Characterization and application of lignocellulose degrading oxidoreductases from *Moniliophthora roreri*

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ABSTRACT

High-redox potential oxidoreductases as laccases and peroxidases are powerful enzymes with high potential for industrial biotechnology. They oxidize a vast variety of substrates, using only O_2 or H_2O_2 as co-substrate, and can be used in the paper and pulp industry, food and pharmaceutical industry, for dye and drug degradation and lignin modification in biorefineries. However, their low availability due to low expression levels, limits their broad application. In the present three-part work, a laccase and a manganese peroxidase from the aggressive pathogenic fungus *Moniliophthora roreri* were produced with the help of the recombinant methylotrophic yeast *Pichia pastoris*. Subsequently the enzymes were purified and their biotechnological potential was explored.

The expression levels achieved in a fed-batch fermentation for both the laccase Mrl2 (1.05 g per L) and peroxidase MrMnP1 (132 mg per L) were among the highest reported so far. In the first part of this doctoral thesis, biochemical characterization of the Mrl2 laccase was performed. The laccase showed a redox-potential of 0.58 V which categorizes it as a mid-redox potential laccase. Still, it showed high k_{cat} values towards common laccase substrates and was able to degrade micropollutants 17α-ethinyl estradiol, estrone, 17β-estradiol, estriol and bisphenol A at neutral pH faster than high-potential laccases from *Trametes versicolor*. In the second part, the manganese peroxidase MrMnP1 from *M. roreri* was expressed and mutants thereof were constructed. Subsequently the mutated enzyme variants were characterized, and their biotechnological potential was examined. By replacing alanine 172 with tryptophane, an additional superficial active center was introduced, which enabled the oxidation of high redox potential substrates such as a non-phenolic lignin dimer. Such a superficial tryptophan is present in versatile peroxidases but not in manganese peroxidases. Thus, the original manganese peroxidase was redesigned into a versatile peroxidase. The mutants turned out to be more stable

and had a broader substrate spectrum compared to the wild type. MnPs accept only Mn^{2+} as a substrate, which in turn can act as a redox mediator and oxidize other substrates. But also, MnPs that accept other substrates than Mn^{2+} have been described. Therefore, the role of manganese in dye degradation, catalyzed by MrMnP1 wild type and mutants thereof was evaluated. Interestingly, the degradation of crystal violet and Reactive Black 5 at pH 5 was inhibited by the addition of manganese, in contrast to pH 3, where manganese did not have an inhibitory effect but was even required for degradation by the wild-type enzyme. In the third part, both laccase and manganese peroxidase, were applied for enzymatic pretreatment of lignocellulose to enhance subsequent saccharification of this biomaterial by cellulases. The obtained glucose contained in the cellulose of lignocellulose from fast-growing perennial *Miscanthus x giganteus* could be used as a renewable raw material source in biorefineries. It is known that lignin prevents cellulases from degrading cellulose and pre-treatment is necessary. The mechanism of the inhibition however is not fully understood. Either the lignocellulose itself or liberated phenolic compounds may inhibit the cellulases. Treatment of *Miscanthus* with laccase and/or the peroxidase (with addition of H_2O_2 or a source thereof) resulted in higher released sugar amounts compared to non-pretreated *Miscanthus*. Analysis of the reaction mixture showed that the concentration of phenolic compounds was lower in the samples with laccase but not with the peroxidase. This suggests that the effect of the oxidoreductases may be related to the oxidation of the lignin in the lignocellulose rather than oxidation of the free phenolic compounds.

ZUSAMMENFASSUNG

Oxidoreduktasen mit hohem Redoxpotential wie Laccasen und Peroxidasen sind sehr leistungsfähige Enzyme, mit hohem Anwendungspotenzial in der industriellen Biotechnologie. Sie oxidieren eine Vielzahl von Substraten, wobei sie nur O_2 oder H_2O_2 als Co-Substrat verwenden. Sie können in der Papier- und Zellstoffindustrie, in der Lebensmittel- und Pharmaindustrie, im Farbstoff- und Arzneimittelabbau und bei der Ligninmodifizierung in Bioraffinerien eingesetzt werden. Ihre geringe Verfügbarkeit, bedingt durch die geringe Expression, macht ihre industrielle Anwendung jedoch recht schwierig. In dieser dreiteiligen Arbeit wurden eine Laccase und eine Manganperoxidase aus dem aggressiven pathogenen Pilz *Moniliophthora roreri* mit Hilfe der methylotrophen Hefe *Pichia pastoris* rekombinant hergestellt. Anschließend wurden die Enzyme aufgereinigt und ihr biotechnologisches Potenzial untersucht.

Das Expressionslevel in einer Fed-Batch-Fermentation sowohl für die Laccase Mrl2 (1,05 g pro L) als auch für die Peroxidase MrMnP1 (132 mg pro L) gehörte zu den höchsten, die bisher berichtet wurden. Im ersten Teil der Arbeit wurde die Laccase Mrl2 biochemisch charakterisiert. Mrl2 zeigte ein Redoxpotential von 0,58 V, was sie in die Gruppe der Laccasen mit mittlerem Redoxpotential einreiht. Dennoch zeigte sie hohe k_{cat}-Werte gegenüber gewöhnlichen Laccasesubstraten und war in der Lage, Mikroverunreinigungen wie 17α-Ethinylestradiol, Estron, 17β-Estradiol, Estriol und Bisphenol A bei neutralem pH-Wert schneller abzubauen als die hochpotenziellen Laccasen von *Trametes versicolor*. Im zweiten Teil wurde die Manganperoxidase aus *M. roreri* exprimiert und es wurden Mutanten davon hergestellt. Anschließend wurden die mutierten Enzymvarianten charakterisiert und ihr biotechnologisches Potenzial untersucht. Die Mutation A172W, die einem Trp gleicht, das in versatile Peroxidasen, aber nicht in Manganperoxidasen vorkommt, führte ein zusätzliches

aktives Zentrum in das Enzym ein und ermöglichte die Oxidation von Substraten mit einem hohen Redoxpotential wie z.B. einem nichtphenolisches Lignindimer. Die Mutanten erwiesen sich als stabiler und hatten im Vergleich zum Wildtyp ein breiteres Substratspektrum. In der Fachliteratur wurden verschiedene Typen von MnPs beschrieben. MnPs, die nur Mn^{2+} als Substrat akzeptieren, das wiederum als Redox-Mediator fungieren und andere Substrate oxidieren kann. Aber auch MnPs, die andere Substrate als Mn^{2+} akzeptieren, wurden beschrieben. Aus diesem Grund wurde die Rolle von Mangan beim Farbstoffabbau untersucht. Interessanterweise wurde der Abbau von Kristallviolett und Reactive Black 5 bei pH 5 durch die Zugabe von Mangan gehemmt, im Gegensatz zu pH 3, wo Mangan keine hemmende Wirkung hatte, sondern sogar für den Abbau mit dem Wildtyp-Enzym erforderlich war. Im dritten Teil wurden sowohl Laccase als auch Manganperoxidase zur enzymatischen Vorbehandlung von Lignocellulose eingesetzt, um die nachfolgende Verzuckerung dieses Biomaterials durch Cellulasen zu verstärken. Die gewonnene Glukose, die in der Cellulose der Lignocellulose aus dem schnell wachsenden, mehrjährigen *Miscanthus x giganteus* enthalten ist, könnte als erneuerbare Rohstoffquelle in Bioraffinerien verwendet werden. Es ist bekannt, dass Lignin die Cellulasen daran hindert, Cellulose abzubauen, so dass eine Vorbehandlung erforderlich ist. Der Mechanismus der Hemmung ist jedoch nicht vollständig verstanden. Entweder die Lignocullulose selbst oder phenolische Verbindungen, die in die Flüssigkeit freigesetzt werden, können die Cellulasen hemmen. Miscanthus, der mit Laccase und/oder der Peroxidase (unter Zusatz von H_2O_2 oder einer Quelle davon) inkubiert wurde, führte im Vergleich zu nicht vorbehandeltem Miscanthus zu höheren freigesetzten Zuckermengen. Die Analyse der Flüssigkeit zeigte, dass die Konzentration der phenolischen Verbindungen in den Proben mit Laccase, aber nicht mit der Peroxidase, niedriger war. Dies deutet darauf hin, dass die Wirkung der Oxidoreduktasen eher mit der Oxidation des Lignins in der Lignocellulose zusammenhängt als mit der Oxidation der freien phenolischen Verbindungen.

1 TABLE OF CONTENT

2 INTRODUCTION

2.1 GREEN CHEMISTRY AND A SUSTAINABLE WORLD

The ever-growing world population and its ever-increasing material needs require actions to ensure an acceptable standard of living. In the 1990's the term "green chemistry" was introduced when people began to address the question how to make chemistry sustainable. There are twelve principles which were developed and published by Anastas and Warner in 1998 that are a guideline how sustainable chemistry might be accomplished [1]. Within these principles the use of renewable raw materials and catalysts in chemical reactions is suggested [2].

Regarding the use of renewable raw materials, lignocellulose (plant dry matter) is considered sustainable as its cultivation does not compete with that of edible plants. However, pretreatment is required to release the valuable glucose in a process called saccharification. In non-pretreated lignocellulose the recalcitrant polymer lignin prevents cellulases from hydrolyzing the cellulose. In nature white rot fungi are well known to degrade lignin. They secrete enzymes as laccases and peroxidases, which are responsible for lignin modification and degradation.

Although it is not regarded as a principle of "green" chemistry, biological remediation plays a major role in preserving the environment in a "green" state. The first principle is to avoid waste. However, sometimes it is difficult to avoid waste when textiles are dyed and the dyes end up in sewage, or even more so when drugs such as painkillers or contraceptives pass through the body, sometimes even unchanged. Fungi are known to detoxify soils by secreting laccases and peroxidases, which are involved in this process [3].

In addition, laccases and peroxidases can be used for the production of chemicals, where "green" catalysts are needed. "Green" catalysts are environmentally safe and allow to avoid

toxic heavy-metal catalysts. These catalysts are supposed to work for many catalytic cycles, in environmentally friendly solvents, have high activity and selectivity and cause minimum waste. Enzymes, which consist mainly of protein could meet all those requirements. Laccases are often referred to as "green catalysts" because they only require oxygen as a co-substrate and produce water as the only by-product. They can oxidize a large number of substrates via electron abstraction and are therefore of interest for biocatalysis. Peroxidases, which perform similar reactions to laccases but are also able to oxidize substrates with high redox potential that are not accessible to laccases, require hydrogen peroxide as electron acceptor and leave water as the only by-product. However, there are still some drawbacks such as low stability, activity and availability which need to be addressed to ensure broad application of both enzyme groups, also at high scale.

2.2 Fungal Oxidoreductases

Fungi are key players in environmental carbon recycling. While plants fixate carbon for structural stability in a three-dimensional, recalcitrant polymer called lignin, fungi are able to break it down and re-introduce it into the carbon cycle. Lignin is composed of different aromatic phenols which are polymerized to a recalcitrant, three-dimensional protective and stability providing amorphous matrix [4]. After cellulose, lignin is the second most abundant polymer and the most abundant aromatic polymer on earth, thus a promising source of renewable raw materials. Due to its high recalcitrance lignin is not fully utilized and mostly left to decompose. In nature the most efficient degraders of lignin are white rot fungi [5, 6] which belong to the phylum Basidiomycota. These fungi have developed a very powerful enzyme consortium, most belonging to the group of oxidoreductases, which is capable of modifying and breaking down the recalcitrant lignin polymer. However, since lignin always and without exception occurs together with cellulose, lignin degrading enzymes were recently included into the Carbohydrate-Active enZYmes (CAZy) database which previously only comprised enzymes

active on carbohydrates. Now the CAZy database includes enzymes acting on cellulose namely glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs) and carbohydrate esterases (CEs) and oxidoreductases called auxiliary activities (AAs) which include carbohydrate active lytic polysaccharide monooxygenases (LPMOs) and lignin active oxidoreductases [7]. The AAs are divided into several subfamilies. The subfamily AA1 comprises multicopper oxidases including laccases; the subfamily AA2 comprises lignin-modifying class II peroxidases including manganese peroxidases (MnPs), lignin peroxidases (LiPs) and versatile peroxidases (VPs) [7]. AA1 and AA2 enzymes can modify lignin directly or through small redox mediators as carboxylic acid chelated Mn^{3+} or small phenolic substrates. In addition, other AA subfamilies contain enzymes that do not directly act on lignin, but are necessary for its complete degradation as they provide H_2O_2 as electron acceptor for peroxidases for example. More recently dye decolorizing peroxidases (DyPs) and unspecific peroxygenases (UPOs) were discovered which might also contribute to lignin degradation but their role still has to be clarified [8, 9].

2.2.1 Laccases

The enzyme laccase was first described about 137 years ago in 1883 [10]. Yoshida extracted this enzyme from the exudates of the Japanese lacquer tree *Toxicodendron vernicifluum* formerly known as *Rhus vernicifera*. In 1896 laccase was also described in fungi [11]. Laccases (*p*-diphenol:dioxygen oxidoreductase, EC1.10.3.2) are multi-copper oxidases (MCO) which catalyze the one electron oxidation of diverse phenols, aromatic amines or inorganic ions and the four electron reduction of molecular oxygen to water. Their substrate spectrum can be extended by the use of redox mediators. Redox mediators are small molecules that can be oxidized by laccase and then oxidize sterically inaccessible to laccase or recalcitrant non-phenolic compounds which have a redox potential too high to be directly oxidized by the laccases [12, 13]. In 1990 Bourbonnais first showed that 2,2'-azino-bis (3-ethylbenzothiazoline-

6-sulfonic acid (ABTS) can serve as redox mediator and enable veratryl alcohol (VA) oxidation in the presence of laccase. Ideally, a redox mediator can perform many cycles without side reactions and its oxidized and reduced forms are quite stable [14].

Since the substrate spectrum of laccases is rather broad and overlaps with other enzymes (e.g. tyrosinases and peroxidases), they can be identified by their ability to oxidize syringaldazine, inability to oxidize tyrosine and their independence from H_2O_2 [15-17].

2.2.1.1 Fungal Laccases

In fungi, laccases occur in ascomycetes and deuteromycetes, but especially in lignin-degrading basidiomycetes [18]. Due to their higher redox potential (up to 0.8 V) than laccases originating from plants, insects or bacteria, biotechnologists have focused on fungal laccases for many years. Although studied for decades, their role in lignin degradation is rather enigmatic. Laccases are not able to oxidize the non-phenolic part of lignin directly, which constitutes about 80 % of the total lignin, but use small natural redox mediators that play a crucial role since they can expand the substrate spectrum of these enzymes towards the non-phenolic part of lignin [13]. It has been hypothesized that laccases may also have an indirect function in lignin degradation. On the one hand, laccase-free fungi for example *Sporotrichum pulverulentum* were found incapable to degrade kraft lignin or wood [19]. Only after laccase addition lignin degradation could be reconstituted. On the other hand, it was demonstrated that the inhibition of the laccase in *Trametes versicolor* (*Coriolus versicolor*) cultures by antibodies did not affect the rate of lignin degradation [20]. Although the genome of the basidiomycete *Phanerochaete chrysosporium* does not contain a laccase gene, the fungus is still able to completely mineralize lignin.

Obviously, laccases have also other functions in fungi, for instance lignin degradation produces reactive and therefore highly toxic products for the fungal hyphae, which could be captured by

laccases [15]. Besides that, laccase was also connected to pigment formation [21, 22], fruit body development [23-25] and virulence [26, 27] in fungi.

2.2.1.2 Structure and Biochemistry of Fungal Laccases

Fungal laccases are sometimes intra- but mainly extracellular globular, mostly monomeric multi-copper enzymes that are produced as isozymes. When secreted, they are glycosylated which provides them stability due to higher solubility in water [28]. It is also assumed that glycosylation ensures adequate conformational stability and protects against radical and protease attacks [14]. Fungal laccases have a molecular weight of 60-70 kDa, a pI around 4-5 [16] and are due to their glycosylation quite heterogenic when purified. The glycosylation consists of mannose, N-acetyl glucosamine and galactose units [29]. In 1939 it was shown by Keilin and Mann [30] and in 1948 by Tissieres [31] that laccases contain copper ions. Ten years later, in 1959, Malmström and colleagues showed that the enzyme contains a Cu(II) ion in the resting state [32].

The first crystal structure of a laccase from *Coprinus cinereus* was published in 1998 [33], and shortly thereafter many other laccases were crystalized [34]. Fungal laccases show high structural similarity among each other. They are three-domain MCOs which consist of a single peptide chain (ca. 500 amino acids) that composes three sequentially arranged cupredoxin-like domains with Greek key ß-barrels (**Figure 1**) [35, 36]. Laccases include four copper(II) ions per laccase molecule, which are mostly coordinated by histidines and bundled in different copper centers. These histidines are highly conserved and located within four motives (L1-L4) in the enzymes' sequence [37]. While L1 and L3 are distinct to laccases, L2 and L4 are also found in other MCOs. These motifs can be helpful when searching for new, uncharacterized laccases in newly sequenced genomes.

Laccases contain three types of copper centers that are characterized by their optical and electron paramagnetic resonance (EPR) spectroscopic features: the T1, T2 and T3 type copper.

In fungal laccases the coppers are located in domain 1 and 3. The T1 copper is only coordinated by amino acids from the first domain and the other three coppers (one T2 and two T3 coppers) are located between domain 1 and 3.

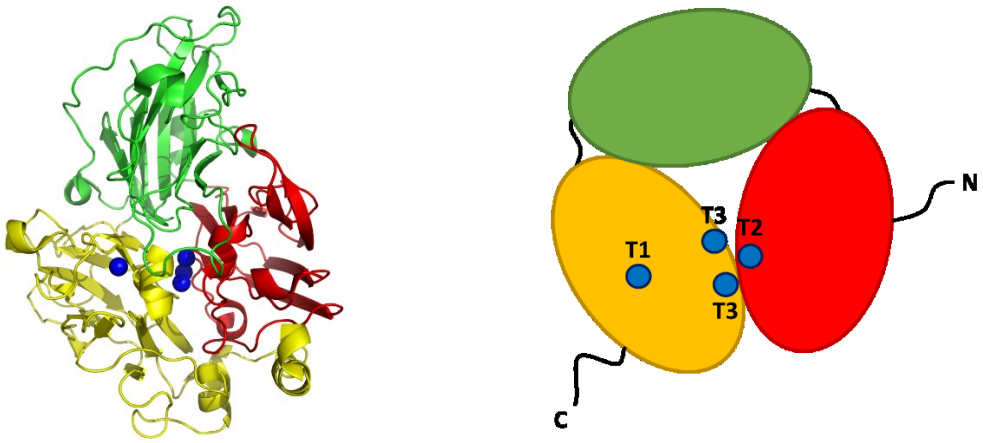

Figure 1 Structure of a three-domain laccase. Left) Cartoon model of a laccase from *Trametes vesicolor* (PDB: 1GYC). Right) Scheme of a three-domain laccase. Red depicts the first, N-terminal domain, yellow the third, C-terminal domain and the second, green domain links the first and third domains. Blue spheres resemble different copper centers.

The T1 copper, which is located close to the surface of the protein at the bottom of the substrate binding site formed by four binding loops [38], is the oxidation site of an organic substrate. Through a charge transfer between the T1 Cu(II) and a neighboring sulfur atom of a cysteine (S→Cu charge transfer), which is caused by a highly covalent nature of the Cu-S(Cys) bond, the protein appears blue [39]. The T1 copper has a distorted tetrahedral coordination system which is built by one cysteine and two histidines, which are strong metal ion ligands, and a weaker ligand which is usually phenylalanine or leucine in fungal laccases [34, 37]. Two T2 and one T3 copper compose the so-called trinuclear cluster (TNC) which is located about 13Å away from the T1 copper (**Figure 2**). The Type 2 copper is coordinated by two histidines and an oxygen, which is bound in a water molecule. The two T3 coppers are coordinated by three histidines each. The T1 and the trinuclear cluster are connected by a His-Cys-His motive with Cys being the T1-coordinating cysteine and the two histidines each coordinating one T3 copper.

