolic compound syringaldazine is often used to identify laccases. Therefore, activities of *AfLacc1* and *AfLacc1:CBD* were tested on all different substrates (**Table 4**).

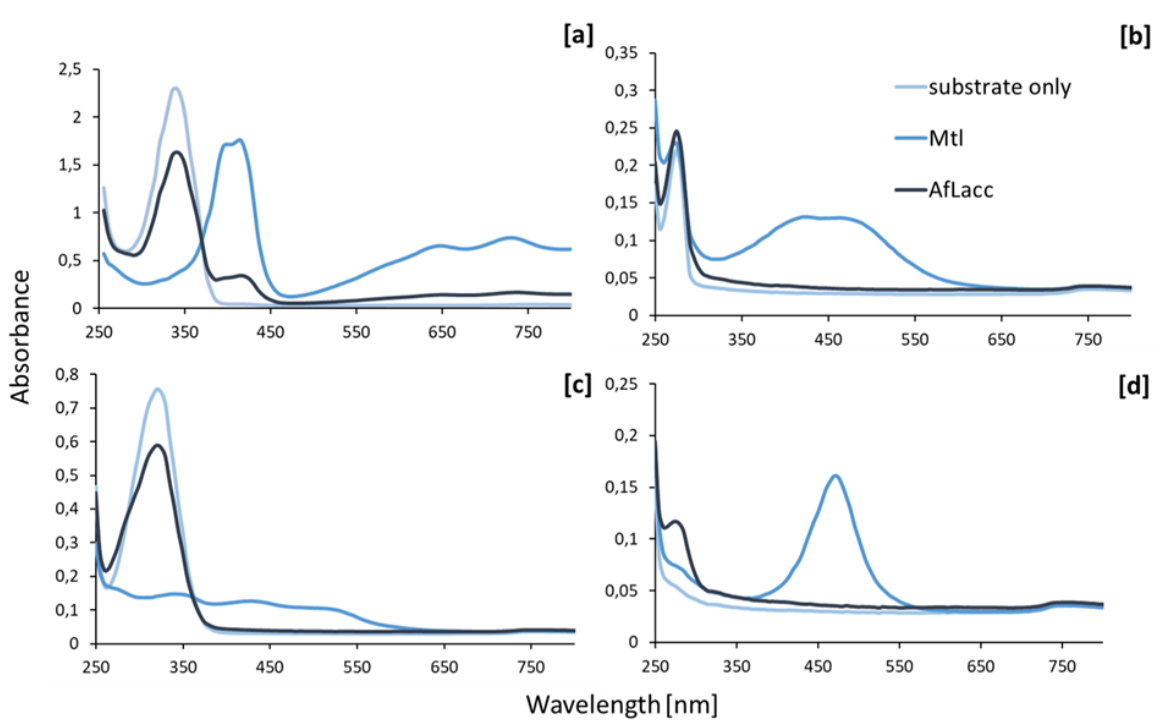



**Figure 21: Substrate specificity:** UV/VIS-Spectra of [a] ABTS, [b] guaiacol, [c] sinapic acid and [d] 2,6 dimethoxyphenol after 30min incubation with *MtL*, *AfLacc1* ([a],[c]) or *AfLacc1:CBD* ([b], [d]).

Incubation with *AfLacc1* and *AfLacc1:CBD* changed the absorbance spectra of ABTS, indicating oxidation. With all other substances no change in the absorbance spectrum after incubation with *AfLacc1* or *AfLacc1:CBD* could be observed (**Figure 21**).

Due to space limitations the figure shows only a subset of the tested substrates. In contrast, a change in the spectrum of all substances was observed in the reference reactions performed with the laccase *MtL*.