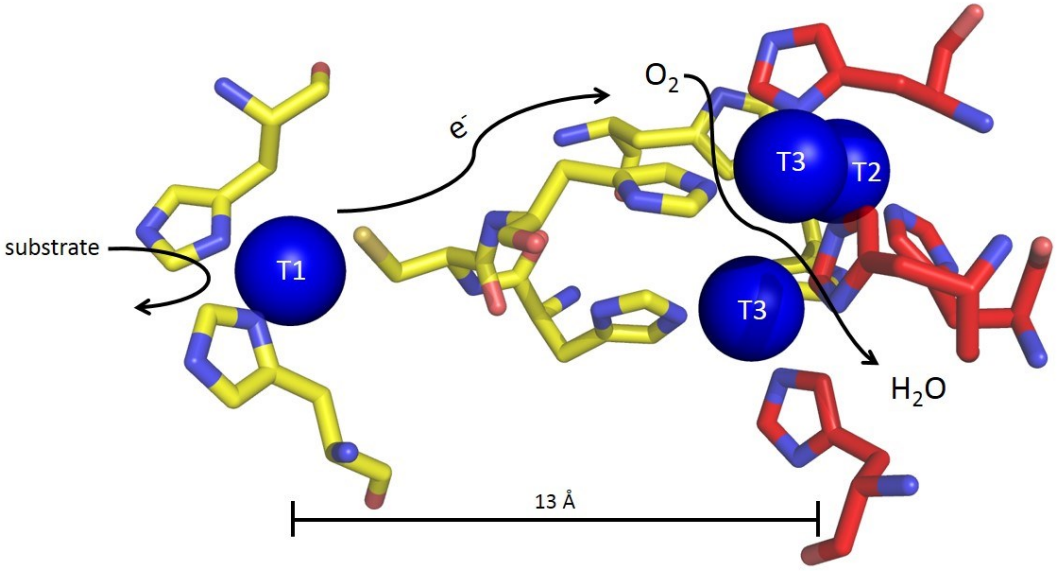

Figure 2 Structure of the active site of a fungal laccase. Arrows show the entry of the substrate and the flow of the electrons and the oxygen. Yellow sticks represent amino acids within the third (C-terminal) domain and red sticks represent amino acids within the first (N-terminal) domain participating in copper coordination. Blue spheres represent copper ions in different T centers.

2.2.1.3 Reactivity and Catalytic Mechanism

Common laccase substrates are phenols, as their redox potential (~0.5-1.0 V *vs*. NHE) is low enough to give electrons to the T1 copper, which has a redox potential of usually 0.5-0.8 V in fungal laccases [35]. It was suggested that the higher the difference in redox potential between substrate and laccase, the higher the catalytic efficiency [40, 41]. However, Tadesse and coworkers also showed that the phenol 2,4,6-tri(But)phenol, though having a redox potential of 0.7 V, is for steric reasons not oxidized by the laccase TviL (E° 0.79 V) from *Trametes villosa* [41]. Phenolic substrates are oxidized to phenoxy radicals, which, depending on the reaction conditions, can polymerize spontaneously via radical coupling or rearrange themselves, which leads to quinones (by disproportionation), alkyl-aryl cleavage, Cα-oxidation or cleavage of the Cα-Cβ bond or aromatic ring [35].

The catalytic mechanism of laccases was reviewed by Jones and Solomon in 2015 very well and is shown in **Figure 3** [42]. Laccases oxidize four substrates at the T1 copper and reduce one oxygen molecule to two water molecules at the T2 and T3 coppers. In the absence of a substrate the enzyme is in its resting oxidized **(RO)** state. All copper ions are oxidized to Cu(II) and the two T3 coppers are bridged by a hydroxo ligand which results in absorption at 330 nm due to μ2-OH → T3Cu(II) charge transfer transitions [42]. The T2 copper is also ligated by a hydroxo ligand. This RO state is actually not the relevant fully oxidized state for catalysis but it's the state of the enzyme seen in crystallography. If a suitable laccase substrate is present, it is oxidized at the T1 copper and the electrons are transferred to the TNC via the His-Cys-His triad to produce a completely reduced enzyme in which all copper is present as Cu(I) which is required for O_2 reduction. The order in which the copper sites are reduced may vary from enzyme to enzyme and reaction conditions but the T3 coppers are reduced as a two electron acceptor and the T2 site is mostly the last one to be reduced [42]. After reduction of the TNC O_2 is being reduced in two 2 e^- reductions steps. In the first step the O_2 molecule binds to the TNC to form a peroxidase intermediate **(PI)** and the T2 and T3ß copper sites are oxidized. Near the T2-T3ß edge is a conserved carboxylate residue, which is responsible for reducing the redox potential of both coppers and thus enabling electron abstraction through the O_2 molecule. Because of this carboxylate and the different behavior the T3 coppers are distinguished as T3α and T3β coppers [43]. In the next step, the O-O bond is cleaved. This step is facilitated by a second conserved carboxylate residue below the T3 copper site, which is necessary for the protonation of the oxygen atom bridging the two T3 copper atoms [42]. The reduced oxygen remains bound in the TNC as μ3-oxo and μ2-hydroxo ligands. Now the native intermediate **(NI)** is formed, which is the fully oxidized catalytically relevant enzyme state. In presence of more substrate molecules the enzyme is rapidly reduced to the fully reduced state while two water molecules are released and another oxygen molecule can be bound. In absence of substrate the NI decays slowly to the RO state.

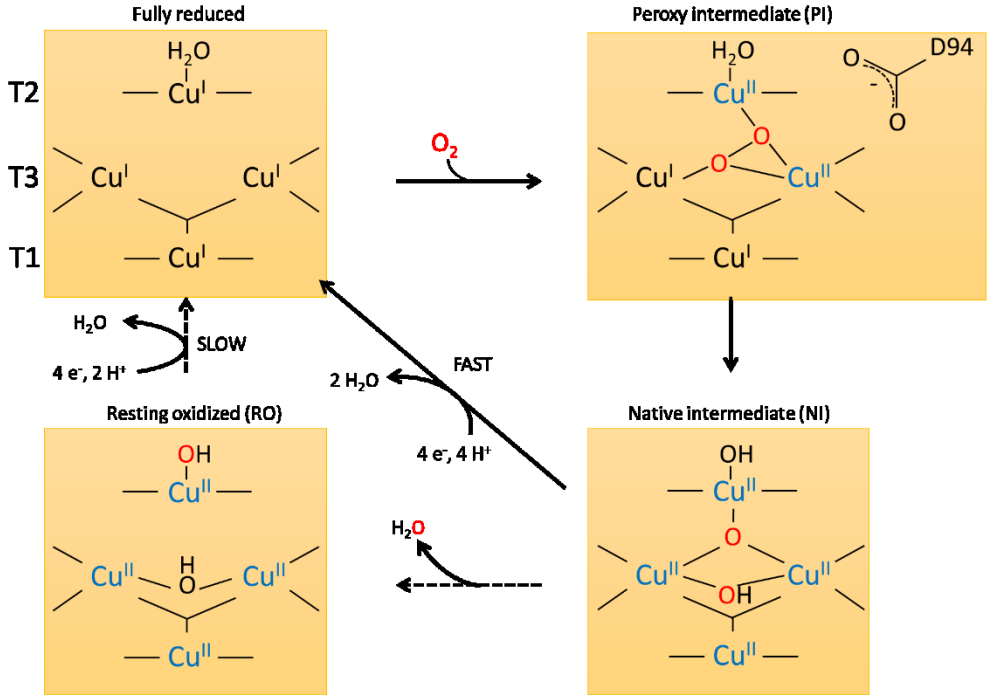

Figure 3 Schematic illustration of O_2 reduction by a laccase. Black arrows indicate the catalytic relevant cycle. Dashed arrows show states which are experimentally observed but are not part of the catalytic cycle, namely the decay of the native intermediate to the resting oxidized enzyme and its reduction to the fully reduced state. The scheme was redrawn and modified from and Solomon 2015 (Permission received from Springer Nature. License Number: 4998870487400) [42, 44].

2.2.2 Fungal Peroxidases

Peroxidases build a diverse group of ubiquitous enzymes that were probably formed billions of years ago to help organisms cope with oxidative stress. Heme-containing peroxidases can be divided into four superfamilies namely the peroxidase-catalase superfamily, peroxidase-cyclooxygenase superfamily, peroxidase-chlorite dismutase superfamily and the peroxidase-peroxygenase superfamily [45]. Earlier, superfamilies were classified based on the origin of the enzymes - plant-fungal-bacterial and animal peroxidases - but as it was shown that peroxidases from different phyla may be very similar it was suggested that classification based on function and structure is more plausible than origin [46]. The peroxidase-catalase superfamily (earlier superfamily of bacterial, fungal and plant heme peroxidases) contains three classes (Class I-III) [47] in which fungal peroxidases belong to Class II. Class II is most probably a monophyletic

gene family [48] and the enzymes are mostly involved in the active mineralization of lignin [46]. There are three types of Class II peroxidases namely the manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) which are well investigated. These three peroxidase types can be distinguished by their active sites which are discussed in the subsequent chapters. Fungal genomes show numerous homologues of peroxidases, which indicates gene duplications [46].

2.2.3 Structure and Catalytic Cycle of MnPs, VPs and LiPs

MnPs, VPs and LiPs are globular glycoproteins formed by 11-12 α-helices [49] (**Figure 4**). Their tertiary structure is highly conserved in contrast to the primary structure [45, 50]. They are constituted by a proximal (C-terminal) and a distal (N-terminal) domain, each harboring a structural calcium ion. Between these two domains is a non-covalently bound high-spin protoporphyrin IX, a heme b, located. The heme pocket is conserved among many peroxidases and contains two conserved histidines. The histidine below the heme plane acts as a fifth ligand (proximal histidine) of the heme iron while the histidine above is more distant (distal histidine). In all structurally characterized ligninolytic peroxidases an arginine and phenylalanine are conserved near the distal histidine, the arginine with the distal histidine being involved in the hydrolysis of H_2O_2 [50]. Close to the proximal histidine an aspartate, which forms H-bonds with the proximal His and stabilizes Compound I, and a phenylalanine are conserved [49, 50]. The roles of both phenylalanines is not well known but the proximal one might play a role in stabilizing the structural Ca-ion integrity [49].

A main heme access channel connects the heme to the solvent. Through this channel H_2O_2 can access and activate the enzyme as well as some low-molecular low redox potential substrates like 2,6-dimethoxyphenol (2,6-DMP) or ABTS enter here.

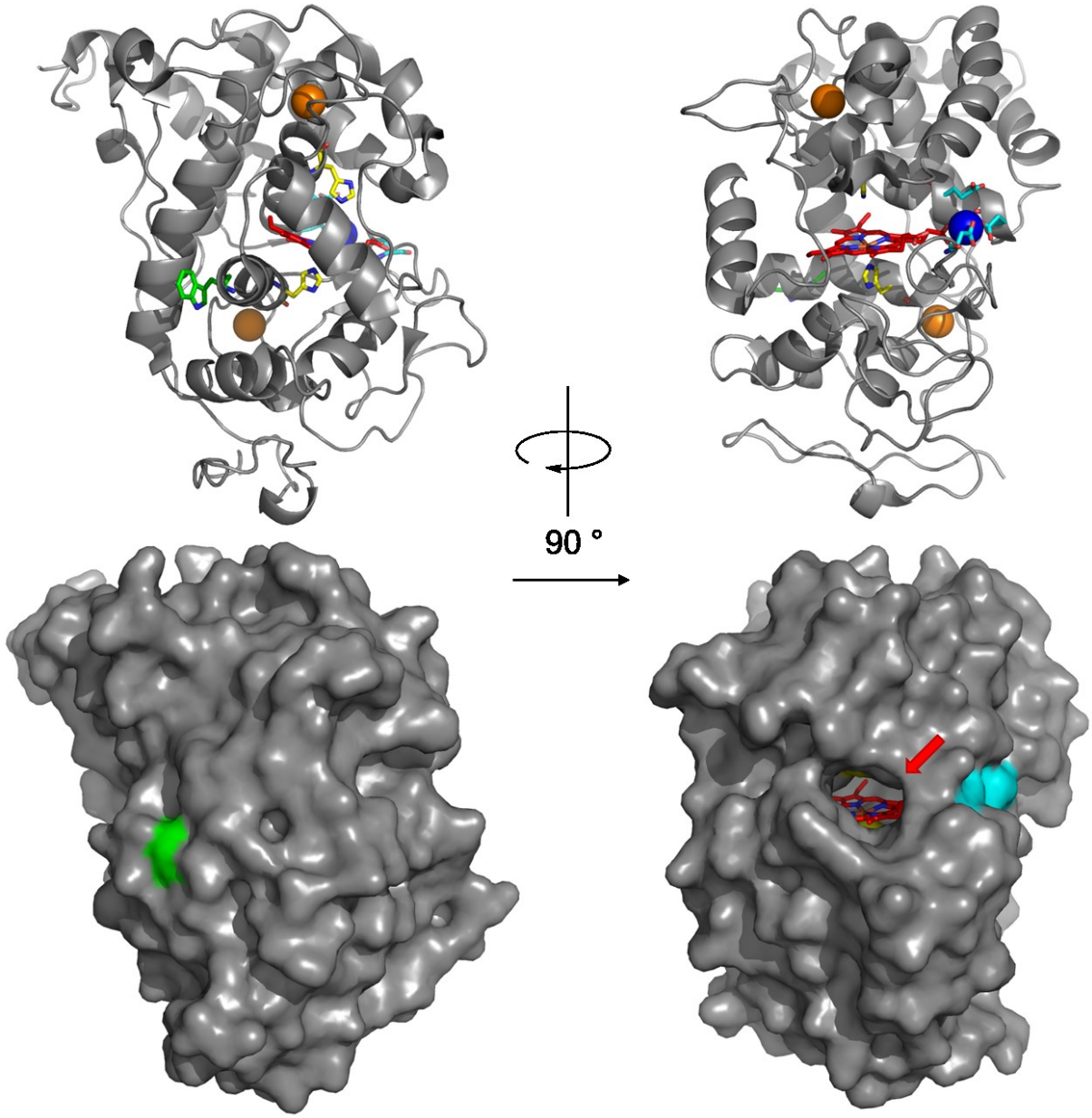

Figure 4 Versatile peroxidase structure. Pymol model of a VP from *P. eryngii* (PDB: 2BOQ). VP includes active sites of MnP and LiP and is therefore shown as an example of a ligninolytic peroxidase. Green sticks and surface: Catalytic Tryptophan; Red sticks: heme; cyan sticks and surface: acidic amino acids forming the manganese oxidation channel; yellow sticks: proximal (bottom) and distal (top) histidine; orange spheres: calcium ions; blue sphere: manganese ion; Top) VP cartoon model. Bottom) VP surface model with the heme access channel (red arrow) in the frontal view on the right side.

The catalytic cycle is very similar among MnPs, LiPs and VPs, but also among other peroxidases such as horseradish peroxidase and varies mainly in the type of electron donors [51]. The overall reaction can be described as follows:

$$2\ RH + H_2O_2 \rightarrow 2\ R^{\cdot} + 2\ H_2O$$

Where RH represents the substrate and $R^{\cdot}$ the radical derived therefrom [51].

In more detail the generic catalytic cycle is depicted in **Figure 5**. The resting enzyme harbors a high spin ferric heme, which is penta-coordinated with five nitrogens, 4 provided by the protoporphyrin IX and one by the proximal histidine [51, 52]. A water molecule is at the distal side between the iron and distal histidine, not coordinating with the iron. In the first step a peroxide, mostly H_2O_2, is bound to the Fe^{3+} and the ferric porphyrin hydroperoxide species Fe^{3+}-OOH, namely Compound 0 is formed [52]. Due to high instability it was only detected at temperatures below 0 °C. There is some evidence that prior to Compound 0, the formation of a peroxide-bond intermediate Fe^{3+}–HOOH may be needed [53-55]. Subsequnt proton transfer to the distal O and successive water cleavage from Compound 0 result in Compound I - oxoiron(IV) porphyrin π-cation radical species. 1980 Poulos and Kraut proposed a mechanism for the heterolytic cleavage of the O-O bond to form Compound I, which is generally referred as the Poulos-Kraut mechanism [56]. It involves acid-base catalysis by conserved His-Arg pair in the distal part of the heme group. It is assumed that the distal histidine is responsible for the deprotonation of the proximal O of the peroxide intermediate in Compound 0 formation and the subsequent protonation of the distal O and subsequent water cleavage. Simultaneously an arginine pulls electrons from the heme to the O_α-O_β to facilitate O_β protonation and homolytic cleavage [55]. It was long questioned if the Fe(IV) in the evolved Compound I is protonated or deprotonated but various (modern) spectroscopic methods support the deprotonated Fe(IV)=O species [57]. In some peroxidases, the porphyrin cation radical of Compound I is short-lived (if formed at all) and the radical spectra are often assigned to a tyrosyl or tryptophan radical of the protein [51]. When the radical is located at the protein and not at the heme, the ferryl oxygen is rather reduced and its spectral properties change [51]. Compound I is usually reduced to Compound II by one electron which is abstracted from one substrate molecule although two-electron reduction from Compound I to the resting enzyme and corresponding two-electron

oxidation of a substrate was observed e.g. for horseradish peroxidase with few substrates [58]. In Compound II (Fe(IV)–OH) the iron is still in its Fe^{4+} state but the porphyrin or protein radical is reduced and the oxygen is single bonded and protonated [59, 60]. After a single electron oxidation of another substrate the heme returns in its resting ferric form.

In presence of excess H_2O_2, Compound III, a peroxyFe(III) porphyrin free-radical is formed, which does not participate in the catalytic cycle. Compound III can be produced either from Compound II by reaction with H_2O_2 or from the resting enzyme by reaction with a superoxide anion [61].

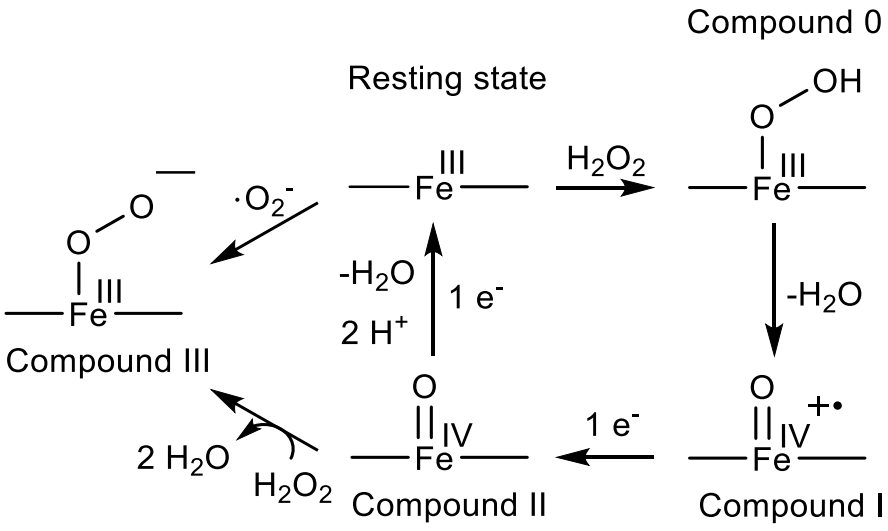

Figure 5 Generic catalytic cycle of a peroxidase. The scheme was redrawn and modified from Valderrama 2010 (Permission received from Springer Nature. License Number: 5001381165734) and de Montellano 2010 (Permission received from Springer Nature. License Number: 5001371469621) [51, 61].

2.2.3.1 Lignin Peroxidase

LiP was first discovered in *P. chrysosporium* and its crystal structure was published in 1993 [62]. LiP isozymes have a molecular weight of 38-46 kDa, pI values of 3.2-4 and an pH optimum for substrate oxidation at around 3 [63]. LiP oxidatively degrades the very recalcitrant polymer lignin [64] with the help of veratryl alcohol (VA) [65]. It was hypothesized that veratryl alcohol acts as a redox mediator that is oxidized to the corresponding cation radical,

which in turn can penetrate into lignin and oxidizes lignin. Alternatively, the veratryl alcohol cation radical can act as a substrate again and is oxidized by LiP to veratryl aldehyde. Formation of veratryl aldehyde can be easily monitored at 310 nm. The redox mediator function of veratryl alcohol during delignification, however, has been questioned due to fast decay of the radical [66]. Also, kinetic studies performed by Koduri and Tien suggested a rather protein stabilizing role of veratryl alcohol as they showed that it can act as a reducing agent for Compound II while other lignin-derived substrates (for instance anisyl alcohol) can only reduce Compound I [67]. Thus, the catalytic cycle can be finished in presence of veratryl alcohol much more efficiently. Another experiment performed by Houtman and colleagues did not confirm the role of veratryl alcohol as a diffusible mediator. They showed that veratryl alcohol and LiP could not oxidize the interior but only the surface of porous beads coated with the fluorescent oxidant sensor BODIPY 581/591, which had pores that were not accessible to the protein but only to veratryl alcohol [68].

LiPs exhibit a superficial catalytically active tryptophan (tyrosine has been described in LiP of *Trametes cervina* [69, 70]) where oxidation of high redox potential substrates as VA takes place (**Figure 6**). It was demonstrated for LiP from *P. chrysosporium* that the catalytically active Trp171 is hydroxylated in an autocatalytic process at the C^{β} next to the indole moiety [71]. This hydroxyl group is only present when the enzyme was in contact with H_2O_2, but is probably not necessary for its oxidation ability at Trp171 [65]. Doyle and colleagues showed with two created mutants of LiPH8 from *P. chrysosporium* namely W171S and W171F that Trp171 is essential for oxidation of the high redox potential substrate veratryl alcohol but not for oxidation of the low redox potential substrates ABTS and 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzenesulfonic acid [72] suggesting the existence of two active sites in LiP. Crystal structures of *P. chrysosporium* lignin peroxidase suggested that the heme access channel in LiP is blocked due to bulky amino acid residues as glutamate, histidine and

phenylalanine and substrates cannot access the heme. MD simulations, however, showed that these residues are flexible, allowing for heme accessibility even for veratryl alcohol [73]. Since VA is not oxidized in Trp171 deficient mutants, it was speculated that the redox potential at the tryptophan must be higher than at the heme directly. Electrons abstracted from the substrate at Trp171 move to the heme through the so called Long Range Electron Transfer (LRET) pathway.

2.2.3.2 Manganese Peroxidase

Like LiP, MnP was discovered in the 1980s in the secretome of *P. chrysosporium*, but at first was less studied than LiP [74]. When it became clear that there are ligninolytic fungi that only secrete MnPs but no LiPs, MnPs also started to attract attention among scientists. MnPs have a molecular weight in the range between 38 - 63.2 kDa, and different isoforms of individual species exhibit different pI values (3-4) [74]. In contrast to LiPs, MnPs are not able to oxidize high redox potential substrates due to the lack of an exposed tryptophan. Instead, MnPs are characterized by their ability to bind and oxidize Mn^{2+} at a manganese oxidation site which is formed by three carboxylates of two glutamates and one aspartate, one heme propionate and two water molecules (**Figure 6**). Afterwards Mn^{3+} is released in complexes with carboxylic acids as tartrate, oxalate or malonate for example. The manganese oxidation site can also bind other di- or trivalent cations inhibiting Mn^{2+} oxidation [75, 76]. The first structure of a MnP from *P. chrysosporium* was published in 1994 as the third crystal structure of a peroxidase after cytochrome c peroxidase and LiP [76, 77]. MnPs can be divided into short (≤347 amino acids), long (348 - 361 amino acids) and extra-long (≥ 362 amino acids) MnPs although the differences between short and long/extra-long MnPs are higher than differences between long and extra-long MnPs. Fernandez-Fueyo and coworkers produced and characterized several MnPs of *Ceriporiopsis subvermispora* which genome contains 13 MnP sequences representing all three lengths [76]. They concluded that long and extra-long MnPs differ from short MnPs in a long

C-terminal tail that surrounds the access channel to the heme and prevents small substrates such as ABTS from accessing and reducing the heme. On the other hand, they showed that this C-terminal tail provides higher acid and thermal stability.

2.2.3.3 Versatile Peroxidase

VP was only discovered at the end of the 20th century, about 15 years later than MnP and LiP [78, 79]. VP can be described as a hybrid of LiP and MnP that possesses a main heme access channel, a manganese binding and oxidation site as well as an active tryptophan on the surface of the protein (**Figure 6**). Its broad substrate spectrum has attracted the attention of many enzymologists. In contrast to LiP the catalytically active Trp is not hydroxylated at the C^{β} position and is described as a neutral radical [80]. In contrast to LiP, which can oxidize different dyes only in the presence of veratryl alcohol, VP is able to catalyze this reaction on its own. This could be explained by the nature of the catalytically active Trp surrounding amino acids [80]. While the catalytic Trp in LiP is surrounded by four acidic amino acids (that can stabilize the veratryl alcohol cation radical resulting in higher affinity), Trp in VP is surrounded by only two acidic amino acids [80]. The fact that VP can be found in nature much more rarely than MnP and LiP (many fungi don't have any VP genes [81]) has probably led to its later discovery.

However, since VPs are of special interest for biotechnology more potential VP sequences have to be discovered or site-directed mutagenesis can be applied on MnPs to create new VPs.

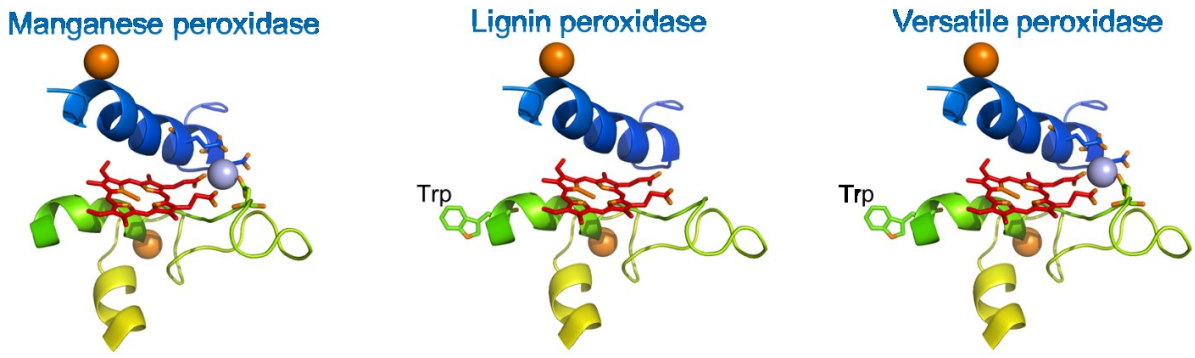

Figure 6 Active sites of Class II peroxidases of the peroxidase-catalase superfamily. Active and oxidation sites of MnP, LiP and VP. Orange spheres depict structural Ca^{2+} ions and purple spheres the Mn^{2+}.

2.3 Fungal Oxidoreductases in Industrial Applications

Laccases can and have been used in a wide variety of applications. They are used as additives in personal care products, in the synthesis of synthons for pharmaceuticals, biosensors, food processing, pulp and paper industry and pretreatment of biomass in biorefineries [27, 82-87]. Due to their ability to detoxify various toxic agents they have been investigated for the biological treatment of waste effluents containing dyes or other micropollutants such as non-steroidal anti-inflammatory drugs (NSAIDs), endocrine disruptors (EDCs) as estrogens and bisphenol A or herbicides [88, 89]. The active substances are di- or polymerized (sometimes followed by precipitation) by the enzyme and therefore lose their toxic effect. Applications for peroxidases have been proposed in similar fields like in biosensors, in the production of cosmetics or natural flavors or in bioremediation [86, 90-93].

2.3.1 Application in Pretreatment of Lignocellulosic Biomass

Biomass valorization involves its growth, harvest, transportation, pretreatment, hydrolysis, fermentation and product separation. Unlike first generation starch-containing biomass feedstock, the recalcitrant nature of lignocellulose in second generation biorefineries makes biomass pretreatment inevitable. Therefore production of second generation biofuels, which are fuels produced from lignocellulose, faces tremendous challenges [94].

Lignocellulose consists of crystalline cellulose, hemicellulose and a considerable amount of lignin, the latter being the most recalcitrant component. Lignin is composed of different aromatic phenols which are polymerized to a recalcitrant, three-dimensional protective and stability providing amorphous matrix [95]. Tight connections between lignin, hemicellulose and cellulose hinder cellulases from efficient cellulose hydrolysis due to steric reasons and by inhibition through non-specific binding of these enzymes to lignin [96]. Various pretreatment methods are used to make lignocellulose more susceptible to hydrolysis. Many pretreatment methods are investigated and tested with different kind of woods. Pretreatment methods can be

divided in three groups, which can be combined in different ways: physical (e.g. disk milling, extrusion, microwave, acidic, alkaline), physicochemical (e.g. steam explosion, supercritical CO_2) and biological (with whole organisms or isolated enzymes) [94].

Biological pretreatment can be performed with microorganisms (especially fungi) found in nature, with isolated lignocellulose-degrading enzymes or with tailored organisms which are able of degrading lignocellulose in a process, that is called consolidated bioprocessing (CBP), and produce high valuable products in one step [97]. Biological pretreatments usually need long incubation times, enzyme costs are high and efficient organisms for CBP capable of lignocellulose degradation and fermentation have to be designed. However, energy consumption is low, as pretreatment can be performed at low temperatures and low pressure.

2.3.2 Production of Ligninolytic Peroxidases and Laccases

Production of peroxidases and laccases at an industrial scale is quite challenging [83, 98, 99]. Homologous expression under strong promoters led to quite high yields (1 g l^{-1}), but only after a long period (24 days) of cultivation [100]. Heterologous expression in the already established hosts such as *Escherichia coli, Pichia pastoris Saccharomyces cerevisiae* or *Aspergillus niger* leads to higher yields in a shorter period of time, but strongly depends on the enzyme and expression host used.

Laccases and peroxidases with a higher redox potential and thus of greater value for industry are mostly of fungal origin and these secreted glycoproteins are more likely to be expressed in a fungal host than in *E. coli*, for which only two cases of expression of a fungal laccase have been reported [101, 102]. However, the expression of peroxidases in *E. coli* is often carried out on a laboratory scale, but the protein usually ends up in inclusion bodies and has to be refolded, which makes industrial application less practical [103]. Ligninolytic enzyme production in *S. cerevisiae*, however, has shown to be rather low and production in *P. pastoris* as alternative yeast host is favored because of much higher expression levels [104-106]. Filamentous fungi

like *Aspergillus niger* are known to have a high protein secretion capacity, however, because of their filamentous nature broth rheology may be problematic in the fermentation process [107]. Additional, low transformation efficiencies and protease activity are observed [108].

Expression in heterologous hosts can be easily modulated by multiple gene insertions and strong and highly regulated promoters. Additionally isozymes or silent genes can be expressed and characterized separately. An easy-to-cultivate expression system with high titers would also help in rational mutagenesis or directed evolution where libraries of laccase and peroxidase mutants have to be screened to find candidates that meet industrial demand for enzymes with high thermal, pH or solvent stability and higher catalytic activity or selectivity.

2.4 Aim and Objectives of the Study

Laccases and ligninolytic peroxidases originating from white rot fungi are of considerable interest for biotechnology due to their high redox potential and broad substrate spectrum. However, their industrial use is mainly hindered by low expression yields or by few known DNA sequences. Microorganisms involved into effective lignocellulose degradation represent a rich source of effective ligninolytic enzymes. In the framework of this thesis *Moniliophthora roreri* was chosen as a target microorganism. *M. roreri* is an aggressive white rot fungus which infects pods of *Theobroma* and *Herrania* species while *Theobroma cacao* (cacao) is of major economic concern [109] and is therefore supposed to express numerous ligninolytic enzymes.

The overall aims of this thesis were i.) To identify ligninolytic enzymes in the genome of *M. roreri*; ii.) To establish their recombinant expression in a heterologous host at high level; iii.) To characterize those ligninolytic enzymes; and iv.) To demonstrate their potential for biotechnological applications.

Therefore, the main tasks of the work were to:

- First, identify laccase and peroxidase genes in the genome of *M. roreri* and produce them in *P. pastoris* X-33 possibly at high titers;
- Purify and thoroughly characterize the enzymes regarding their pH, temperature stability and their K_m and k_{cat} values;
- Evaluate their biotechnological potential to degrade bioactive compounds in waste water, industrial textile dyes and lignin derivatives;
- Furthermore, if needed, use rational protein design to improve the performance of the enzymes;
- Finally test the produced enzymes for the pretreatment of wood of *Miscanthus x giganteus* for subsequent saccharification.

According to these tasks the achieved results are organized in the corresponding chapters

3 RESULTS

3.1 MANUSCRIPT 1: EXPRESSION OF A NEW LACCASE

Title: Expression of a new laccase from *Moniliophthora roreri* at high levels in *Pichia pastoris* and its potential application in micropollutant degradation.

Authors: Agathe Bronikowski, Peter-Leon Hagedoorn, Katja Koschorreck, Vlada B. Urlacher

Contribution: Design of experiments. Carrying out all experiments. Writing the manuscript.

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ORIGINAL ARTICLE

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Expression of a new laccase from *Moniliophthora roreri* at high levels in *Pichia pastoris* and its potential application in micropollutant degradation

Agathe Bronikowski[1,3], Peter-Leon Hagedoorn[2], Katja Koschorreck[1,3] and Vlada B. Urlacher[1,3*]

Abstract

Laccases have gained significant attention due to their emerging applications including bioremediation, biomass degradation and biofuel cells. One of the prerequisites for the industrial application of laccases is their sufficient availability. However, expression levels of recombinantly expressed laccases are often low. In this study Mrl2, a new laccase from the basidiomycete *Moniliophthora roreri,* was cloned in *Pichia pastoris* and produced in an optimized fed-batch process at an exceptionally high yield of 1.05 g l^{-1}. With a redox potential of 0.58 V, Mrl2 belongs to mid-redox potential laccases. However, Mrl2 demonstrated high k_{cat} values of 316, 20, 74, and 36 s^{-1} towards 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP) and guaiacol, respectively. Mrl2 remained stable above pH 6 and in the presence of many metal ions, which is important for application in bioremediation. Mrl2 was investigated for the ability to degrade endocrine disrupting chemicals (EDCs) and non-steroidal anti-inflammatory drugs (NSDAIs) at neutral pH value. The enzyme accepted and converted estrone, 17β-estradiol, estriol, the synthetic contraceptive 17α-ethinyl estradiol and bisphenol A at pH 7 faster than high-potential laccases from *Trametes versicolor*. For example, within 30 min Mrl2 removed more than 90% bisphenol A, 17ß-estradiol, 17α-ethinyl estradiol and estriol, respectively. The concentration of the recalcitrant drug diclofenac dropped by 56% after 20 h incubation with Mrl2.

Keywords: Laccase, Expression, *Pichia pastoris*, Micropollutant degradation

Introduction

Laccases (EC 1.10.3.2) are multi-copper oxidases which catalyze the oxidation of various electron-rich organic and inorganic molecules like mono- and diphenols, polyphenols, diamines, aminophenols, aromatic or aliphatic amines with the four-electron reduction of molecular oxygen to water (Thurston 1994; Xu 1996; Xu et al. 1996). Small compounds, so called redox mediators, can expand the substrate range to non-phenolic lignin derivatives (Bourbonnais and Paice 1990; Eggert et al. 1996) or recalcitrant dyes (Soares et al. 2001). Laccases contain four copper ions. Substrates are oxidized at the mononuclear type 1 (T1) copper site and electrons are transferred to the trinuclear site comprising one T2 and two T3 copper ions, where the reduction of oxygen to water takes place (Solomon et al. 2008). Laccases are ubiquitous and found in plants, fungi, insects (Mayer and Staples 2002), yeast (Kalyani et al. 2015) and bacteria (Dwivedi et al. 2011). The redox potential of laccases is quite different and range from 0.36 to 0.8 V. It is widely accepted that the axial ligand of the T1 copper as well as a tripeptide in the T1 copper site (LEA) roughly indicate the redox potential of laccases (Mot and Silaghi-Dumitrescu 2012; Xu et al. 1998). Low-potential laccases, mainly found in bacteria, with a redox potential from 0.36 to 0.46 V exhibit a methionine at the axial position, while middle-potential laccases, mainly from ascomycete origin, with

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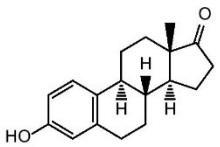
a redox potential of 0.46–0.71 V usually have a leucine. High-potential laccases with a redox potential >0.71 V are mainly found in basidiomycetes. They display a phenylalanine as a non-coordinating axial ligand (Mot and Silaghi-Dumitrescu 2012). High-potential laccases typically possess a broader substrate spectrum than low- or middle-potential laccases and are even able to oxidize substrates with a redox potential of up to 1.2 or 1.4 V (Tadesse et al. 2008). Regarding their broad substrate spectrum while only relying on water as a cosubstrate, laccases often are called "green" biocatalysts (Pardo and Camarero 2015). Therefore, these enzymes, and high-potential laccases in particular, are attractive for industrial applications as in the pulp and paper, food and textile industry, nanobiotechnology, synthetic chemistry or bioremediation (Rodriguez Couto and Toca Herrera 2006).

Micropollutants comprising endocrine disrupting, toxic, persistent and bioaccumulative substances, like EDCs and NSAIDs (Table 1), are one of the key problems facing humanity (Schwarzenbach et al. 2006). They are ubiquitous in aquatic environments, since e.g. human or veterinary pharmaceuticals are only partially metabolized and eventually end up in the wastewater system (Hamid and Eskicioglu 2012). In an aging society it is very likely that more and more pharmaceuticals will be released in the environment. Human hormones as 17β-estradiol or the contraceptive 17α-ethinyl estradiol have been reported to lead to feminization or sexual disruption of fish (Chen et al. 2016a; Orn et al. 2016). Other estrogens as estrone which is predominant in menopausal women and estriol, a metabolite of estrone and 17β-estradiol, are also found in wastewaters (Auriol et al. 2007a, b). The diphenylmethane derivatives bisphenol A and bisphenol S used in plastics have endocrine mimicking properties (Ji et al. 2013; Vinas and Watson 2013). Diclofenac and naproxen are NSAIDs which show analgesic, antipyretic and anti-inflammatory effects. Because of more and more emerging concerns about the fate of EDCs and NSAIDs much effort has been made to develop alternative

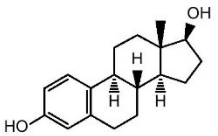
Table 1 Chemical structures of endocrine disrupting chemicals and nonsteroidal anti-inflammatory drugs investigated in this study

Endocrine disrupting chemicals (EDCs)

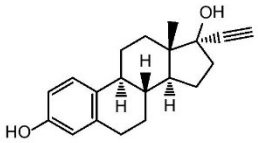
E1



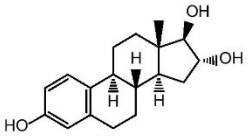
E2



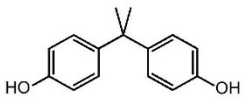
EE2



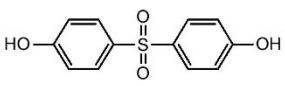
E3



BPA

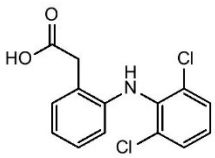


BPS

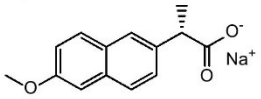


Nonsteroidal anti-inflammatory drugs (NSAIDs)

Diclofenac



Naproxen



E1 estrone, *E2* 17ß-estradiol, *EE2* 17α-ethinyl estradiol, *E3* estriol, *BPA* bisphenol A, *BPS* bisphenol S

strategies to remove those micropollutants since conventional wastewater treatment processes do not meet the demands (Schroder et al. 2016). Among others laccases are capable of reducing estrogenic activity in wastewater (Suzuki et al. 2003; Tsutsumi et al. 2001). However, most high-redox potential laccases demonstrate low activity under neutral or basic pH conditions found in wastewater (Baldrian 2006). Another limitation for a broader laccase application is their rather low expression level in recombinant hosts.

In this study, we identified and characterized a new laccase, Mrl2, from *Moniliophthora roreri*. Mrl2 was produced at exceptionally high levels in *Pichia pastoris* (1.05 g l^{-1}) in a 3 l fed-batch fermentation process. High stability above pH 6 and resistance to many metal ions make this enzyme suitable for application in wastewater treatment. Amongst others, 17β-estradiol, 17α-ethinyl estradiol and diclofenac, which are listed by the European Union (EU) as dangerous compounds which should be monitored by the EU members (Schroder et al. 2016) could be degraded by Mrl2.