The oxidation of ABTS to the radical $ABTS^{+}$ represents one cornerstone of this thesis, therefore spectra of the oxidation products using the various laccases at different timepoints were looked at in detail. The characteristic peek of the reduced ABTS at 340 nm can be clearly seen in the spectrum of the

unreacted substrate. After incubation of 30 min with *AfLacc1* and *AfLacc1:CBD* a peek at this wavelength remains (**Figure 22**). In the case of ABTS incubated with *AfLacc1:CBD* this peak is the same height as for the unoxidized substrate. Still the spectrum shows characteristic peaks for oxidized ABTS at 420 nm and 734 nm. The spectrum used as reference for complete ABTS oxidation with *MtL* looked as expected. No peak at 340 nm remained and the peaks at 420 nm and 734 nm are easily visible. The same trend was observed with *AfLacc1* as oxidizing enzyme after 30 min, but the peak at 340 nm only decreased to approximately half its size, whereas the characteristic peaks for $ABTS^+$ showed half the height of that of *MtL* oxidized ABTS. This indicates that after incubation for 30 min with *MtL* the ABTS in the reaction mixture is already completely oxidized, whereas after that time *AfLacc1* and *AfLacc1:CBD* are only able to partly oxidize the ABTS. For that reason longer reaction times were chosen for following experiments.

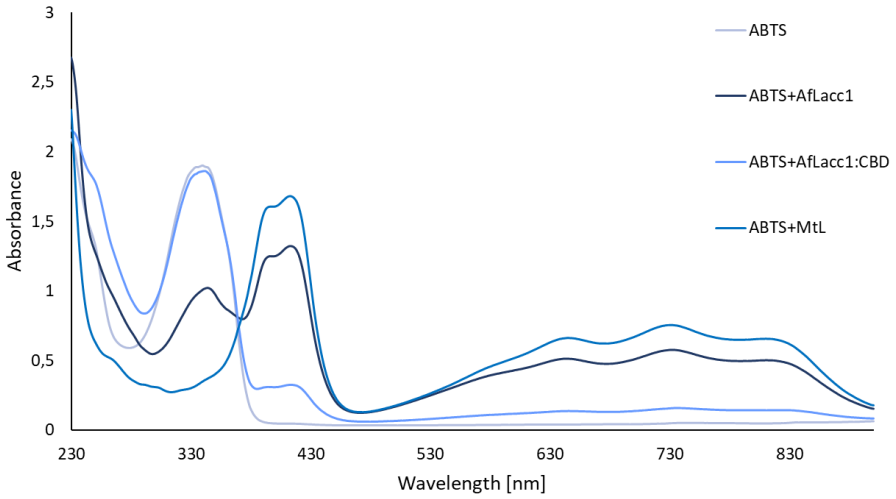



**Figure 22: Oxidation of ABTS**: spectra of ABTS oxidized with various enzymes after 30 min incubation

To sum up the biochemical characterization of *AfLacc1* and *AfLacc1:CBD*, the results are virtually the same for both enzymes. The fusion of the cellulose-binding-domain to the enzyme *AfLacc1*, gave the advantage of a simple way of immobilization to nanocellulosic material. This is why *AfLacc1:CBD* was used for further experiments regarding the application as a biosensor.

## 4.3 Enzyme immobilization

Many expectations according the reaction conditions of an antioxidant capacity biosensor have to be considered while designing the assay. The prevention of interactions of the enzyme used to create the radical with the present antioxidants, as well as re-oxidation or further oxidation of ABTS during the reaction have to be guaranteed. To immobilize the enzyme to a solid support material, represents an easy and fast way to remove the enzyme from the solution after oxidation of ABTS.

During the incubation time of 24 h at 4 °C a decrease in enzyme activity of around 13 % was observed (**Figure 23**). After centrifugation of the nanocellulose-enzyme solution the activity of the supernatant was measured to compare its activity to that of the enzyme alone. A strong decrease of activity in the supernatant would suggest that enzyme is attached to the nanocellulose and therefore no longer measurable in the solution. The activity of the supernatant was approximately half of the activity of the enzyme, which indicated that half of the enzyme was immobilized on the gel.

The enzyme that was not strongly attached to the nanocellulose was washed off. After the third washing step there was hardly any activity measurable in the supernatant (**Figure 23**).

Additionally, the reusability of the nanocellulose-immobilized *AfLacc1:CBD* was tested. The results indicate that the gel can be reused for at least one time. The ABTS oxidized with the once reused nanocellulose-immobilized *AfLacc1:CBD* showed still 96% of the initial absorbance. When washing and reusing the gel again, 64% of initial absorbance could be measured. With further reuses this absorbance decreased until only 18% of absorbance were left at the fourth reuse (**Figure 24**). The decrease in the absorbance of oxidized ABTS could be a result either of dissolution of enzyme from the nanocellulose during the washing, or inactivation of the still attached enzyme due to shear stress by repeated centrifugation steps. Therefore, the nanocellulose gel was reused only 3 times.

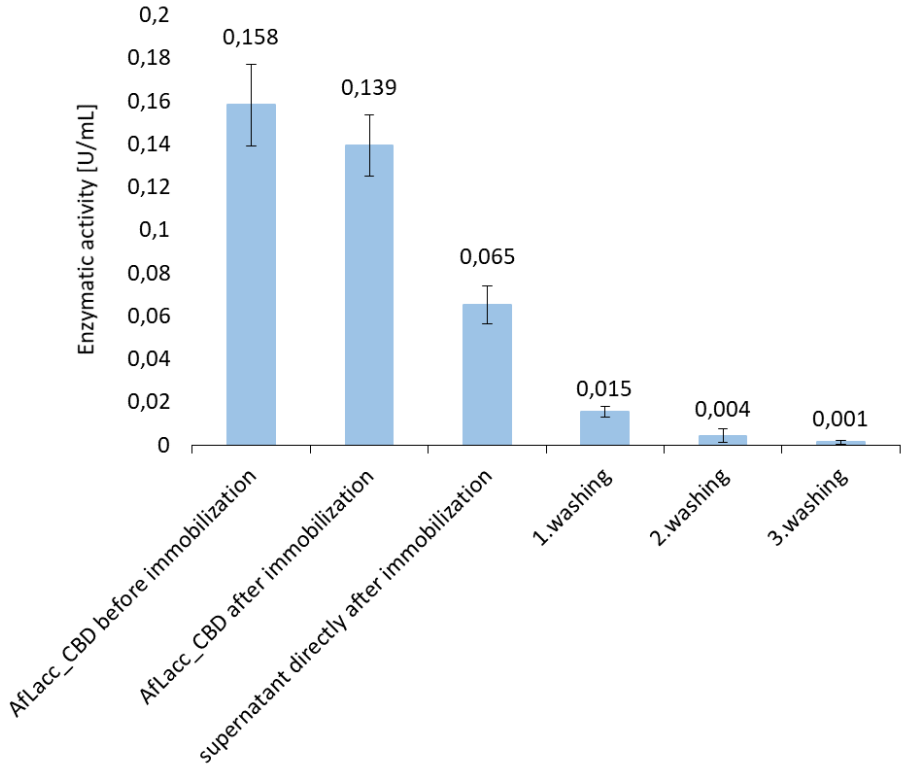



**Figure 23: Enzyme immobilization:** Enzymatic activities of *AfLacc1:CBD* used for immobilization on nanocellulose before and after, and enzymatic activities of washing supernatants after the immobilization.

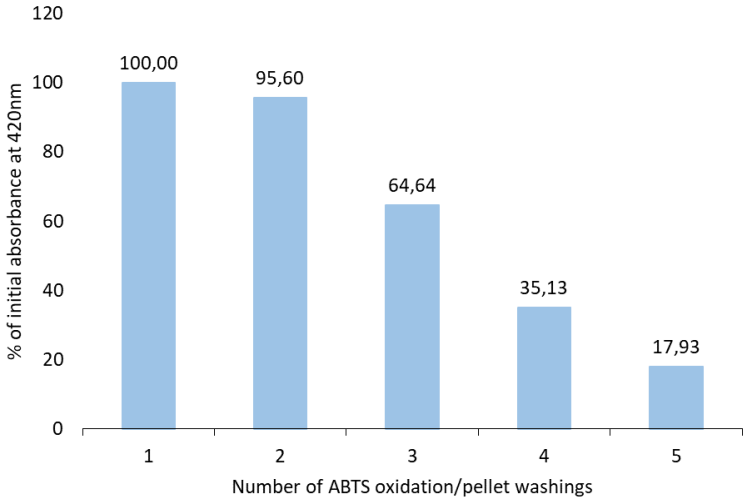



**Figure 24: Reusability of nanocellulose pellets:** Absorbances of ABTS solutions oxidized with immobilized *AfLacc1:CBD* shown as % of the initial absorbance which shows the activity of 1100 µL solved pellet in a 1 cm pathlength cuvette at 420 nm.