Materials and methods

Materials

Unless specified otherwise, all chemicals (of analytical grade or higher) and commercial proteins were acquired from AppliChem. (Darmstadt, Germany), Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA), Sigma-Aldrich (Schnelldorf, Germany), VWR (Darmstadt, Germany), Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Ipswich, Massachusetts, USA).

Cloning of *mrl2*

The gene for Mrl2 (NCBI Reference Sequence: XP_007855001.1) was codon optimized (GenBank accession number: KY111767) with JCat for expression in yeast (http://www.jcat.de/) and synthesized by Eurofins (Ebersberg Germany). The gene was cloned in the pPICZαA vector from Invitrogen™ (Carlsbad, California, USA) with both the native signal peptide and the α-factor secretion signal from *Saccharomyces cerevisiae*. Mrl2 was amplified with primers mrl2_BstBI_fw (GATA*ttcgaa*ATGGCTAGATTGCAATTC) and mrl2_XbaI_rev (CA*agatct*TTACAAGTCGTCGTCAG) and mrl2_*Xho*I_fw (GTAT*ctcaga***AAAAGA**TCTATCGGTCCAATCG) and mrl2_*Xba*I_rev for the native signal sequence and the α-factor construct, respectively. Recognition sites for the endonucleases are underlined. The forward primer contained the Kex2 signal cleavage site (highlighted in bold), thus *mrl2* starts right behind Kex2 at the end of the alpha factor pre pro leader sequence. The vector and the amplified genes were subjected to cleavage with respective restriction enzymes and ligated into pPICZαA to generate pPICZαAmrl2 and pPICZAmrl2. The sequence of the constructs was confirmed by sequencing (GATC Biotech, Konstanz, Germany). pPICZαAmrl2 and pPICZAmrl2 were linearized with *Pme*I and inserted in *P. pastoris* X-33 (Invitrogen, Carlsbad, California, USA) by electroporation. After transformation, cells were selected at 30 °C on Yeast Extract Peptone Dextrose medium with sorbitol (YPDS; 10 g l^{-1} yeast extract; 182.2 g l^{-1} sorbitol; 20 g l^{-1} peptone; 15 g l^{-1} agar) agar plates with 100 µg ml^{-1} Zeocin™. For further assessment clones were streaked out on 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-Buffered Minimal Methanol (BMM; 13.4 g l^{-1} Yeast nitrogen base w/o amino acids; 15 g l^{-1} agar; 0.00004% biotin, 0.5% methanol; 100 mM potassium phosphate buffer pH 6; 0.3 mM $CuSO_4$; 0.2 mM ABTS) agar plates and incubated at 30 °C. Clones which formed greenish halos were chosen for further experiments.

Expression of Mrl2 in shaking flasks

Pichia pastoris transformants were grown in 10 ml Buffered Complex Glycerol medium (BMGY; 10 g l^{-1} yeast extract; 20 g l^{-1} peptone; 100 mM potassium phosphate buffer pH 6; 1% glycerol; 0.00004% biotin) over night at 30 °C and 180 rpm. Overnight cultures were used for inoculation of 50 ml BMM medium in baffled flasks to an OD_{600} of 0.1. The cultures were shaken at 30 °C and 180 rpm for 3 days. The medium was supplemented with methanol every day to 0.5% (v/v) final concentration. Samples were taken daily for cell density and laccase activity monitoring.

Expression of Mrl2 in a 7.5 l bioreactor

Pichia pastoris transformants were grown in 10 ml BMGY (preculture) over night at 30 °C. A starter culture of 200 ml BMGY medium was inoculated with the preculture to an OD_{600} of 0.01–0.05 and grown over night at 30 °C and 180 rpm. The starter culture was used to inoculate a 7.5 l bioreactor (Infors, Bottmingen, Switzerland) containing 3 l fermentation basal salt medium (0.47 g l^{-1} $CaSO_4 \cdot 2\ H_2O$, 9.1 g l^{-1} K_2SO_4, 7.5 g l^{-1} $MgSO_4 \cdot 7\ H_2O$, 4.2 g l^{-1} KOH, 8 ml H_3PO_4 (85%), 50 g l^{-1}, glycerol (87%), 0.87 mg l^{-1} biotin, 4.35 ml l^{-1} *Pichia* trace metals (PTM_1, 6 g l^{-1} $CuSO_4 \cdot 5\ H_2O$, 0.08 g l^{-1} NaI, 3 g l^{-1} $MnSO_4 \cdot H_2O$, 0.5 g l^{-1} $CoCl_2$, 20 g l^{-1} $ZnCl_2$, 0.02 g l^{-1} H_3BO_3, 0.2 g l^{-1} $Na_2Mo_4 \cdot 2\ H_2O$, 65 g l^{-1} $FeSO_4 \cdot 7\ H_2O$, 0.2 g l^{-1} biotin, 5 ml l^{-1} H_2SO_4) to an OD_{600} of 0.5. pH 6 was adjusted with 10% phosphoric acid and 25% ammonium hydroxide. In the first growth phase on glycerol the temperature was maintained at 30 °C. When glycerol was used up (noticeable in pO_2 increase) expression was induced by addition of methanol (0.5% (w/v) containing 12 g l^{-1} trace metal salts PTM_1) and temperature was shifted to 25 °C.

Methanol was added automatically when the C-source was depleted, indicated by a sharp increase in pO_2 value. 0.9 ml 1 M $CuSO_4$ was added daily to the fermentation broth. Samples were taken daily for monitoring OD_{600}, laccase activity and determining protein concentration by the Bradford assay using bovine serum albumin (BSA) as standard.

Purification and characterization of Mrl2

The fermentation broth was centrifuged (10,000*g*; 15 min; 4 °C) and the supernatant was concentrated by Crossflow ultra-filtration with a cut-off membrane of 10 kDa (Pall, East Hills, NY, USA). The concentrated supernatant was centrifuged (22,000*g*; 20 min; 4 °C) and filtered through a 0.22 µm pore size filter. 5–10 ml of the concentrated supernatant was purified by DEAE FF anion exchange chromatography with an Äkta FPLC (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). After protein application the column was washed with 50 mM potassium phosphate buffer pH 6 and 200 mM NaCl. Mrl2 was eluted with 50 mM potassium phosphate buffer pH 6 and 250 mM NaCl. Blueish, active fractions (towards ABTS as substrate) were pooled and concentrated with a MILLIPORE Amicon® stirred ultrafiltration cell 8200 (Bedford, Maine, USA) with a cut-off membrane of 10 kDa and desalted with a PD Midi Trap G-25 desalting column (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). Mrl2 was stored in 50 mM potassium phosphate buffer pH 6 at −20 °C until further use.

Protein concentration was estimated by the Bradford Assay with BSA as standard. Deglycosylation was conducted with PNGase F as described in the manual instruction. SDS-PAGE was conducted according to Laemmli (1970). Samples used for the zymogram were not heated at 95 °C. For activity staining the SDS-PAGE gel was incubated in 100 mM sodium acetate buffer pH 5 supplemented with 0.5 mM ABTS.

Copper content of Mrl2 was determined by atomic absorption spectroscopy on a Perkin Elmer AAnalyst 100 (Waltham, USA) equipped with an air-acetylene burner. Mrl2 was diluted in water for copper measurement. The same sample was used for protein concentration determination by Bradford assay and at 280 nm. For determining the protein concentration the molar extinction coefficient was calculated with http://web.expasy.org/protparam/as 80,580 M^{-1} cm^{-1}.

Redox potential measurements were performed in a nitrogen flushed glove box under the absence of oxygen. For the titration redox mediator couples $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ and $[Fe(bipy)_2]^{3+}$/$[Fe(bipy)_2]^{2+}$ were used with a standard redox potential of 0.433 V (O'Reilly 1973) and 0.78 V versus the standard hydrogen electrode, respectively. For the bipyridine complex solutions, iron(III) chloride hexahydrate and iron(II) chloride were mixed in 1:2 ratio with 2,2′-bipyridine. The reduction status of the T1 copper was monitored at 600–750 nm. Measurements were performed in 50 mM potassium phosphate buffer, pH 7.5 at room temperature in duplicate. The normalized absorbance was plotted against the redox potential with the software Igor Pro (Wavemetrics; https://www.wavemetrics.com/) and the midpoint was determined using the Nernst equation for a one electron redox reaction: $Y = \frac{A}{1+e^{\frac{nF}{RT}(E_m-E)}} + B$ with n = 1, F = 96,486 C mol^{-1}, R = 8.314 J mol^{-1} K^{-1}, T = 293 K, E_m = midpoint potential.

Enzyme activity determination and kinetic parameters

Enzyme activity was determined in 100 mM sodium acetate buffer, pH 5 with 0.5 mM ABTS at room temperature. The increase of absorbance resulting from ABTS oxidation was monitored at 420 nm (ε = 36,000 M^{-1} cm^{-1}). Kinetic parameters of Mrl2 with the substrates ABTS, 2,6-dimethoxyphenol (2,6-DMP; 468 nm; ε = 49,600 M^{-1} cm^{-1}), guaiacol (470 nm; ε = 26,000 M^{-1} cm^{-1}) and syringaldazine (SGZ; 530 nm; ε = 65,000 M^{-1} cm^{-1}) were determined in 100 mM citrate phosphate buffer at their optimal pH. The substrate concentration ranges were 0.00781–1 mM, 0.000976–0.5 mM, 0.001–10 mM and 0.0001–10 mM for ABTS, SGZ, 2,6-DMP, and guaiacol, respectively. One Unit is defined as the amount of enzyme that converts 1 µmol substrate per minute.

Effect of pH, temperature, metal ions and inhibitors on enzyme activity

For determining the optimal pH the assay was conducted at different pH values: pH 2 and 8–10 in 100 mM Britton Robinson buffer and pH 3–7 in 100 mM citrate phosphate buffer. For temperature stability investigations the enzyme was diluted to 500 µg ml^{-1} in 50 mM potassium phosphate buffer pH 7 in 0.2 ml reaction tubes. The tubes were incubated at 20, 30, 40, 50, 60, 70 and 80 °C in a PCR Cycler and the residual activity was measured with the standard ABTS activity assay. For pH stability study Mrl2 was diluted in 100 mM Britton Robinson buffer at pH 2-10 and incubated at room temperature. Aliquoted samples were used directly for measuring residual activity with the ABTS assay. The activity in the presence of metal ions as indicated in Table 4 was determined with ABTS as substrate at pH 3 in 100 mM citrate phosphate buffer. The enzyme was incubated with the metal ions for 5 min before the addition of ABTS. The metal ions were added as sulfates or in the case of calcium as nitrate. To assess the metal chelating effect of the citrate buffer used, enzyme activity was tested also in 100 mM HEPES buffer,

pH 3 in the presence of 100 mM calcium, nickel, cobalt or zinc. Enzyme stability in the presence of 10 and 100 mM glycerin, acetonitrile, methanol, ethanol, 2-propanol, acetone, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF) was determined as described for metal ion tolerance but in sodium acetate buffer pH 5. For determining activity in the presence of potential inhibitors the protein was incubated in 100 mM sodium acetate buffer pH 5 including 10–100 mM NaCl, 0.1–1% SDS or 0.1% sodium azide for 5 min at room temperature. After incubation time ABTS was added to the reaction mixture and oxidation was followed at 420 nm.

EDC and NSDAI degradation

Stock solutions of 100 mM estrone, 17ß-estradiol, 17α-ethinyl estradiol, estriol, bisphenol A, bisphenol S, naproxen and diclofenac, respectively, were made in DMF. Degradation studies were carried out in 2 ml tubes at room temperature with a total reaction volume of 750 µl. The reaction solution contained 50 mM potassium phosphate buffer, pH 7 containing 10% ethanol (Sei et al. 2008), 100 µM EDCs or NSDAIs and 20 U ml^{-1} Mrl2. The same samples but containing heat-inactivated Mrl2 were used as a control. The reaction tubes were shaken in a rotating over-head incubator at room temperature. 100 µl samples were taken after 0, 0.5, 1, and 20 h and mixed with 3 µl 6 M HCl to stop the reaction. 100 µl Methanol was added, samples were centrifuged (12,300 *g*, 10 min) and analyzed by HPLC. Laccases from *Trametes versicolor* (Sigma Aldrich) were used for comparative degradation studies. The laccases were dissolved in 50 mM potassium phosphate buffer, pH 7, and protein concentration was determined with the Bradford assay using BSA as standard. The solution was stored at −20 °C until use. Degradation reactions with 30 µg ml^{-1} laccase (Mrl2 and from *T. versicolor*, respectively) were conducted in a total volume of 500 µl 50 mM potassium phosphate buffer, pH 7 containing 10% ethanol, 100 µM EDCs or NSDAIs. For HPLC analysis samples were prepared as described above. Samples were analyzed immediately.

HPLC analysis

Samples were analyzed on a Shimadzu HPLC (Shimadzu, Duisburg, Germany) with a Chromolith® C18 100 mm, 4.6 mm reversed phase column (Merck, Darmstadt, Germany). As mobile phase a mixture of 100% methanol (mobile phase A) and water supplemented with 0.1% formic acid (v/v) (mobile phase B) was used. 10–25 µl of sample were injected for analysis. Steroids were analyzed with an isocratic elution method. The methanol concentration was 55% for estrone, 60% for 17ß-estradiol and 17α-ethinyl estradiol and 33% for estriol. For the NSAIDs the following gradients were used: for bisphenol A in 10 min from 50% mobile phase A to 75% mobile phase A, from 10 to 12 min 100% mobile phase A, from 12 to 15 min 50% mobile phase A; for diclofenac in 10 min from 70% mobile phase A to 85% mobile phase A, from 10 to 12 min 100% mobile phase A, from 12 to 15 min 50% mobile phase A; for bisphenol A in 10 min from 25% mobile phase A to 75% mobile phase A, from 10 to 12 min 100% mobile phase A, from 12 to 15 min 50% mobile phase A and for naproxen in 10 min from 50% mobile phase A to 75% mobile phase A, from 10 to 12 min 100% mobile phase A, from 12 to 15 min 50% mobile phase A. The flow of the mobile phase was 1–2 ml min^{-1}. The compounds were evaluated at 280 nm. Stock solutions of EDCs and NSAIDs dissolved in 50% methanol to a concentration of 100 mM were used as standards. Degradation of the chemical compounds was calculated as percentage of the initial (time point 0 h) peak area size.

Results

Identification of a putative high-potential laccase

The catalytic activity of laccases is influenced by the difference in the redox potential of a substrate and the type 1 copper (Xu 1996; Xu et al. 1996). Thus, in most cases, high-potential laccases accept a broader range of substrates than low-potential laccases (Tadesse et al. 2008). To identify a laccase with a high redox potential, a BLAST search was done with the motif HCHIDWHLEAGF, containing the coordinating histidines of the T1 and T3 copper ions, the LEA tripeptide and the non-coordinating axial ligand phenylalanine, that is highly conserved within high-potential laccases (Mot and Silaghi-Dumitrescu 2012). A gene with the NCBI Reference Sequence XP_007855001.1 was identified as a putative laccase from a fungus *Moniliophthora roreri* causing frosty pod rot in cacao. The new gene was designated *mrl2*.

Cloning and recombinant expression of Mrl2 in shaking flasks

The gene *mrl2* was cloned with either its native signal sequence or with the α-mating factor signal sequence from *Saccharomyces cerevisiae* for secretion into the vector pPICZαA (Invitrogen, Carlsbad, California, USA) resulting in pPICZAmrl2 (nMrl2) and pPICZαAmrl2 (αMrl2), respectively. The constructs were used to transform *P. pastoris* X-33. Transformants were screened for laccase production on buffered minimal methanol agar plates containing ABTS. After overnight incubation at 30 °C transformants of both constructs formed strong green halos. Selected *Pichia* transformants were cultivated in buffered minimal methanol medium in shaking flasks at 30 °C. Activity of Mrl2 (towards ABTS) increased over 3 days of expression and reached 1850

U l^{-1} for nMrl2 and 2630 U l^{-1} for αMrl2. Since Mrl2, secreted via the α-factor, provided higher activity towards ABTS, this construct was used for laccase production in a 3 l fed-batch fermentation process.

Fermentation and purification of Mrl2

Laccase production was evaluated in a 3 l high cell-density fed-batch fermentation process. The highest volumetric activity of 281,000 U l^{-1} was reached on day seven after induction with methanol. The fermentation broth was harvested on day eight after induction when a volumetric activity of about 272,800 U l^{-1} was measured. Cell-density reached $OD_{600} = 460$. Mrl2 was purified from concentrated supernatant by DEAE FF anion exchange chromatography to a specific activity of 248 U mg^{-1} towards ABTS which resulted in 1.33-fold purification. The yield of laccase calculated on the basis of the specific activity was 1.05 g l^{-1}.

SDS-PAGE of purified Mrl2 revealed a strong band around 90 kDa (Fig. 1). Deglycosylation with PNGase F resulted in a shift of the 90 kDa band to around 60 kDa. The calculated molecular weight of Mrl2 is 54 kDa. The discrepancy to the calculated molecular weight might be due to additional O-glycosylation of Mrl2, which is not cleaved off by PNGase F. Laccase zymogram in the gel supplemented with ABTS revealed a strong band at ~55 kDa (Fig. 1). Migration of Mrl2 in the zymogram at a molecular weight of ~55 kDa might result from faster migration of properly folded, unboiled Mrl2 sample loaded onto the gel. Boiled Mrl2 samples completely lost their activity in the zymogram.

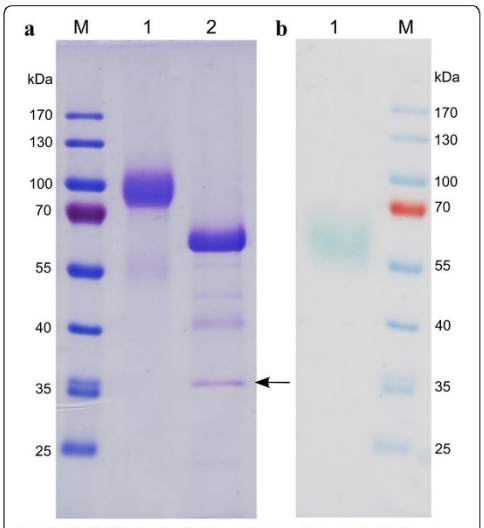


Fig. 1 12.5% SDS-PAGE of Mrl2 purification. 5 µg total protein was loaded in each *lane*; *M* pre-stained protein markers; **a** Coomassie staining; *1* Mrl2 after DEAE FF purification, *2* Mrl2 after DEAE FF purification and PNGase F (indicated by an *arrow*) treatment, **b** zymogram with ABTS *1* purified Mrl2

Biochemical characterization

Atomic absorption spectroscopy revealed fully copper loaded Mrl2 (4.8 copper ions per molecule). The pH optimum of Mrl2 was determined with four typical laccase substrates: ABTS, SGZ, 2,6-DMP and guaiacol. Mrl2 was rather active at acidic pH values (Fig. 2). For ABTS oxidation the optimal pH was 2, for SGZ and 2,6-DMP oxidation the pH optimum was 4 and the highest guaiacol oxidation was found at pH 5 (Fig. 2). Kinetic parameters were determined for all four substrates at their optimal pH value (Table 2). Mrl2-catalyzed reactions followed Michaelis–Menten kinetics. The highest k_{cat} value was determined with ABTS as substrate (316 s^{-1}) and the lowest with SGZ (21 s^{-1}). K_m values ranged from 12.45 µM for syringaldazine to 2235 µM for guaiacol.