## 4.4 Development of an antioxidant activity assay

### 4.4.1 Screening for interactions of enzyme with antioxidants

To exclude any interactions between AfLacc1 and natural occurring human antioxidants and amino acids in blood and plasma, some of the most prominent ones were incubated with *AfLacc1* and *MtL* and changes in UV/VIS spectra were investigated.

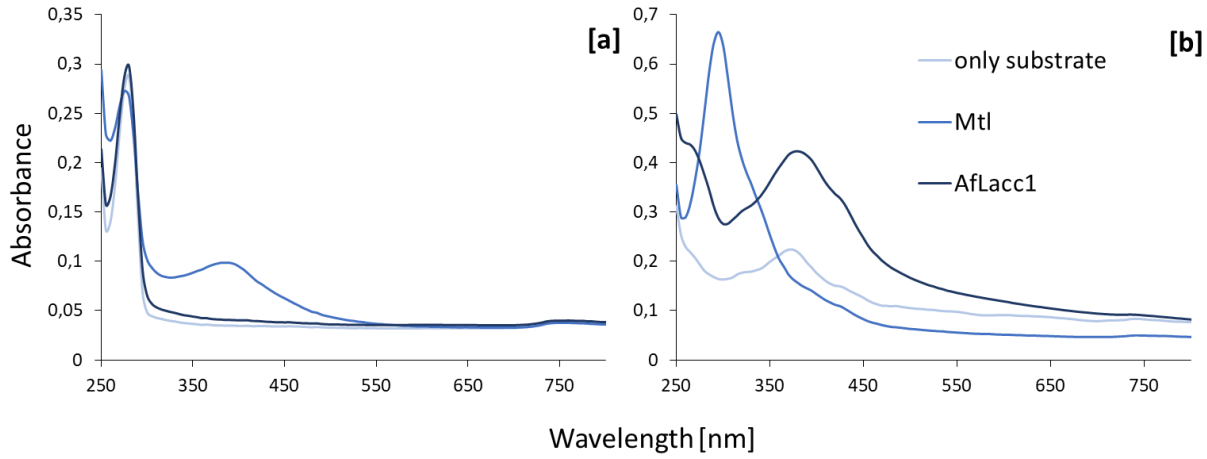



**Figure 25: Interactions with antioxidants**: UV/VIS-spectra of [a] catechin hydrate and [b] quercentin after 30 min incubation with *MtL*, *AfLacc1* and without enzyme.

As expected none of the tested amino acids showed interactions with AflLacc1 and *MtL* (data not shown). **Table 8** shows a list of the tested antioxidants and if there were interactions with the enzymes or not. Most antioxidants did not react with any of the enzymes, only catechin hydrate, quercetin and rutin were oxidized by *MtL* (**Figure 25**). Catechin is a natural occurring phenol and antioxidant, and quercetin is a phenol and flavonoid. Furthermore, the flavonoid rutin is known to be oxidized by laccases [110]. Therefore, it was expected that *MtL* is able to oxidize rutin as well as catechin hydrate and queretin. In conclusion, AfLacc1 showed an oxidation behaviour untypical for laccases. Concerning the aim of this thesis, the high substrate specificity could be exploited and unwanted interactions of the enzyme in the antioxidant biosensor could be excluded. Nevertheless, this uncharacteristic behaviour demands for further investigations concerning the class and characteristics of the enzyme.

**Table 8:** Interactions of antioxidants with the enzymes *AfLacc1* and *MtL*

| | ***AfLacc1*** | ***Mtl*** |
|---|---|---|
| **Malvidin Chloride** | - | - |
| **Catechin hydrate** | - | + |
| **Quercetin** | - | + |
| **Rutin** | - | + |
| **alpha-tocopherol** | - | - |
| **Glutathione** | - | - |
| **L-Ascorbic acid** | - | - |

### 4.4.2 ABTS quenching and Trolox standard curves

The quenching of $ABTS^{+}$ with different antioxidants was done to check the time needed for the antioxidants to reduce the ABTS radical. For most antioxidants the decrease in absorbance at 420 nm stopped after only three minutes incubation time, and the measured absorbance values were constant again. Only the reaction with glutathione took longer (**Figure 26**). As a consequence, glutathione was not considered for the further design of the assay, because of the very small impact of glutathione compared to the whole of antioxidants.

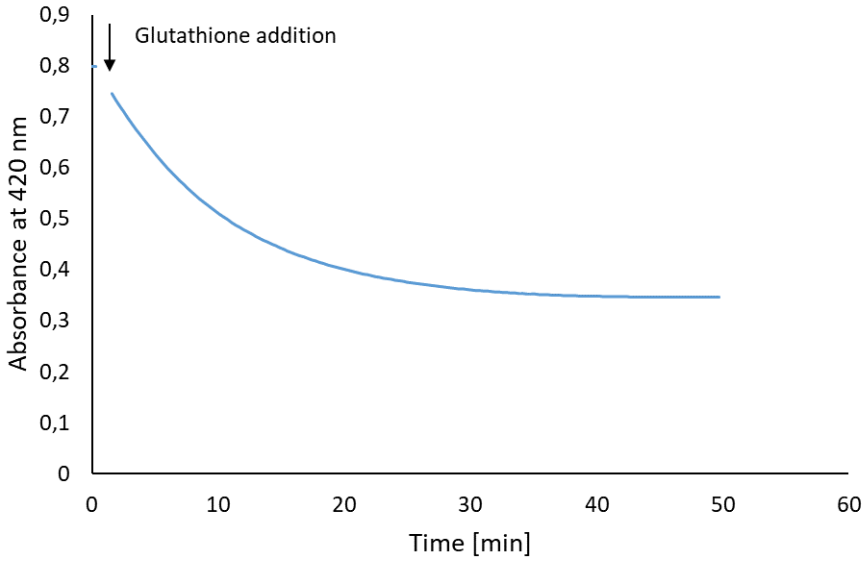



**Figure 26: ABTS quenching with antioxidants:** $ABTS^{+}$ quenching by glutathione over time, the arrow shows the timepoint of glutathione addition.

Accordingly, to the results of the quenching experiments the assay incubation time was chosen to be 3 minutes. Consequently, the same incubation time was used for the Trolox reference measurements. The Trolox standard curve was measured prior to every other experiment with antioxidants or plasma samples. A linear correlation between decrease in absorbance and the amount of added Trolox could be proven (**Figure 27**). From the equation of the linear correlation, Trolox equivalents of other antioxidants or human plasma samples could be calculated.

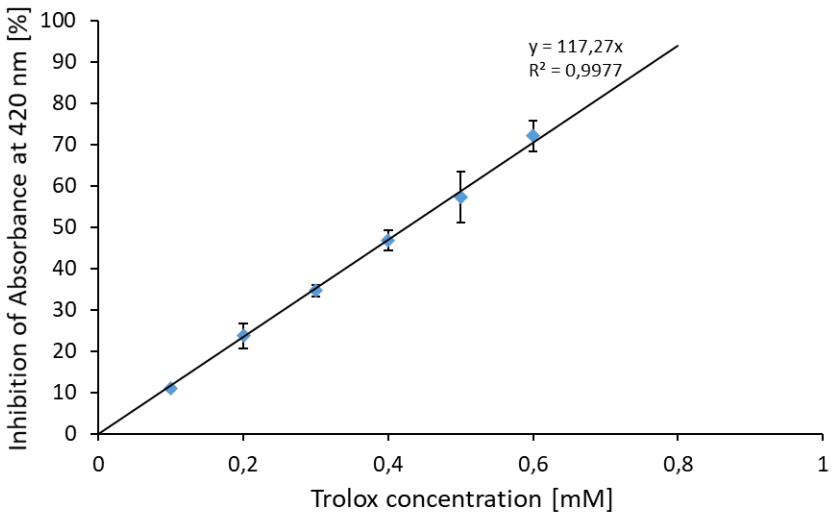



**Figure 27: Trolox standard curve:** Decrease in absorbance at 420 nm is shown against the concentration of Trolox [mM].