Stability of Mrl2 was determined at pH 2–10 and was found to be higher at basic pH values than at acidic ones. After 1 h incubation at pH 2 no activity of Mrl2 was detectable (Fig. 3). At pH 3–6 after 1 h incubation residual activity of 45–75% was found, while after 24 h the activity

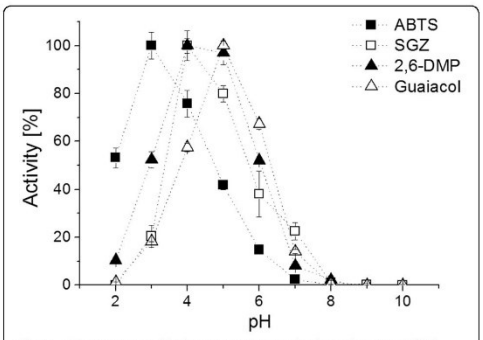


Fig. 2 pH optimum of Mrl2 in reactions with the substrates ABTS, SGZ, 2,6-DMP and guaiacol. The highest measured activity for each substrate was set to 100%

Table 2 K_m and k_{cat} values of Mrl2 with ABTS, SGZ, 2,6-DMP and guaiacol at their corresponding pH optima

	K_m (µM)	k_{cat} (s^{-1})	k_{cat} K_m^{-1} ($µM^{-1}$ s^{-1})
ABTS	24.13 ± 1.9	316	13.076
SGZ	12.45 ± 1.6	21	1.684
2,6-DMP	358 ± 58.5	74	0.207
Guaiacol	2235 ± 117.5	37	0.016

was hardly detectable. At pH 7 the activity was still around 50% after 24 h incubation and at pH 8–10 residual activity was highest with around 70% after 24 h (Fig. 3).

Half-life of Mrl2 at temperatures between 20 and 80 °C was determined at pH 7. Mrl2 was quite stable at 20 °C and pH 7 with a half-life of 13 days (Table 3). At elevated temperature the stability of Mrl2 was quite low with a half-life of 59 min at 50 °C and less than 1 min at 60 °C and above.

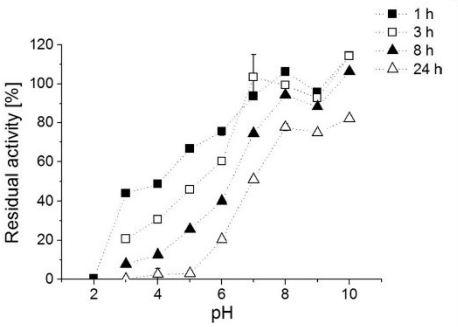


Fig. 3 Stability of Mrl2 at different pH values. After 1 h (*black square*), 3 h (*white square*), 8 h (*black triangle*) and 24 h (*white triangle*) incubation the residual activity (indicated in % of initial activity) was determined

Table 3 Half-life of 500 µg ml^{-1} Mrl2 after incubation in 50 mM potassium phosphate buffer, pH 7 at different temperatures

Temperature (°C)	Half-life
20	13 days
30	3 days
40	12.5 h
50	59 min
60, 70, 80	<1 min

The effect of different metal ions, water-miscible organic solvents as well as some inhibitors on Mrl2 was examined by using ABTS as substrate. The metal ions were applied as sulfates, except Ca^{2+}, which was applied as $Ca(NO_3)_2$ due to low solubility of $CaSO_4$. Activity of Mrl2 was hardly or not affected by Mn^{2+}, Co^{2+}, Cu^{2+}, Na^{2+}, Zn^{2+} and K^{+} even at 100 mM concentration. At a concentration of 10 mM only Ni^{2+} and Ca^{2+} led to a slight inhibition of Mrl2 of 15–18%. At 100 mM metal ion concentration again Ni^{2+} and Ca^{2+} ions led to the highest inhibition of Mrl2 with residual activities towards ABTS of 26.2 and 50.4%, respectively. NO_3^- was not responsible for the inhibitory effect of $Ca(NO_3)_2$ since 100 mM KNO_3 had no effect on the activity of Mrl2 (data not shown). 100 mM Mg^{2+} ions diminished the activity by about 20%, while the other tested metal ions hardly reduced the activity but even increased it in the case of Mn^{2+} (Table 4).

Water-miscible organic solvents more severely affected Mrl2 activity than metal ions. Already with 10% DMSO or acetone residual activity of Mrl2 dropped by about 80% (Table 4). 10% of ethanol and methanol slightly affected activity of Mrl2, while at a concentration of 20% residual activity of Mrl2 was 22 and 39%, respectively. 20% acetonitrile and 2-propanol inhibited Mrl2 almost completely. Activity of Mrl2 was hardly impaired by glycerol with about 90% residual activity at 20% glycerol.

Low concentrations of SDS had little effect on Mrl2 while 0.1% sodium azide almost completely inhibited Mrl2. Cl^- ions had a strong inhibitory effect on Mrl2. In the presence of 10 mM Cl^- Mrl2 showed 37% residual activity and at 50 and 100 mM concentration only 12.2 and 6.74% residual activity towards ABTS could be determined.

Table 4 Residual activity of Mrl2 towards ABTS in presence of different metal ions, cosolvents and inhibitors

Metal ions	Residual activity (%)		Cosolvent	Residual activity (%)		Inhibitor	Concentration	Residual activity (%)
	10 mM	100 mM		10%	20%			
Mn^{2+}	99.3 ± 1.9	119.4 ± 3.8	Ethanol	81.9 ± 1.5	21.7 ± 2.4	SDS	0.1%	100.3 ± 0.7
Co^{2+}	100.9 ± 0.9	104.6 ± 2.9	Methanol	76.5 ± 2.7	39 ± 0.8		1%	77.7 ± 1.1
Ni^{2+}	82.7 ± 1.5	26.2 ± 0.9	2-Propanol	61.9 ± 2.5	5.5 ± 2.5			
Ca^{2+}	85.4 ± 1	50.4 ± 1.9	ACN	52 ± 2.8	0.7 ± 0.4	Sodium azide	0.1%	3.3 ± 1.9
Cu^{2+}	104.9 ± 2.5	95.2 ± 2.7	DMSO	22.9 ± 2.2	26.1 ± 3.6			
Na^{+}	99 ± 2.8	105.1 ± 4.4	Acetone	22.2 ± 2	16.6 ± 2.3	Cl^-	10 mM	37.3 ± 0.9
Mg^{2+}	101.5 ± 1.5	80.1 ± 1.7	DMF	40.2 ± 4.3	16.6 ± 0.4		50 mM	12.2 ± 0.15
Zn^{2+}	104.9 ± 2.1	97.0 ± 1.2	Glycerol	85.7 ± 0.5	89.7 ± 1.9		100 mM	6.7 ± 0.04

ACN acetonitrile, *DMSO* dimethyl sulfoxide, *DMF* dimethylformamid

Redox potential

For redox potential determination we used $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$and $[Fe(bipy)_2]^{3+}$/$Fe[(bipy)_2]^{2+}$ redox couples with a standard redox potential of 0.433 V (O'Reilly 1973) and 0.78 V, respectively. In first titrations with the $[Fe(bipy)_2]^{3+}$/$Fe[(bipy)_2]^{2+}$ redox mediator couple Mrl2 could not be fully reduced (data not shown), therefore the $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ couple with the lower standard potential was applied. The redox titration with ferro- and ferricyanide could not fully oxidize Mrl2. For this reason the sample was aerated and the potential was set to an arbitrary high potential of 1.23 V. Redox titrations with iron(II/III) bipyridine indicated that the redox potential must be lower than 0.7 V. Titrations with the ferri/ferrocyanides confirmed these findings and revealed a redox potential of 0.58 V (Additional file 1: Figure S1).

Degradation of EDCs and NSAIDs

In the next step we examined the degradation of several micropollutants (Table 1) by Mrl2 at pH 7. Mrl2 degraded bisphenol A and the steroids 17ß-estradiol, 17α-ethinyl estradiol and estriol within 30 min below 10% residual concentration. Estrone seemed to be the most recalcitrant among the tested estrogens. After 1 h incubation a residual concentration of about 55% could be determined. After 20 h concentration of estrone decreased below the detection limit. Diclofenac was the most recalcitrant compound that could be degraded. After a reaction time of 20 h still about 42% diclofenac remained. Bisphenol S and naproxen were not affected by Mrl2 treatment within 20 h (Fig. 4).

A comparative EDCs and NSAIDs degradation study was conducted with commercially available laccases preparation from *T. versicolor* (TvL). 30 µg ml^{-1} Mrl2 and TvL were used to degrade 100 µM micropollutants. After 1 h treatment, Mrl2 degraded all four estrogens and bisphenol A to a higher extent than TvL, while for diclofenac hardly any difference could be noticed between the two laccases. The reaction time for diclofenac was extended to 24 h. In this case TvL showed approximately twice the activity of Mrl2 (Fig. 5).

Discussion

Laccases have gained significant attention due to emerging applications including biomass degradation, biofuel cells, biocatalysis and bioremediation (Cannatelli and Ragauskas 2016; Kudanga et al. 2011; Mikolasch and Schauer 2009; Senthivelan et al. 2016; Strong and Claus 2011; Suzuki et al. 2003). However, low expression levels have been recognized as a major obstacle to industrial use of these enzymes (Piscitelli et al. 2010). Our results demonstrate that Mrl2 was expressed at exceptionally high levels in *P. pastoris* in a 3 l fed-batch fermentation process. To our knowledge Mrl2 expression achieved the highest level of a fungal laccase in *P. pastoris* reported so far with 281,000 U l^{-1}, which corresponds to 1.05 g Mrl2/l medium at a specific activity of 248 U mg^{-1}. Similar laccase yield in *P. pastoris* was only reported for a bacterial laccase from *Thermus thermophilus* with 1.2 g l^{-1} (Liu et al. 2015a). Specific activity of this enzyme (1.12 U mg^{-1}) was, however, much lower compared to Mrl2. Comparable laccase yields of 0.8–1 g l^{-1} of the heterologously expressed fungal laccase *T. versicolor* could only be reached using the filamentous fungal host *Trichoderma reesei* (Baker and White 2001). Higher yields were only reported for homologous expression of laccases in

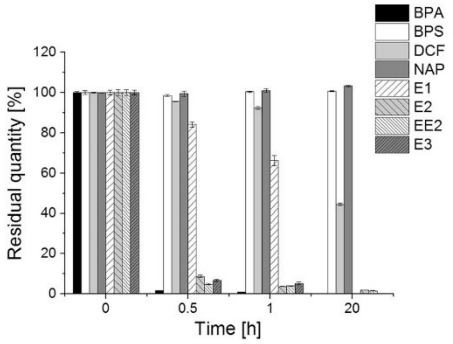


Fig. 4 Residual quantity of EDCs and NSAIDs (%) after 0.5, 1 and 20 h of incubation with 20 U ml^{-1} Mrl2 at room temperature in 50 mM potassium phosphate buffer, pH 7. Measurements were carried out in triplicate

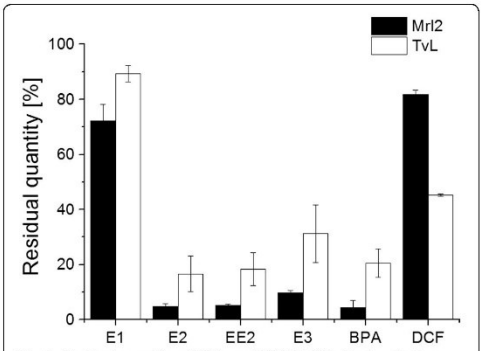


Fig. 5 Residual quantity of EDCs and NSAIDs (%) after incubation with 30 µg ml^{-1} Mrl2 or TvL. The reaction time was set to 1 h except for DCF, for which it was 24 h. Measurements were carried out in triplicate

basidiomycetes (Table 5). Explanations for the exceptional high expression of Mrl2 in *P. pastoris* remain elusive. Both the α-factor and the native secretion signal sequence resulted in high secretion of Mrl2, indicating that high expression yield can rather be traced back to the laccase sequence than signal peptide used.

Mrl2 possesses a phenylalanine as an axial ligand of the T1 copper. A positive correlation between hydrophobicity of this ligand and the redox potential E° has been reported (Marshall et al. 2009). Phenylalanine at this position implicates a high redox potential. Moreover, the hydrogen bond between serine at position 113 and glutamate at position 455 contributes to a long T1 Cu-His ligand distance which, according to Piontek and colleagues, strongly anticipates a high redox potential (Piontek et al. 2002). With a redox potential of 0.58 V, Mrl2, however, belongs to the middle-potential laccases. Besides the nature of the axial ligand and neighboring amino acids, redox potential is obviously influenced by other factors including hydrogen bonding of H(N) backbone and the coordinating SCys as well as protein and solvent dipoles and solvent accessibility (Hong et al. 2011). The reason why Mrl2 has a middle redox potential is under further investigation.

Important prerequisites for application of laccases in bioremediation for example in wastewater treatment are besides their sufficient availability, high stability at pH 7 or higher and the ability of degrading micropollutants including pharmaceuticals under neutral or slightly alkaline conditions even without adding redox mediators. In respect of these factors, Mrl2 looks very promising. Moreover, Mrl2 demonstrated high stability in the presence of metal ions which are often present in wastewater. Even at 100 mM concentration Mn^{2+}, Co^{2+}, Cu^{2+}, Na^{2+}, Zn^{2+} and K^{+} had almost no effect on laccase activity, examined in citrate phosphate buffer pH 3. A chelating effect of citrate on metal ions and thus their weaker effect on the laccase activity could be excluded since measurements in HEPES buffer provided comparable results (data not shown).

Despite middle redox potential, Mrl2 was able to degrade bisphenol A to 100% and all tested estrogens to more than 97% at neutral pH, with estrone being the most recalcitrant one. Diclofenac that is considered as poorly degradable in studies dealing with its removal (Barbosa et al. 2016), was degraded to 56%. Several reports describe effective removal of EDCs and NSAIDs by other laccases as well. Those experiments were carried out at acidic pH values, due to higher laccase activities at these pH values (Asadgol et al. 2014; Garcia-Morales et al. 2015; Macellaro et al. 2014; Sei et al. 2008; Tsutsumi et al. 2001). However, wastewater of plant effluents, which are considered the main source of estrogens (Snyder et al. 2001), usually show a neutral or basic pH. For this reason we used pH 7 for removal experiments with Mrl2. Despite the fact that fungal laccases usually demonstrate very low activities at that pH, Mrl2 still showed reasonable activity, thus representing an effective biocatalyst for the use in bioremediation. For example, Saito et al. (2004) used 50,000 U l^{-1} purified laccase from an ascomycete fungus belonging to *Chaetomiaceae* family to degrade 93.7% bisphenol A within 1 h, whereas 20,000 U l^{-1} Mrl2 degraded 98% bisphenol A within 30 min. To degrade the recalcitrant NSAID diclofenac Lloret et al. (2010, 2013) used 2000 U l^{-1} mid-redox potential laccase MtL from *Myceliophthora thermophila* and 2000 U l^{-1} (147 mg l^{-1}) of high-redox potential *T. versicolor*. After 8 h incubation with MtL and 24 h incubation with *T. versicolor* laccase 2 and 27% diclofenac was removed, respectively, while 20,000 U l^{-1} (80 mg l^{-1}) Mrl2 decreased diclofenac concentration by 8 and 56% after 1 and 20 h incubation, respectively. Lloret et al. (2010) also tested 2000 U l^{-1} MtL with the estrogens estrone, 17ß-estradiol and 17α-ethinyl estradiol at pH 7. While removal of 17ß-estradiol with Mrl2 and MtL was comparable (96 and 99% decrease for Mrl2 and MtL after 1 and 8 h, respectively), the contraceptive 17α-ethinyl estradiol was degraded by Mrl2 (95%, 1 h) much faster than by MtL (85%, 8 h).

Yet comparison of different micropollutant removal experiments might be misleading in regard to different specific activities of the laccases and thereby quite different amounts of applied enzyme as e.g. Mrl2 has an exceptionally high specific activity towards ABTS. This is why we applied commercial TvL and Mrl2 in a degradation experiment of several micropollutants at equal enzyme amounts. Though TvL possess a high redox potential (Reinhammar 1972), Mrl2 showed faster degradation of estrogens and bisphenol A within 1 h. Besides laccases' redox potential, amino acids involved in substrate binding near the T1 copper site contribute to high substrate turnover rates. Our findings suggest better binding of hydroxylated compounds like estrogens and bisphenol A by Mrl2 than TvL. With the more recalcitrant, non-hydroxylated diclofenac, TvL displayed about 2.5-fold higher activity than Mrl2. In this case the higher redox potential of TvL might afford faster degradation of diclofenac compared to Mrl2. Another aspect might be different stability of the enzymes during 24 h incubation.

In concordance with previous studies, bisphenol S and naproxen were not degraded by laccase alone (Lloret et al. 2010). Laccase-mediated removal of bisphenol S and naproxen seems to require addition of small redox mediators, applied to expand the substrate spectrum of laccases to e.g. compounds with higher redox potentials like non-phenolic substances. These mediators are, however, mostly expensive and often not environmentally

Table 5 Comparison of reported laccase yields and volumetric activity in different expression hosts

Source	Expression host	Laccase	Yield (mg l^{-1})	Vol. activity (U l^{-1})	Reference
Heterologous expression in yeast					
Trametes versicolor	*Pichia pastoris*			2.8	Bohlin et al. (2006)
Cryphonectria parasitica	*Saccharomyces cerevisiae*	lac3		41.5	Kim et al. (2010)
Pleurotus eryngii	*Saccharomyces cerevisiae*			146	Bleve et al. (2008)
Melanocarpus albomyces	*Saccharomyces cerevisiae*		3	168	Kiiskinen and Saloheimo (2004)
Bacillus licheniformis	*Pichia pastoris*			227.9	Lu et al. (2013)
Moniliophthora perniciosa FA553	*Pichia pastoris*	LacMP		232	Liu et al. (2015b)
Melanocarpus albomyces	*Saccharomyces cerevisiae*	MaL	7	270	Andberg et al. (2009)
Ganoderma lucidum	*Pichia pastoris*		580	685.8	You et al. (2014)
Pycnoporus cinnabarinus	*Yarrowia lipolytica*		19.84	1024	Madzak et al. (2005)
Yarrowia lipolytica	*Pichia pastoris*	YlLac		1290	Kalyani et al. (2015)
Bacillus subtilis	*Pichia pastoris*	CotA		1648.15	Wang et al. (2015)
Ganoderma weberianum TZC-1	*Pichia pastoris* GS115	GwLac1		2260	Zhou et al. (2014)
Trametes trogii	*Pichia pastoris*	Lcc1	17	2520	Colao et al. (2006)
Botrytis aclada	*Pichia pastoris*		517	3220	Kittl et al. (2012a)
Ganoderma fornicatum 814	*Pichia pastoris*	rLac1		3460	Huang et al. (2011)
Lenzites gibbosa	*Pichia pastoris*			5406	Zheng et al. (2014)
Trametes sp. AH 28-2	*Pichia pastoris*	lacA	4	5470	Hong et al. (2006)
Rigidoporus microsporus (*Fomes lignosus*)	*Pichia pastoris*		9.03	5950	Liu et al. (2003)
Thermus thermophilus SG0.5JP17-16	*Pichia pastoris*	LacTT	1200	6130	Liu et al. (2015a)
Cerrena sp. HYB07	*Pichia pastoris*	Lac1	19.3	6300	Yang et al. (2015)
Pleurotus sajor-caju	*Pichia pastoris*	lac4	110	10,200	Soden et al. (2002)
Trametes versicolor	*Pichia methanolica*			12,600	Guo et al. (2006)
Trametes versicolor	*Pichia pastoris*	lccA		18,123	Li et al. (2014)
Botrytis aclada	*Pichia pastoris*		495	51,000	Kittl et al. (2012b)
Trametes versicolor	*Pichia pastoris*			140,000	Hong et al. (2002)
Trametes sp. 420	*Pichia pastoris*		136	239,000	Zhou et al. (2007)
Pycnoporus cinnabarinus	*Pichia pastoris*		8		Otterbein et al. (2000)
Myceliophthora thermophila	*Saccharomyces cerevisiae*		18		Bulter et al. (2003)
Heterologous expression in filamentous fungi					
Trametes versicolor	*Aspergillus niger*			2700	Bohlin et al. (2006)
Pycnoporus coccineus	*Aspergillus oryzae*			3000	Hoshida et al. (2005)
Trametes hirsute	*Penicillium canescens*			3000	Abianova et al. (2010)
Myceliophthora thermophila	*Aspergillus oryzae*	r-MtL	19		Berka et al. (1997)
Phlebia radiate	*Trichoderma reesei*		20		Saloheimo and Nikupaavola (1991)
Pycnoporus cinnabarinus	*Aspergillus niger*		70		Record et al. (2002)
Melanocarpus albomyces	*Trichoderma reesei*		920		Kiiskinen et al. (2004)
Trametes versicolor	*Trichoderma reesei*		800–1000		Baker and White (2001)
Heterologous expression in bacteria					
Bacillus pumilus W3	*Bacillus subtilis* WB600	CotA		373,100	Guan et al. (2015)
Natural production host					
Trametes versicolor 1017	*Trametes versicolor* 1017	Tvlac		10,000	Chen et al. (2016b)
Trametes sp. AH 28-2	*Trametes* sp. AH 28-2	rLacB	31.6	32,000	Li et al. (2007)
Trametes pubescens MB 89	*Trametes pubescens* MB 89			65,000	Galhaup and Haltrich (2001)
Trametes multicolor MB 49	*Trametes multicolor* MB 49			85,000	Hess et al. (2002)
Cerrena sp. WR1	*Cerrena* sp. WR1	Lcc3	200	202,000	Chen et al. (2012)
Cerrena sp. HYB07	*Cerrena* sp. HYB07	LacA	108	210,800	Yang et al. (2014)
White rot fungus WR-1	White rot fungus WR-1			692,000	Revankar and Lele (2006)

friendly, and their application in wastewater treatment thus doubtful.