### 4.4.3 Spiking of human plasma samples

Human plasma contains many substances in varying concentrations, especially numerous so called plasma proteins, responsible for the transport of glucose, lipids and hormones through the body [111]. To assure that none of these components interfere with the assay, human plasma samples were examined..

The absorbance decrease after 3 minutes reaction time in the oxidized ABTS was measured with human plasma samples as well as spiked human plasma samples. The values for the unspiked plasma samples were then substracted from the values of the spiked samples, to see if the impact of the Trolox concentration was comparable to the Trolox reference measurements. Thereby it was proven that no effects of plasma components interfere with the assay.

At the lower used Trolox concentrations the difference between the spiked samples and the pure Trolox samples is only 1-3%. Contrary at the higher Trolox concentration (0.2 mM) the decrease in absorbance of the spiked plasma samples deviates from the pure Trolox even to 10%, which seems significant (**Figure 28**).

The high antioxidant level in human plasma samples lead, however, to a complete reduction of $ABTS^{+}$ present in the assay. Therefore, it was not possible to measure different antioxidant levels in the human plasma samples. The spiked Trolox could not be measured either. This showed that the assay needed improvement to determine antioxidant levels in human samples. This challenge could be

overcome by higher $ABTS^+$ concentrations, which could not be reached by oxidation with *AfLacc1:CBD*, however.

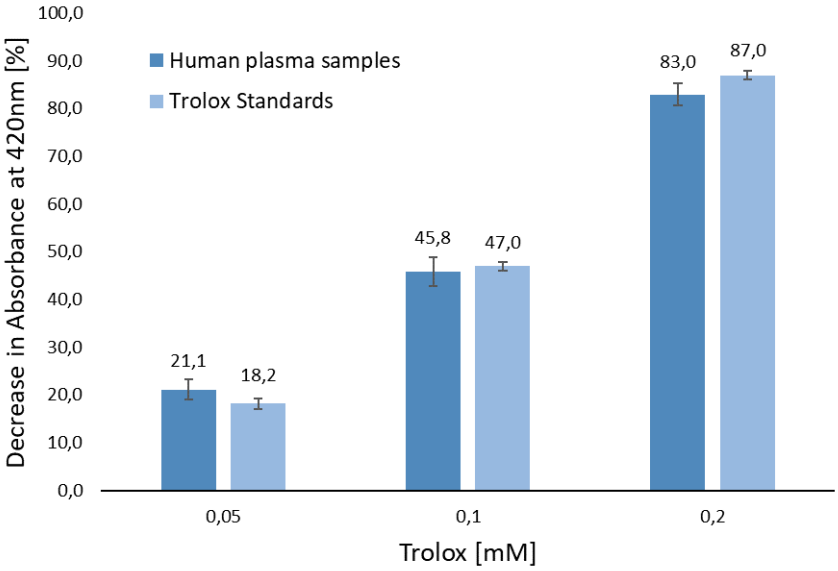



**Figure 28: Spiked human plasma samples:** Comparison of decrease in absorbance at 420 nm of the Trolox standard curve and the Trolox spiked samples

To sum up the concept for the antioxidant assay with ABTS oxidized by *AfLacc1:CBD* could be proven. There were, however, some drawbacks. Very high $ABTS^+$ concentrations would be needed to be quenched by human plasma antioxidants without total loss of colour and resulting inability of measurement. Additionally, the plasma samples contained antioxidant concentrations too high to be used undiluted. Consequently, a dilution step seems necessary, which increases the demand of work and time.

# 5 Conclusion and Outlook

The aim of this thesis was the biochemical characterization of two heterologously expressed fungal laccases, to compare their properties to that of other commercially available laccases and data of already published heterologously expressed fungal laccases.

Following the characterization results one of the enzymes, namely *AfLacc1:CBD*, was chosen to be used in the application of an antioxidant biosensor. First experiments to proof the concept were performed. Especially the high substrate specificity for ABTS of *AfLacc1:CBD* makes the enzyme highly interesting for the design of an application as an antioxidant biosensor.

It was demonstrated that *AfLacc1* and *AfLacc1:CBD* were able to oxidize ABTS. Due to the inactivity of the enzyme at pH 7, an easy way to inactivate the enzyme after the oxidation was available. Moreover, *AfLacc1:CBD* was successfully immobilized onto a nanocellulose gel, which represented another way to separate the enzyme from the oxidized ABTS in the reaction mixture, and enabled the enzyme to be used up to 3 times, which makes applications more economical. Furthermore, a linear relationship in the decrease of absorbance at 420 nm and 734 nm of $ABTS^+$ to the amount of Trolox, was established. Besides that, $ABTS^+$ quenching after addition of other antioxidants was proven. These preliminary experiments showed promising results towards the applicability of the assay.

First experiments using human plasma samples were performed and showed that the antioxidant concentration in biological samples was too high for the assay. A dilution of the samples prior to antioxidant capacity testing was necessary, which makes it hard to employ in the clinical routine work, since there are methods which use the samples directly. Nonetheless, it was proven that no matrix effects of the plasma samples interfere with the assay, and the general functionality of the assay was confirmed.

Higher $ABTS^+$ concentrations for the reaction would be preferable and could either be reached by improving the activity of the enzyme or by higher enzyme concentrations, to make it easier to use it in a high amount. With increase of the radical concentration also the challenge of high antioxidant concentrations in biological samples could be overcome.

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# 7 Appendix

## 7.1 Sequence of *AfLacc1* and *AfLacc1:CBD*

HLVLHDDSFQPDHILRVTAQDVNQACMDRYSVLINGSLPGPQLNIQEGKVNWIRVYNDMEDLNVTMHWHGLSAFTAP
FSDGTPMASQWPIPPGHFFDYEVRPEVGYAGTYFYHSHVGFQALTAWGALIVESAQPSPYQYDEERIIALSDFFTKTDEEI
ENGLTSTNFTWSGETSAVLVNGQGRLATNATGSCKLAAISVEPGKTYRLRFIGATALSFVSISLESHDVLEIIEADGHYTKPV
NTSYLQISSGQRYSVLLKAKTEAELQQAKSRQFYFQLTTMGRPTVLTTFAVLEYPSPTTTDLITVPVTPPLPVANITYGWLDY
TLEPYYPDLDFPTVEEVTRRIIINVHQNISDRTVWLQNGYDWVETFPKSPYLVDIYAGTLDLDASYKRAIASGYAFDNQTRL
FPAKMGEVLEIVWQNQGAVSNGGVENHPFHAHGRHFYDIGGGDGLYNLTENEARLKGTHPVIRDTTMLYAYRKTTTAL
EPSGWRAWRIRVTAAGVWMVHCHVLQHMLMGMQTAFAFGDQTAIKAQSGTPAEGYLTYGGSAYGNVTHFPPVKHF
FNPPGGNRGTTTTRRPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL*

Red: *AfLacc1*
Blue: Linker of Cellobiohydrolase I aus *Trichoderma reesei*
Green: cellulose-binding domain of Cellobiohydrolase I from *Trichoderma reesei*