In conclusion, Mrl2, a new laccase from *M. rorerі* was identified and showed exceptionally high expression levels during fed-batch fermentation of recombinant *P. pastoris* (280,000 U l^{-1}; 1.05 mg l^{-1}) matching those of filamentous fungi. The enzyme is stable up to 30 °C, at alkaline pH values and active in the presence of several water-miscible organic solvents and metal ions. Despite its redox potential of 0.58 V Mrl2 degrades estrogens like estrone, 17β-estradiol, estriol and the contraceptive 17α-ethinyl estradiol and an estrogenic active substance bisphenol A faster than TvL at neutral pH. This makes Mrl2 a promising candidate for application in wastewater treatment.

Additional file

Additional file 1: Figure S1. Additional information.

Abbreviations

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa: kilo Dalton; PNGase F: peptide-*N*-Glycosidase F; HPLC: high performance liquid chromatography; BMGY: buffered complex glycerol medium; BMM: buffered minimal methanol; DMSO: dimethyl sulfoxide; DMF: dimethylformamide; TvL: laccases preparation from *Trametes versicolor*; PTM_1: *Pichia* trace metal salts; ABTS: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); SGZ: syringaldazine; 2,6-DMP: 2,6-dimethoxyphenol; HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid; DEAE FF: diethylaminoethyl-Sepharose Fast Flow; BPA: bisphenol A; BPS: bisphenol S; DCF: diclofenac; NAP: naproxen; E1: estrone; E2: 17ß-estradiol; EE2: 17α-ethinyl estradiol; E3: estriol; EDC: endocrine disrupting compound; NSAID: non-steroidal anti-inflammatory drug.

Authors' contributions

AB designed and conducted the experiments, evaluated the results and drafted the manuscript. KK drafted the manuscript and helped in research design and data analysis. PLH helped in redox potential titrations, evaluated the redox potential titration results, and revised the manuscript. VBU gave advices in the research work, helped in drafting the manuscript, and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data on which the conclusions are made are all presented in this paper.

Ethical approval and consent to participate

This paper does not contain any studies with human participants or animals performed by any of the authors.

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Expression of a new laccase from *Moniliophthora roreri* at high levels in *Pichia pastoris* and its potential application in micropollutant degradation

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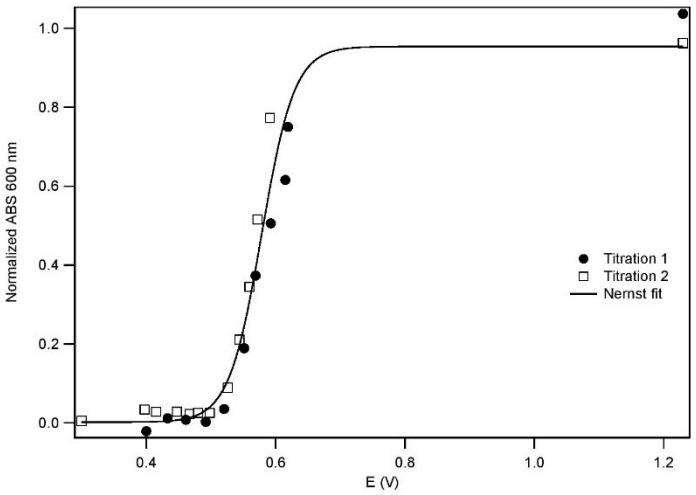

Figure S1. Results of two independent redox titrations with Mrl2 (21-26 µM) and the $[Fe(CN)_6]^{3-}$ / $[Fe(CN)_6]^{4-}$ redox mediator couple. The fully oxidized sample was set to 1.23 V. Fit to Nernst curve: $Y = \frac{A}{1+e^{\frac{nF}{RT}(E_m - E)}} + B$. Fixed parameters: n = 1; T = 293 K. Fit parameters: A = 0.95 ± 0.04; B = 0.00 ± 0.02; E_m = 0.578 ± 0.004 V.

3.2 Manuscript 2: Redesign of a New Manganese Peroxidase

Title: Redesign of a New Manganese Peroxidase Highly Expressed in *Pichia pastoris* towards a Lignin-Degrading Versatile Peroxidase

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Contribution: Design of experiments. Carrying out the experiments. Writing the manuscript.

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Redesign of a New Manganese Peroxidase Highly Expressed in *Pichia pastoris* towards a Lignin-Degrading Versatile Peroxidase

Agathe Bronikowski, Katja Koschorreck, and Vlada B. Urlacher*[a]

Manganese peroxidases, lignin peroxidases, and versatile peroxidases secreted by white rot fungi are supposed to play an essential role in lignin degradation. Thus, these enzymes have attracted significant attention as potential biocatalysts. Versatile peroxidases are the most interesting ones, since they comprise activities of manganese and lignin peroxidases. Herein, we demonstrate how the properties of a new manganese peroxidase from *Moniliophthora roreri*, designated MrMnP1, were shifted towards those of a versatile peroxidase. MrMnP1 was cloned in *Pichia pastoris* X-33 and highly expressed in a fed-batch fermentation, yielding 132 mg L^{-1} of active enzyme. To extend the substrate spectrum of MrMnP1, a catalytically active tryptophan present in lignin and versatile peroxidases was first introduced. Additionally, the role of five amino acids at positions adjacent to the catalytic tryptophan was elucidated through their replacement by those found in a versatile peroxidase from *Pleurotus eryngii*. The resulting mutants demonstrated new activities towards high-redox-potential substrates, such as lignin dimers, veratryl alcohol, and the azo dye Reactive Black 5.

Introduction

One of the most urgent problems to solve in the near future is the substitution of the finite feedstock petroleum. Lignocellulose, consisting of cellulose, hemicellulose, and lignin, is a renewable source of fermentable sugars and small aromatic compounds; thus representing a good replacement for crude oil.[1] The main bottleneck in the valorization of lignocellulose is the high recalcitrance of the biopolymer lignin. On the one hand, lignin surrounds the readily usable cellulose, which hampers the access of cellulases and, on the other hand, it adsorbs those enzymes, which are then no longer available for cellulose depolymerization.[2] Ligninolytic enzymes, such as manganese peroxidases (MnPs), lignin peroxidases (LiPs), versatile peroxidases (VPs), or laccases, secreted by white rot fungi, are supposed to play a major role in biomass and particularly lignin degradation.[3] MnPs (EC 1.11.1.13) are the most abundant ligninolytic peroxidases expressed by basidiomycetes.[4] They belong to class II heme-containing peroxidases that are characterized by the ability to oxidize Mn^{2+} to Mn^{3+}, which then is chelated by dicarboxylic acids that, in turn, can act as diffusible mediators capable of attacking phenolic constituents of lignin.[4] Low-redox-potential substrates, such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 2,6-dimethoxyphenol (2,6-DMP), can be oxidized by some MnPs at the main heme access channel.[5] In contrast, LiP has no catalytic activity on manganese. It possesses a catalytic tryptophan on the enzyme surface, which enables oxidation of some high-redox-potential compounds, such as veratryl alcohol (VA), through long-range electron transfer. The VA cation radical, in turn, can act as a diffusible mediator and oxidize highly recalcitrant non-phenolic lignin components.[6] However, LiPs catalyze the oxidation of simple phenols quite inefficiently, and are not able to oxidize high-redox-potential dyes and many other substrates that VPs are capable of oxidizing.[7] VP comprises catalytic activities of both MnP and LiP. VP can oxidize Mn^{2+} at its Mn oxidation site, whereas low-redox-potential substrates, such as ABTS or 2,6-DMP, are oxidized at the main heme access channel, as well as at the catalytic tryptophan, which, more importantly, enables oxidation of high-redox-potential substrates, such as non-phenolic lignin dimers, VA, or the azo dye Reactive Black 5 (RB5) without the help of diffusible mediators.[8] This makes VP quite valuable and interesting for biotechnological purposes.[8,9] However, VPs are less abundant than MnPs and LiPs, so that a lower diversity of VP can be expected.[4,10] Generally, the industrial application of ligninolytic peroxidases is hampered by their low expression titers.

In our previous work, we showed that the laccase Mrl2 from *Moniliophthora roreri*, a ligninolytic enzyme exhibiting a relatively low redox potential (0.57 V), could be expressed under the AOX1 promoter in easy-to-cultivate *Pichia pastoris* at a remarkably high level of 1.05 g L^{-1}.[11] *M. roreri* causes frosty pod rot in cacao, and thus, is supposed to produce a number of lignocellulose-degrading enzymes. Indeed, apart from laccase genes, *M. roreri* genome contains several genes that encode for ligninolytic peroxidases, such as MnPs, which are, due to their higher redox potential, more powerful in lignin degradation than laccases, but no VPs. In this work, one of those

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genes, namely, *mrmnp1*, was cloned and expressed in *P. pastoris* at a high level. To extend the substrate spectrum of MrMnP1, a catalytic tryptophan was introduced at the enzyme surface, as shown previously for other MnPs.[12] Whereas the catalytic tryptophan is considered to be essential for oxidizing substrates with a high redox potential, much less is known about the effect of other positions in its surrounding. Site-specific mutagenesis of MrMnP1 at positions adjacent to the catalytic tryptophan towards those present in VP from *Pleurotus eryngii* allowed us to elucidate their role in substrate selectivity, and to design MrMnP1 variants with new activities not present in the wild-type (WT) enzyme.

Results and Discussion

Cloning and expression

The codon-optimized gene of putative MnP ESK95360.1, designated MrMnP1, was cloned without its native secretion signal into the *P. pastoris* expression vector pPICZαA, with the α-factor secretion signal from *Saccharomyces cerevisiae* and integrated into the *P. pastoris* X-33 genome under the control of the *AOX1* promoter. Several clones were screened for activity towards ABTS in the culture medium in shaking flasks. The clone that showed the highest activity was chosen for production in a 7.5 L bioreactor. During a fed-batch fermentation, the MrMnP1 enzyme was secreted into the culture medium upon induction with methanol. Seven days after induction, an activity of (17530±324) U L^{-1} towards ABTS at pH 3 was measured, which corresponded to a peroxidase concentration of about 132 mg L^{-1} in the culture medium. Because the total protein concentration in the medium was 295 mg L^{-1}, about 45% of the total protein was assigned to the peroxidase. This value was corroborated by densitometric analysis of Coomassie-stained proteins after SDS-PAGE (Figure S1 in the Supporting Information). A large-scale utilization of fungal peroxidases is often hampered by their low expression in native and heterologous expression hosts (e.g., *Escherichia coli*, *S. cerevisiae* or *P. pastoris*).[13] Many research groups use *E. coli* as an expression host, but the peroxidases mostly aggregate in inclusion bodies and have to be tediously refolded.[13a,14] Peroxidase expression levels in heterologous hosts have not yet exceeded 100 mg L^{-1} in the culture medium, but are mostly in the range of 1–20 mg L^{-1}.[9a,13b] Thus, the concentration of 132 mg L^{-1} achieved in this work is, to the best of our knowledge, the highest yield of ligninolytic peroxidase obtained so far. A yield of 100 mg L^{-1} peroxidase LiPH8 has been reported as well; however, because the total protein amount was 2500 mg L^{-1}, only 4% could be assigned to the peroxidase.[15]

MrMnP1 was purified by hydrophobic-interaction chromatography (HIC) and size-exclusion chromatography (SEC). After purification by SEC, SDS-PAGE revealed a band at around 65 kDa; the calculated molecular weight is 35.8 kDa. After deglycosylation by EndoHF, this band shifted to around 45 kDa, which indicated that the protein was probably not only N-, but also O-glycosylated (Figure S1). In some cases, incorporation of the heme in the apoprotein or the incorporation of iron in the heme is not sufficient during overexpression of hemoproteins.[16] Thus, we examined the integrity of the heme by measuring UV/Vis spectra of oxidized and reduced MrMnP1. The enzyme showed characteristic features of peroxidases and the Soret band shifted from $\lambda = 408$ to 438 nm upon reduction with sodium dithionite; thus indicating correct heme binding (Figure S2). The spectrum indicates proper heme incorporation. The RZ value of about 3.9 indicates high purity of the enzyme. These results indicate that *P. pastoris* is an appropriate recombinant host for expression of fungal hemoproteins.

Biochemical characterization and site-directed mutagenesis

MnPs can be divided into groups of short, long, and extra-long MnPs.[5] The length of long MnPs is 348–361 amino acids, extra-long MnPs are longer than 362 amino acids, and short MnPs are no longer than 348 amino acids. The long and extra-long MnPs are characterized by a C-terminal tail that surrounds the main heme access channel; thus preventing oxidation of low-redox-potential substrates (ABTS, 2,6-DMP). MrMnP1 is 343 amino acids long, and thus, can be categorized as a short MnP. Indeed, MrMnP1 was able to convert ABTS, 2,6-DMP, and Mn^{2+}, but not high-redox-potential substrates, such as RB5 or VA. The kinetic parameter k_{cat} was determined to be 70, 106, and 22 s^{-1} for Mn^{2+}, ABTS, and 2,6-DMP, respectively, and K_m values for these substrates were 0.022, 0.68, and 89.57 mM, respectively. For Mn^{2+}, the K_m value was in a similar range as that for other natively expressed MnPs.[17] The ability of MrMnP1 to oxidize ABTS and 2,6-DMP, as well as Mn^{2+}, confirmed that MrMnP1 shared characteristics of short MnPs.

To facilitate the conversion of high-redox-potential substrates, such as non-phenolic lignin dimers, site-directed mutagenesis was applied. First, a catalytically active superficial tryptophan, which is present in LiP and VP, but not in MnP and that enables direct oxidation of high-redox-potential substrates, was introduced (mutation A172W).[8,18] This mutation site was identified by multiple sequence alignment with numerous LiPs, VPs, and MnPs. The area surrounding the catalytically active tryptophan is also of importance for high activity of LiPs and VPs. For example, LiPs exhibit a partially negatively charged tryptophan environment, which is supposed to stabilize the VA cation radical ($VA^{\bullet+}$) formed after VA oxidation.[19] In contrast, VPs possess several positively charged residues at the surface around the catalytically active tryptophan. Unlike LiP, VP is able to oxidize polymeric lignin and the high-redox-potential dye RB5, but VA oxidation is less efficient.[8] To compare the surroundings of the catalytically active Trp164 in the VP VPL of *P. eryngii* with the corresponding area around Ala172 in MrMnP1, a homology model based on VPL of *P. eryngii* (PDB ID: 2BOQ) was created by Swiss-model (Figure 1). Five amino acids in close proximity (4 Å) to Ala172 were different in both enzymes, particularly regarding the distribution of hydrophobic and charged amino acids. By mutagenesis at the corresponding positions of the A172W mutant towards those of VPL from *P. eryngii*, respective double mutants carrying one of the K168V, I171V, F259M, A269R, and A273T mutations were constructed and individual effects of each position were assessed.

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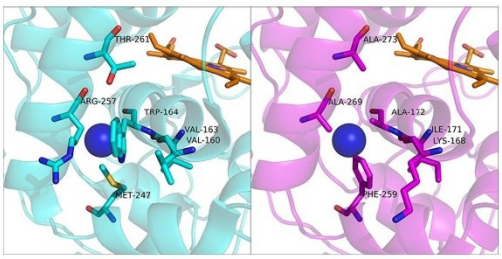

Figure 1. Image of the crystal structure of VPL from *P. eryngii* (PDB ID: 2BOQ) (left)[20] and a homology model of MrMnP1 based on 2BOQ (VPL of *P. eryngii*) from the Protein Data Bank (right). The blue sphere and orange sticks represent a calcium ion and a heme, respectively.

All mutants were purified to homogeneity by means of HIC and SEC. The introduced tryptophan 172 seems to be essential for the oxidation of high-redox-potential substrates. Activity measurements revealed that all mutants gained new activities towards high-redox-potential substrates RB5 and VA, compared with the WT (Table 1). Moreover, high catalytic efficiency during 2,6-DMP oxidation was measured for the mutants accompanied by K_m values in the micromolar range. Additional substitutions at adjacent positions to A172W enhanced catalytic efficiencies for 2,6-DMP, in most cases, due to lower K_m and higher k_{cat} values. For RB5, K_m values did not change significantly by the surrounding mutations, but k_{cat} doubled in most cases. Catalytic efficiency for VA barely changed because the K_m and k_{cat} values either did not change or doubled or tripled simultaneously. Interestingly, additional substitutions at K168V, I171V, F259M, and A273T resulted in increased k_{cat} values for all substrates, whereas A269R did not influence k_{cat} values at all. In general, higher catalytic efficiencies were determined for RB5 than those for VA. This is in agreement with earlier reports on the importance of an acidic tryptophan environment, which is present in LiPs, but not in VPs, for efficient VA oxidation.[21] Because we tried to mimic the Trp environment of VP, no acidic amino acids were introduced to enhance VA oxidation.

Two homologous positions to the chosen adjacent positions in MrMnP1 have been reported previously. Ruiz-Dueñas and co-workers showed that exchanging methionine 247 for phenylalanine in VP from *P. eryngii* (corresponds to F259 in MrMnP1) led to decreased catalytic efficiencies,[22] which was in accordance with our finding that the catalytic activities of the double mutant A172W F259M towards 2,6-DMP and RB5 increased, relative to that of the single mutant A172W. The same group also demonstrated that replacement of Arg257 (corresponds to Ala269 in MrMnP1) by Asp led to loss of activity towards RB5, whereas with Ala at that position the enzyme activity towards RB5 was retained. In our work, the exchange of Ala269 for positively charged Arg barely affected the K_m and k_{cat} values towards RB5.

We exchanged Lys168 and Ile171 in MrMnP1, which were very close to Trp172, for the smaller amino acid valine, which might have led to better substrate accessibility and thereby to higher catalytic efficiencies for 2,6-DMP. Furthermore, a more hydrophobic environment of the catalytically active tryptophan 172 might increase the redox potential of the peroxidase, thereby facilitating substrate oxidation.

The catalytic parameters listed in Table 1 were determined at pH 3. Furthermore, we investigated the activity of the mutants at higher pH values (up to pH 8) towards several substrates (ABTS, 2,6-DMP, RB5, and VA; Figure S3). The highest activity of all enzymes with the tested substrates was at pH 3, except for the WT with 2,6-DMP as a substrate, for which the activity was almost twice as high at pH 8 than that at pH 3. Although the activity of the mutants towards ABTS dropped to 5–10% at pH 5 and higher, the residual activities of the mutants towards RB5 and VA dropped less at higher pH values (20–40% residual activity at pH 5). For 2,6-DMP as a substrate, the activities of MrMnP1 and mutants were lowest at pH 6, but increased at pH 7 and 8. Nevertheless, the activity at high pH values was transient, presumably due to Ca^{2+} loss and inactivation of the enzyme. Autoxidation of 2,6-DMP in Britton Robinson buffer at pH 8 in the presence of H_2O_2 was not detected, so that the observed 2,6-DMP oxidation could be attributed to enzyme activity at this pH. Increased oxidation of, for example, textile dyes at high pH values by peroxidases and their relatively fast inactivation was reported previously.[23] The pK_a of the hydroxyl group of 2,6-DMP is about 9.98,[24] which leads to partial deprotonation at higher pH values, and hence, facilitates oxidation. Because the specific activities for 2,6-DMP did not differ much between the WT (0.4 U mg^{-1}) and mutants (0.3–1.6 U mg^{-1}) at pH 8, we assume that oxidation rather takes

Table 1. Kinetic parameters of MrMnP1 mutants towards 2,6-DMP, RB5, and VA. For 2,6-DMP, only high-efficiency oxidation at Trp172 is shown.

Substrate		WT	A172W	A172W/K168V	A172W/I171V	A172W/F259M	A172W/A269R	A172W/A273T
2,6-DMP	k_{cat} [s^{-1}]	[a]	0.5	1.0±0.1	1.1±0.1	0.8±0.1	0.4±0.1	0.7±0.1
	K_m [μM]	[a]	52.8±3.7	16.3±2.9	37.3±4.3	14.4±2.4	11.6±6.2	10.4±2.2
	k_{cat}/K_m [s^{-1} mM^{-1}]	[a]	10.0	58.8	28.6	55.6	33.3	71.4
RB5	k_{cat} [s^{-1}]	n.d.	8.6±0.2	16.7±0.7	13.4±0.7	12.1±0.3	7.9±0.3	16±1
	K_m [μM]	n.d.	0.4±0.1	0.5±0.1	0.7±0.1	0.4±0.05	0.5±0.1	0.5±0.1
	k_{cat}/K_m [s^{-1} mM^{-1}]	n.d.	21 500	33 400	19 143	30 250	15 800	32 000
VA	k_{cat} [s^{-1}]	n.d.	0.3±0.04	1.3±0.1	0.7±0.6	1.1±0.7	0.3±0.1	1.1±0.1
	K_m [μM]	n.d.	0.3±0.01	1.0±0.02	0.6±0.02	1.0±0.02	0.3±0.01	1.1±0.03
	k_{cat}/K_m [s^{-1} mM^{-1}]	n.d.	1	0.77	0.86	0.91	1	1

[a] not determined because of lack of high-efficiency oxidation. n.d.: not detected.

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place at the main heme access channel at higher pH value than at the catalytic tryptophan.

Usually the highest activities were measured at pH 3, but, since most described peroxidases are not very stable at acidic pH values,[25] we tested the stability of MrMnP1 WT and mutants at pH 3–7 by incubating the enzymes in Britton Robinson buffer for 24 h (Figure 2). All enzymes were most stable at around pH 5, which is in agreement with the data for other reported peroxidases.[5,26] The results demonstrated that, although mutation A172W had a destabilizing effect on the enzyme, mutation K168V had a stabilizing effect (Figure 2). MrMnP1 A172W K168V was the most stable mutant and retained about 54% of its initial activity after 24 h incubation at pH 3, whereas the WT only showed 26% residual activity. The highest stability of MrMnP1 A172W K168V was found at pH 6, with a residual activity of 72% after 24 h, whereas the WT showed only 38% residual activity. An explanation for the stabilizing effect of the mutation K168V remains elusive.

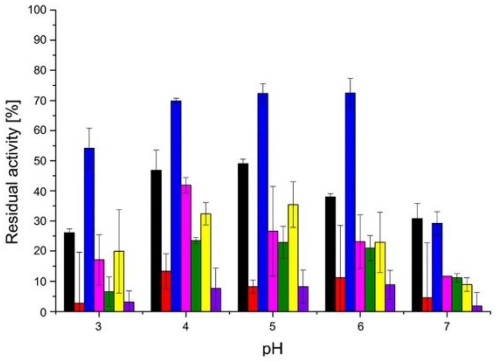

Figure 2. The pH stability of MrMnP1 WT (■) and mutants A172W (■), A172W K168V (■), A172W I171V (■), A172W F259M (■), A172W A269R (■), A172W A273T (■). The stability of MrMnP1 WT and mutants was evaluated in Britton Robinson buffer at pH 3–7 for 24 h. The residual activity was measured with ABTS as a substrate.

Substrate scope of MrMnP1 mutants

To evaluate the substrate scope and applicability of the mutants in lignin degradation, the phenolic and non-phenolic lignin dimers guaiacylglycerol-β-guaiacyl ether (Ge) and veratrylglycerol-β-guaiacyl ether (Ve) were tested as substrates at pH 3–5 (Figure 3). The phenolic lignin dimer Ge was barely oxidized by the WT (2% conversion), but after introduction of the A172W mutation around 33% could be degraded at pH 3 and 4, and around 15% at pH 5. All additional mutations (except for A269R) further increased the activity towards Ge at pH 3 and 4, with the A172W K168V mutant showing the highest conversion of up to 56% at pH 3.

The more recalcitrant non-phenolic lignin dimer Ve was oxidized only by the mutants, but not WT. Generally, lower conversions (1–6.5%) were achieved relative to the oxidation of Ge at the same experimental settings. Again, the A172W K168V mutant showed the highest activity and resulted in conversions of about 6.5 and 5.5% at pH 3 and 4, respectively, which might be explained by higher k_{cat} values of the double mutant than that of the single mutant (0.32 s^{-1} for A172W; 1.56 s^{-1} for A172W K186V). K_m values of 1.91 and 1.62 mM were similar for the single and double mutant, respectively. Although Ve was much better oxidized by the A172W K168V mutant compared with the other three double mutants (A172W I171V, A172W F259M, and A172W A273T), the activity of these four double mutants towards Ge was more or less the same. It can be assumed that the proposed higher redox potential at Trp172 in the A172W K168V mutant (relative to the other double mutants) plays an important role in oxidizing high-redox-potential substrates, such as Ve, but is less pronounced in oxidizing substrates with lower redox potentials, such as Ge.

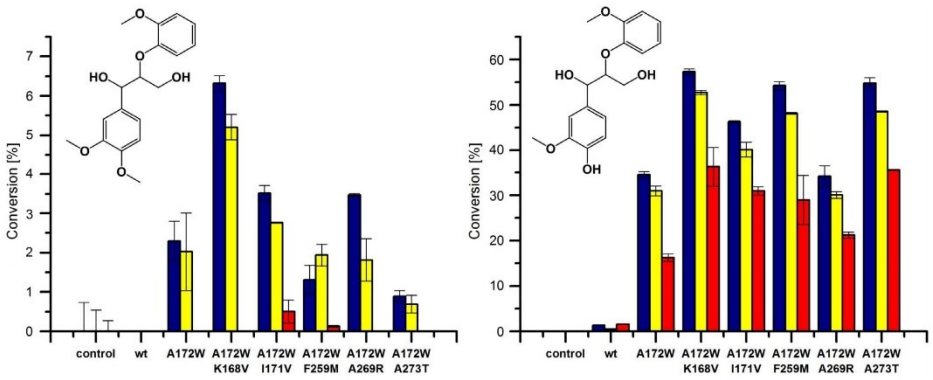

Figure 3. Lignin dimer degradation by MrMnP1 WT and mutants. The lignin dimers Ve (left) and Ge (right) were incubated with MrMnP1 WT and mutants at pH 3 (blue), 4 (yellow), and 5 (red).

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In addition to lignin dimers, we also tested the degradation of aromatic compounds, which may resemble aromatic units present in lignin. For the unsubstituted aromatic compound *trans*-cinnamic acid, and the single methoxy-substituted aromatic compounds 3-methoxycinnamic acid and 4-methoxyphenylacetic acid, no product formation with either WT or mutants could be detected. For the two and three methoxy-substituted aromatic compounds 3,4,5-trimethoxycinnamic acid and 3-(3,4-dimethoxyphenyl)propionic acid, no product formation with WT, but with the mutants was detected, whereby A172W K168V was the best mutant (Figure S4). In general, compounds with two or three methoxy-substituted aromatic rings were better substrates than the un- or once substituted aromatic compounds. These findings are consistent with those published by other groups.[27] Better performance of the A172W K186V variant might be due to better accessibility of the substrates to Trp172 and a higher redox potential, as stated before.

To further investigate the influence of the introduced mutations on the oxidative power of MrMnP1 variants towards high-redox-potential compounds, several dyes were tested as substrates (Figure 4). The anthraquinone dyes Remazol Brilliant Blue R and Alizarin Red S were completely oxidized by all mutants within 20–40 min. The WT enzyme showed no activity towards Alizarin Red S, but accepted Remazol Brilliant Blue R as a substrate, although showing a lower activity than the mutants. The second azo dye, Methyl Orange, was almost completely oxidized in the presence of mutants after 60 min, but only to 50% by the WT. Overall, the constructed MrMnP1 mutants were able to oxidize Alizarin Red S, which was not accepted by the WT enzyme, and showed higher activity towards other tested dyes, with MrMnP1 A172W K168V being again the most powerful variant.

Dye decolorization was often performed with MnP in the presence of manganese.[28] However, Tinoco and co-workers showed that manganese-independent decolorization with VP from *Bjerkandera adusta* was superior at pH 4 to that of manganese-mediated decolorization at pH 3.[29] Thus, we investigated the effect of Mn^{2+} on the oxidation of Crystal violet and RB5 by the single mutant A172W and the double mutant A172W I171V, as well as by the WT enzyme. Crystal violet can be slightly oxidized by the WT at pH 3 without added manga-

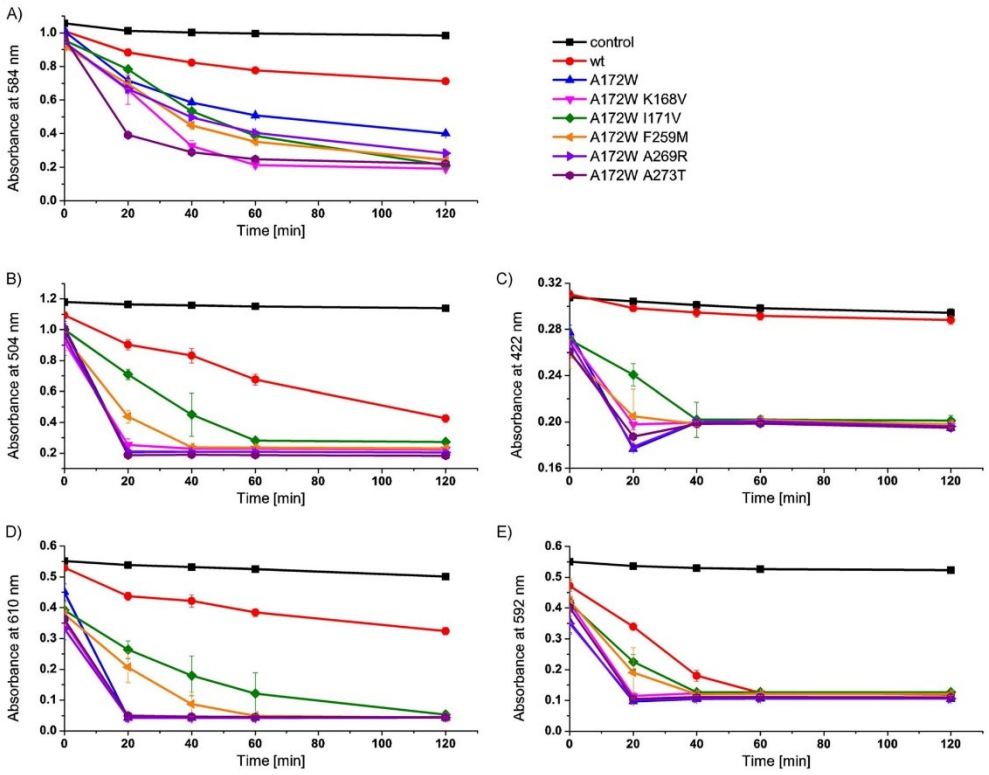

Figure 4. Dye decolorization by MrMnP1 WT and mutants. A) 25 μM Crystal violet; B) 50 μM Methyl Orange; C) 100 μM Alizarin Red S; D) 50 μM Indigo carmine; E) 100 μM Remazol Brilliant Blue R.

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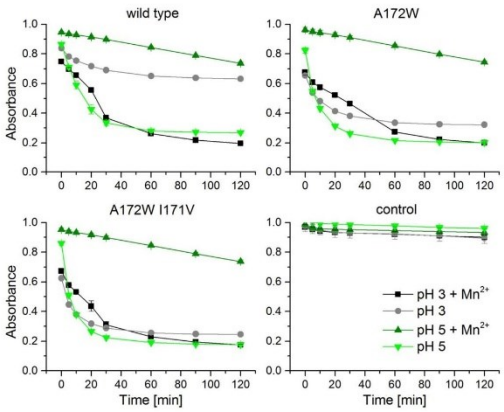

Figure 5. Influence of 1 mM Mn^{2+} on Crystal violet oxidation catalyzed by MrMnP1 WT and mutants.

nese, but not RB5. Crystal violet was oxidized by the WT at pH 3 better in the presence of manganese, whereas at pH 5 it was the opposite (Figure 5). The mutants showed similar tendencies; however, decolorization at pH 3 was stronger than that with WT, as also shown in experiments without added Mn^{2+} (Figure 4). We hypothesize that Mn^{2+} oxidation at pH 5 competes with Crystal violet oxidation, which is probably oxidized at the main heme access channel by the WT (and at Trp172 in mutants), but very inefficiently by Mn^{3+}–tartrate complexes. At pH 3, however, the presence of manganese increases oxidation of Crystal violet. We assume that Mn^{2+} oxidation at pH 3 is slower than that at pH 5; thus competing less with Crystal violet oxidation at other active sites. Furthermore, protein stabilization by slow Mn^{2+} oxidation might play an important role, as suggested by Wariishi and co-workers for a MnP from *Phanerochaete chrysosporium*.[30] The same group also showed that compound II was only efficiently reduced by Mn^{2+}, and thus, obligatory for a complete catalytic cycle. Koduri and Tien reported similar results for VA and LiP.[31] Higher total decolorization at pH 5 than that at pH 3 seems to be in contrast to the optimal pH determined for other substrates (Figure S3). However, decolorization after 5 min shows that the initial decolorization rate at pH 3 is faster, but, perhaps due to the higher enzyme stability at pH 5, the total decolorization at this pH value was higher than that at pH 3.

In the case of RB5, the addition of Mn^{2+} could enable WT oxidation at pH 3, but not at pH 5 (Figure S5). Indeed, the mutants could oxidize RB5 under all tested conditions, except if Mn^{2+} was added at pH 5. Thus, in contrast to Crystal violet, for which some decolorization was measured at pH 5 in the presence of manganese, no oxidation was observed for RB5 under these conditions. Probably, the redox potential of Mn^{3+}–tartrate complexes at pH 5 is high enough to slowly oxidize Crystal violet, but not the recalcitrant dye RB5. Similar to Crystal violet oxidation, the mutants decolorized RB5 faster at pH 3 than at pH 5 (Figure S5), but the final decolorization at pH 3 and 5 was equally efficient.

It has already been shown that the presence of Mn^{2+} can increase or reduce dye oxidation, depending on the dye used.[29,32] Our findings corroborate the reported observations and indicate that this process might be strongly dependent on pH and time.

Our results illustrate that, apart from the catalytically active tryptophan, its surroundings are also of importance for fast oxidation of high-redox-potential substrates, such as lignin model substrates and recalcitrant dyes.

Analysis of protein expression of WT MrMnP1 and mutants

Although the catalytic efficiency of MrMnP1 was clearly improved by the mutations introduced, the enzyme yields obtained in fed-batch fermentations for the corresponding mutants have been reduced from 132 to 5–33 mg L^{-1}, which is equivalent to or even higher than that of yields reported for other VPs in *E. coli* or *S. cerevisiae*.[33] Enhanced heterologous expression in *P. pastoris* might result from, for example, multiple insertion of the heterologous gene into the *P. pastoris* genome or a high mRNA level. To investigate how a single or double amino acid substitution could affect expression to such a high extent, the expression yields, gene copy numbers, and mRNA levels of several *P. pastoris* clones producing MrMnP1 WT, A172W, and A172W K168V were analyzed in shake flask experiments. No direct correlation could be found between gene copy number, mRNA level, and peroxidase yield (Figure S6). Most WT, single-mutant, or double-mutant clones contained one to three MrMnP1 peroxidase genes per genome and mRNA expression levels were around five to ten times as high as that of the reference actin gene. It can be concluded that neither the copy number nor the mRNA level are responsible for the decreased expression of the constructed mutants.

To check if the newly introduced activity at Trp172 might be responsible for the decreased expression by, for example, the formation of toxic compounds during cultivation, we expressed the WT and A172W mutant as apoenzymes in an inactive form in shake flasks and then reconstituted the activity by heme supplementation. Krainer and co-workers showed that peroxidases could be expressed in *P. pastoris* as apoenzymes in the absence of heme and be reconstituted after its supplementation.[34] In our work, the WT could be reconstituted to a volumetric activity of about 40 U L^{-1} (compared with 105 U L^{-1} for the holoenzyme), whereas the A172W mutant was reconstituted to around 4 U L^{-1}, which did not exceed enzyme activity if the expression took place in the presence of heme. This indicates that the new enzyme activity related to Trp172 introduction probably did not cause lower expression yields. The question as to why expression levels of the mutants decreased still needs to be answered and is under further investigation.

Conclusion

The wild-type manganese peroxidase MrMnP1 from *M. roreri* was produced in a fed-batch fermentation of recombinant *P. pastoris*, yielding 132 mg L^{-1} of active enzyme. To the best of our knowledge, this is the highest reported expression level of

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a fungal class II peroxidase from the superfamily of plant, fungal, and bacterial heme peroxidases so far.[9a] Furthermore, the introduction of a catalytically active tryptophan at the enzyme surface was, as expected, an essential step for introducing new activities against lignin model substrates, as well as recalcitrant dyes. Importantly, the gained activities could be significantly increased by redesigning the space surrounding the catalytic tryptophan. Particularly, replacement of lysine at position 168 by hydrophobic valine had a positive effect on activity towards high-redox-potential substrates, such as VA, the lignin dimers Ge and Ve, and dyes. Apart from increased activity, mutation K168V, along with mutation A172W, had a clear influence on the stability of MrMnP1. The double mutant A172W K168V demonstrated high stability over a pH range from 3.0 to 7.0. Our results demonstrate that the toolbox of ligninolytic peroxidases active on recalcitrant lignin can be expanded by site-directed mutagenesis of MnP. Combining MnP or VP with other cellulolytic enzymes, as isolated enzymes or in a single expression host, will be necessary for efficient valorization of lignocellulose in the biorefinery concept.

Experimental Section

Materials: Chemicals and enzymes were purchased from Sigma–Aldrich, New England Biolabs, Thermo Fisher Scientific, Fluka, Carl Roth, Alfa Aesar, Fluorochem (Hadfield, UK), AppliChem (Darmstadt, Germany), VWR (Darmstadt, Germany), and ABCR GmbH (Karlsruhe, Germany). RB5 was purchased from Sigma with the grade "Dye content $\geq$50%" and was considered to be 50% when kinetics were measured.

Cloning, site-directed mutagenesis, and transformation: The gene encoding MrMnP1 (GenBank database ID: ESK95360.1) was codon optimized by JCat for expression in *S. cerevisiae* (http://www.jcat.de/, GenBank database ID: MG646281), and cloned in pPICZαA without its native secretion signal but with the α-factor secretion signal. MrMnP1 was amplified with the primers MrMnP1_XhoI_fw and MrMnP1_XbaI_rev (Table S1). Restriction sites are indicated in italics. The pPICZαA vector and the amplified *mrmnp1* genes were cleaved with respective restriction endonucleases (Table S1). After ligation, the pPICZαAMrMnP1 construct was obtained. The sequence of the construct was confirmed by sequencing (GATC Biotech, Konstanz, Germany). Site-directed mutagenesis of MrMnP1 was performed according to the QuikChange protocol from Stratagene. First, the A172W mutation was introduced with MrMnP1_A172W_Fw and MrMnP1_A172W_Rev primers (Table S1), and the resulting plasmid pPICZαAMrMnP1A172W was used as a template for the construction of the double mutants. For the double mutants, the primers indicated in Table S1 were used. The sequence of the constructs was confirmed by sequencing. Eventually, plasmids were linearized with PmeI, and *P. pastoris* X-33 was transformed with the linearized constructs. Clones were selected on YPDS Agar-Plates, including 100 μg mL^{-1} Zeocin™.

Gene expression: For expression in shake flasks, a preculture in buffered complex glycerol medium (BMGY; 10 mL; 10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 100 mM potassium phosphate buffer pH 6; 10 g L^{-1} glycerol; 0. 4 mg L^{-1} biotin) was grown overnight at 200 rpm and 30 °C. The next day, buffered minimal methanol medium (BMM; 10 mL; 13.4 g L^{-1} yeast nitrogen base without amino acids; 0.4 mg L^{-1} biotin, 0.5% methanol; 100 mM potassium phosphate buffer pH 6) was inoculated with the preculture to an OD_{600} of 0.5. The cultures were grown at 200 rpm, 25 °C, and methanol was supplemented daily to a final concentration of 0.5%.