MW *AfLacc1*: 62.8 kDa
MW *AfLacc1*-CBD: 68.8 kDa

## 7.2 Extinction coefficient of ABTS at various pH values

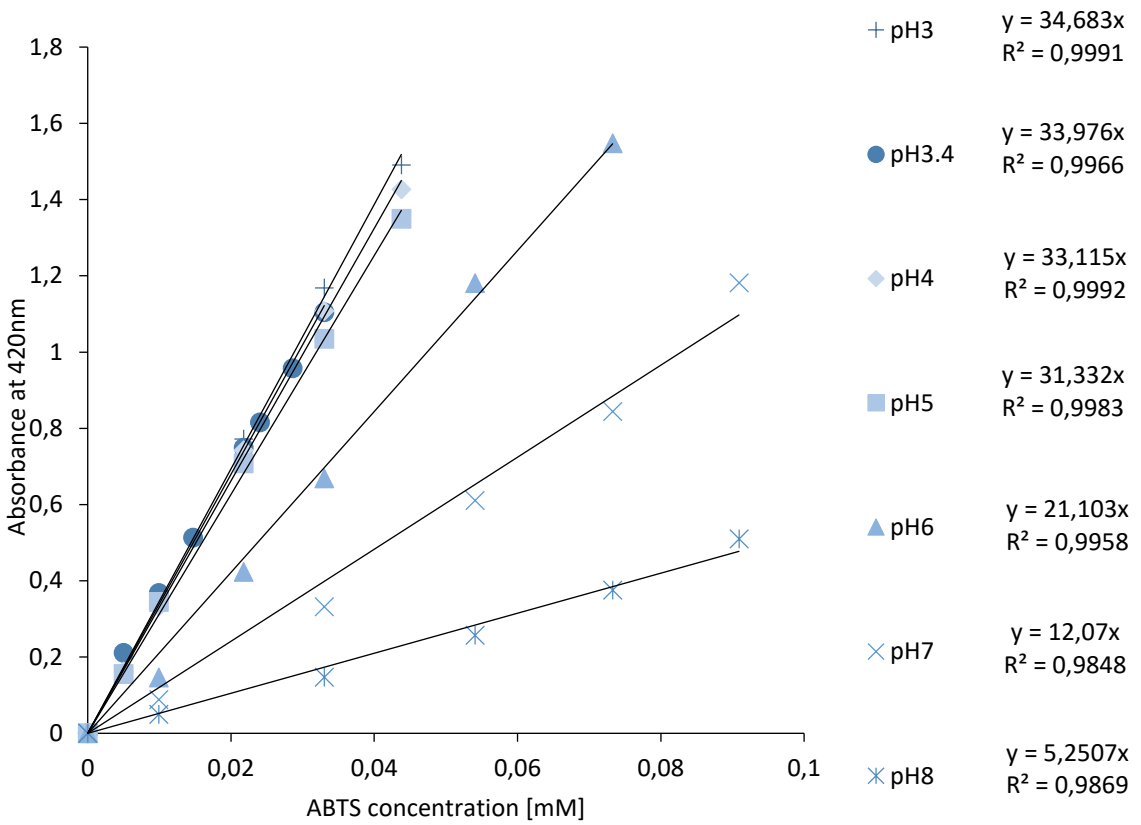


**Figure 29:** Determination of extinction coefficients of ABTS at 420nm and different pH values.

# 8 Figures

# 9 Tables



# 10 Equations

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# 12 List of Abbreviations

| | |
|---|---|
| AAPH | 2,2-azobis-(2-amido-propane)dihydrochloride |
| ABAP | 2,2-diazobis-(2-amidino-propane)dihydrochloride |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) |
| B-PE | B-phycoerythrin |
| CAT | Catalase |
| CBD | Cellulose-binding-domain |
| CVD | Cardiovascular Disease |
| DPPH | 2,2 diphenyl-1-picrylhydrazyl |
| EPR | Electro Paramagnetic Resonance |
| ET | Electron Transfer |
| FRAP | Ferric Reducing Antioxidant Power |
| GPx | Glutathione Peroxidase |
| GSH | Glutathione |
| HAT | Hydrogen Atom Transfer |
| LDL | Low-Density-Lipoprotein (Cholesterin) |
| MCO | Multicopper Oxidases |
| *MtL* | Laccase from *Myceliophthora thermophila* |
| ORAC | Oxygen Radical Absorption Capacity |
| ROS/RNS | Reactive Oxygen/Nitrogen Species |
| SCP | Single cell proteins |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamid Gel Electrophoresis |
| TEAC | TEAC |
| UV/VIS | Ultraviolet/Visible spectrum |