Fed-batch fermentations were performed as described earlier for the laccase Mrl2 from *M. roreri*, with the exception that the pH was set to 5 instead of 6, and no additional $CuSO_4$ was supplemented, but hemin was added after induction to a final concentration of 10 μM.[11]

Protein purification: After harvesting, the cells were separated from the supernatant by centrifugation (10000 *g*; 15 min; 4 °C) and the supernatant was concentrated by means of Crossflow ultrafiltration with cutoff membranes of 10 kDa (Pall, East Hills, NY, USA). The concentrated supernatant was supplemented with solid ammonium sulfate to a final concentration of 1.5 M. After 30 min of centrifugation at 50000 *g*, 4 °C, and filtration through a 0.22 μm filter, the solution was applied on a ButylHP column on the Äkta purifier system (GE Healthcare). The protein was washed with 2 column volumes (CVs) of solution B (50 mM sodium acetate buffer pH 5; 1 mM $CaSO_4$; 1.5 M ammonium sulfate) before it was eluted with a linear gradient over 3 CVs to 100% solution A (50 mM sodium acetate buffer pH 5; 1 mM $CaSO_4$). The active fractions towards ABTS were pooled and concentrated with a Vivaspin 20 with a 10 kDa cutoff membrane. The concentrate was applied on a size-exclusion column (Superdex 200 Increase 10/300 GL); 20 mM sodium acetate buffer, pH 5, supplemented with 1 mM $CaSO_4$ was used with a flow rate of 0.75 mL min^{-1}. Again, the active fractions towards ABTS were pooled and the protein concentration was estimated by the Bradford assay with bovine serum albumin as a standard. SDS-PAGE analysis was performed according to the procedure reported by Laemmli.[35] Peroxidase concentration estimation in the fermentation supernatant was performed by using GelAnalyzer2010 (http://www.gelanalyzer.com/). The UV/Vis spectra of the purified enzyme were determined by using a Lambda 35 spectrophotometer from PerkinElmer. The enzyme was reduced with 10 mg mL^{-1} sodium dithionite. The RZ value (for Reinheitszahl) was a ratio of absorbance at $\lambda = 408$ (due to the heme group) to that at 275 nm (due to protein).

Activity measurements, pH stability, and dye decolorization: Volumetric activity of MrMnP1 WT and mutants during expression and specific activity during purification were determined by using 1 mM ABTS ($\varepsilon_{420} = 36000\ M^{-1}cm^{-1}$) and 0.4 mM H_2O_2 in 50 mM Na-K-tartrate buffer, pH 3, at room temperature. One unit was defined as the amount of enzyme that oxidized 1 μmol of substrate in 1 min under the described conditions. Kinetic constants were determined towards 2,6-DMP ($\varepsilon_{468} = 49600\ M^{-1}cm^{-1}$), RB5 ($\varepsilon_{598} = 30000\ M^{-1}cm^{-1}$), VA ($\varepsilon_{310} = 9300\ M^{-1}cm^{-1}$), and Mn^{2+} ($\varepsilon_{238} = 6500\ M^{-1}cm^{-1}$) in 50 mM Na–K–tartrate buffer, pH 3, and 0.4 mM H_2O_2 (except for Mn^{2+} Na–K–tartrate buffer, pH 5 was used). The optimum pH was determined in Britton Robinson buffer, pH 3–8, towards 1 mM ABTS, 1 mM 2,6-DMP, 20 μM RB5, and 10 mM VA. Reaction rates were determined spectrophotometrically at room temperature and were fitted to the Michaelis–Menten equation in OriginPro 9.0G.

For pH stability studies, the enzyme was diluted in Britton Robinson buffer, pH 3–7, at 4 °C, and the value measured immediately after dilution was set to 100%. After 24 h incubation at 4 °C, the residual activity was determined. The dye decolorization studies were conducted with 0.1 μM enzyme in 50 mM Na–K–tartrate buffer, pH 3, with 25 μM Crystal violet (at $\lambda = 585$ nm), 50 μM Methyl Orange (at $\lambda = 504$ nm), 100 μM Alizarin Red S (at $\lambda = 422$ nm), 50 μM Indigo carmine (at $\lambda = 610$ nm), or 100 μM Remazol Brilliant Blue R (at $\lambda = 592$ nm) and 0.4 mM H_2O_2 at RT. In the con-

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trol sample, the enzyme was omitted. The absorbance for each sample (triplicate) was measured immediately (about 1 min) after mixing and set as the "0 min sample". The influence of Mn^{2+} on Crystal violet and RB5 oxidation was performed with 25 μM Crystal violet or 50 μM RB5, 0.4 μM enzyme, and 0.4 mM H_2O_2 in the presence or absence of 1 mM Mn^{2+} at pH 3 and 5.

Degradation of lignin dimer model substrate and other non-phenolic compounds: 0.4 mM lignin dimer (Ve and Ge) or 1 mM aromatic monomer compounds were incubated in 50 mM Na–K–tartrate buffer (200 μL), pH 3, 4, and 5 for the dimers and pH 3 for the monomers, respectively, with 0.1 μM enzyme and 0.4 mM H_2O_2 for 2 h at RT. The samples were stopped with 6 M HCl (6 μL; 180 mM final concentration). Substrate conversion was analyzed on a Shimadzu HPLC (Shimadzu, Duisburg, Germany) equipped with a Chromolith C_{18} 100 mm, 4.6 mm reversed-phase column (Merck). The lignin model dimers were eluted with a gradient from 25% eluent B (75% eluent A) to 100% eluent B (0% eluent A) in 10 min. Then for 3 min, 100% eluent B was applied and the column was re-equilibrated at 25% eluent B for another 4 min. For the aromatic monomer compounds, the elution gradient started with 5% eluent B and increased to 100% eluent B within 15 min. Then for 3 min, 100% eluent B was applied and the column was re-equilibrated at 5% eluent B for 5 min. The flow rate was set to 1 mL min^{-1}. Methanol was used for the dimers as eluent B and acetonitrile for the monomers. Eluent A was, in both cases, water supplemented with 0.1% formic acid. Product formation for aromatic monomer compounds was followed in the UV range and degradation of Ve and Ge was determined at $\lambda = 280$ nm. Conversion of the substrates was calculated as the percentage of the peak area of the control sample without enzyme.

Enzyme kinetics with the non-phenolic lignin dimer Ve were performed by following the formation of veratraldehyde ($\varepsilon_{310} = 9300\ M^{-1}cm^{-1}$) in 50 mM Na–K–tartrate buffer, pH 3, in the presence of 0.4 mM H_2O_2. The substrate concentrations used ranged from 0.0156 to 2 mM. Initial reaction rates were fitted to the Michaelis–Menten equation in OriginPro 9.0G.

Gene copy number and mRNA level determination: Several *P. pastoris* clones producing MrMnP1 WT and mutants were selected for gene copy number and mRNA determination. The cultures were grown as described above. Cells from the BMGY preculture were used for genomic DNA extractions. After growth for 48 h in BMM (1 mL), an OD_{600} corresponding to 1 was obtained. The samples were centrifuged (30 s, RT, 10000 rpm) and stored at −80 °C for RNA extraction.

Genomic DNA was extracted with the Quick-DNA™ Fungal/Bacterial Microprep Kit from Zymo Research, and total RNA was extracted by using the RNeasy Mini Kit from Qiagen, according to the manufacturer's instructions. The integrity of the gDNA and RNA was verified by 0.8% agarose gel. cDNA was transcribed by using the DyNAmo cDNA Synthesis Kit from Thermo Scientific, according to the manufacturer's instructions. RNA (100 ng) was used as the template. After cDNA synthesis, H_2O (60 μL) was added to the sample (20 μL) and an aliquot (2 μL) was further used for mRNA analysis. For qPCR, the DyNAmo Flash SYBR Green qPCR Kit was used, according to the manufacturer's instructions. PCR was conducted in a PikoReal real-time PCR system from Thermo Scientific. qPCR conditions were as follows: after 7 min denaturation at 95 °C, 40 amplification cycles were performed: denaturation (95 °C for 5 s); annealing/extension (60 °C for 30 s) and fluorescence data collection, final extension (60 °C for 30 s), and a melting curve between 60 and 95 °C was recorded.

Primer efficiency was determined according to Rasmussen and considered in gene copy number and mRNA levels.[36] As a reference, the housekeeping gene actin was chosen.[36,37] The primers used for quantitative PCR for MrMnP1 WT and mutants were 2qPCR-MrMnP1-fw 5′-CCAGG TACTC CATTC GACTC TA-3′ and 2qPCR-MrMnP1-rev 5′-GTAGA GTTTG GACCT GGGAA AG-3′ and for actin as the reference gene ActinTool-Fw 5′-GAACC CAAAG TCCAA CAGAG A-3′ and ActinTool-Rev 5′-CGGCC TGAAT AGAAA CGTAG AA-3′. The relative amounts of mRNA and the gene copy number were calculated by the PikoReal 2 Software with the actin gene as a reference.

Reconstitution of activity of MrMnP1 WT and MrMnP1 A172W: MrMnP1 WT and the A172W mutant were expressed as described above in the presence and absence of hemin. After expression without hemin, the culture media were supplemented with 10 μM hemin and incubated at 4 °C. Volumetric activities were measured up to 6 h of incubation.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · enzymes · mutagenesis · renewable resources · structure–activity relationships

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Supporting Information

Redesign of a New Manganese Peroxidase Highly Expressed in *Pichia pastoris* towards a Lignin-Degrading Versatile Peroxidase

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Supporting Information

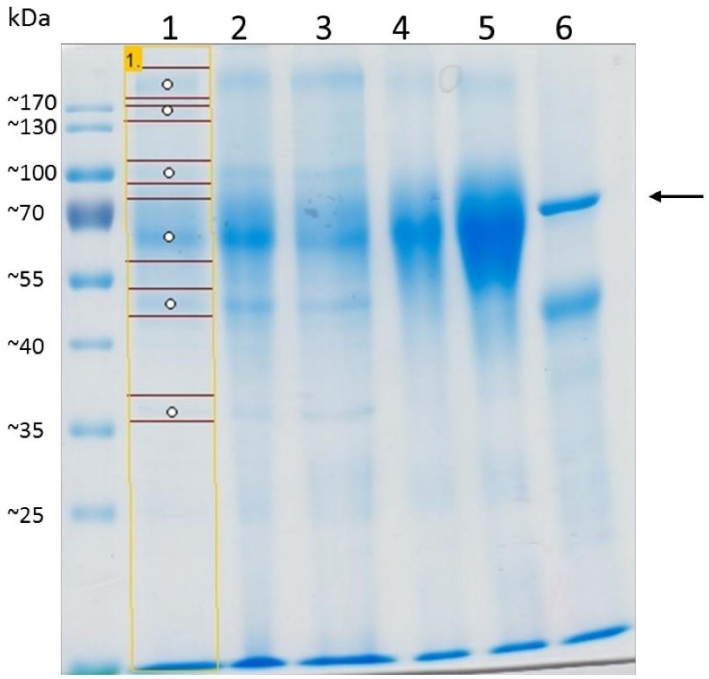

Figure S1. SDS-PAGE of MrMnP1 purification, deglycosylation and quantitative analysis. 1) Fermentation supernatant analyzed by GelAnalyzer2010; 2) Cross-flow concentrated supernatant; 3) after ammonium sulfate supplementation; 4) after hydrophobic interaction chromatography; 5) after size-exclusion chromatography; 6) N-deglycosylated MrMnP1; arrow indicates EndoHF in lane 6

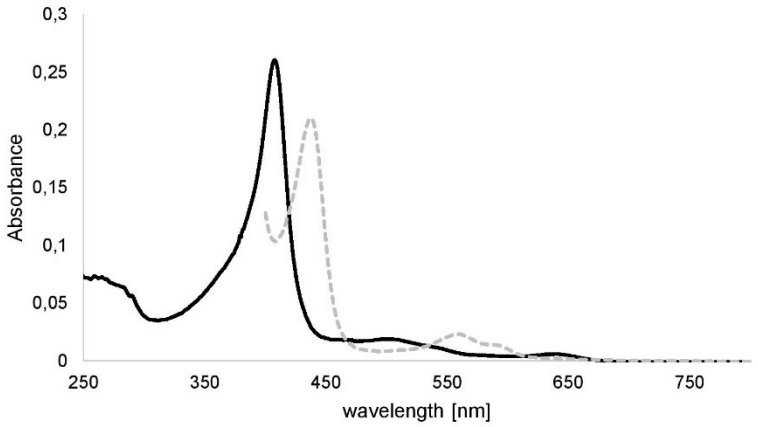

Figure S2. UV-Vis absorption spectra of MrMnP1 in the oxidized (black line) and reduced (gray dashed line) form.

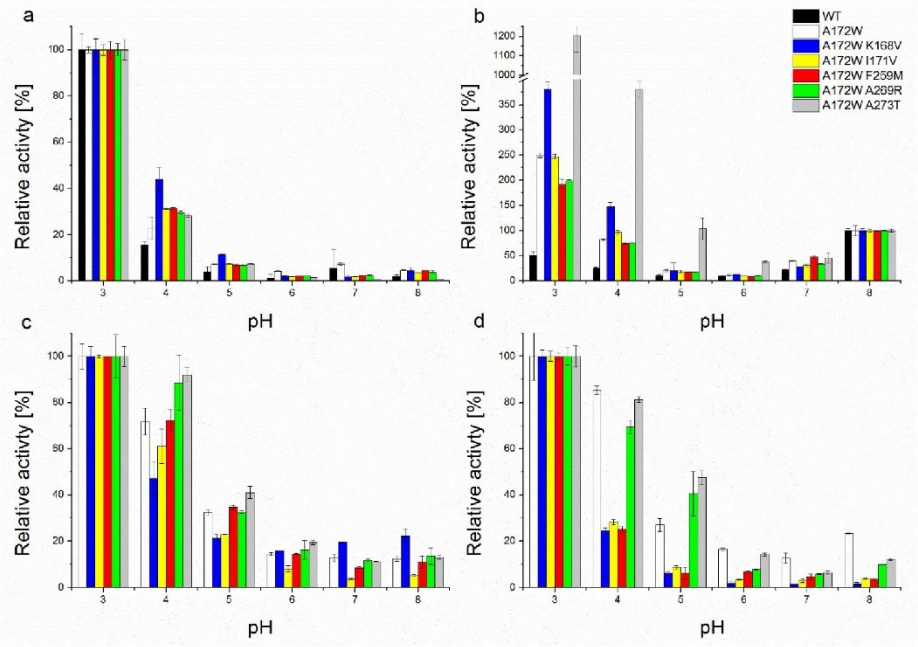

Figure S3. Influence of pH on substrate conversion. Activities of MrMnP1 wild type and mutants were evaluated towards ABTS (a), 2,6-DMP (b), RB5 (c) and VA (d). Activities at pH 3 were set to 100 %, except for 2,6-DMP values were set to 100 % at pH 8 because specific activities were similar at that pH value.

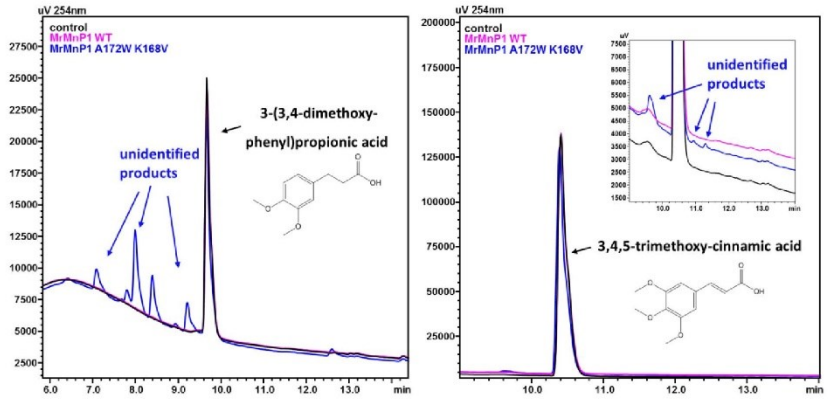

Figure S4. Degradation of aromatic monomeric compounds. 1 mM compound was incubated with 0.1 µM enzyme in 50 mM Na-K-Tartrate buffer, pH 3 for 2 h.

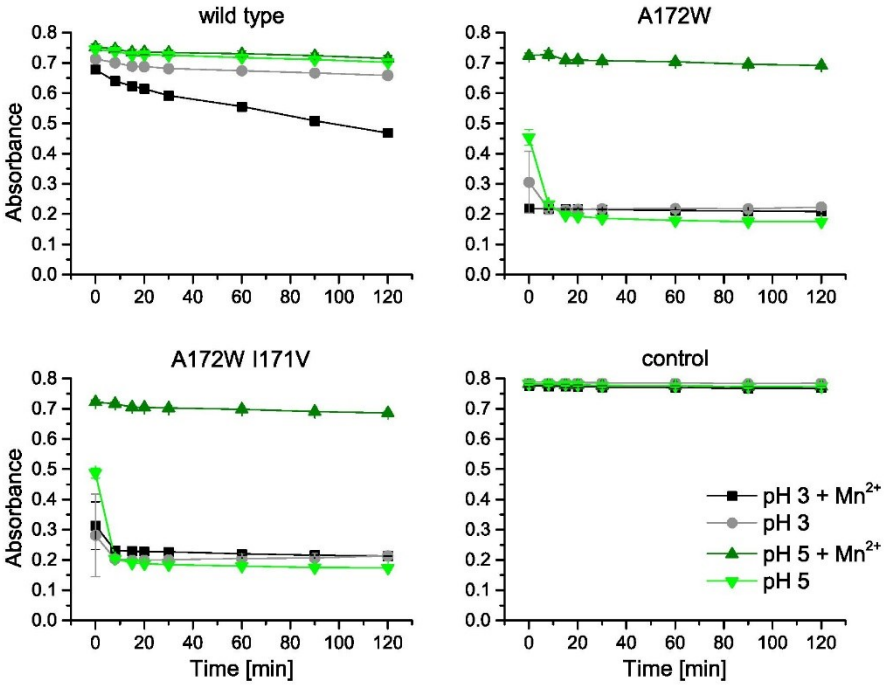

Figure S5. Influence of Mn^{2+} on RB5 oxidation by MrMnP1 wild type and mutants.

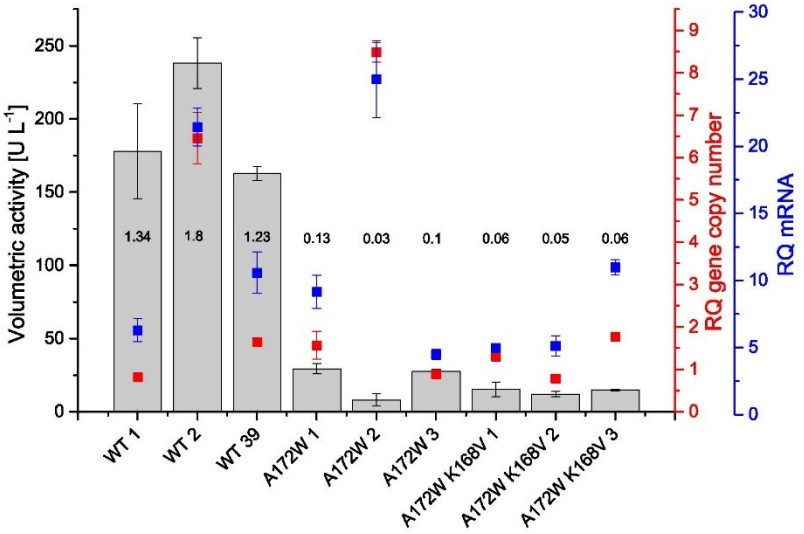

Figure S6. Analysis of different *P. pastoris* clones expressing MrMnP1 wild type and mutants. RQ=relative quantity; numbers in the diagram indicate peroxidase concentration (mg L^{-1}); calculation based on specific activity.

Table S1. Oligonucleotides used for cloning and site-directed mutagenesis. Restriction sites in the sequences are indicated in italics.

Name	Nucleotide sequence (5‘ – 3‘)
MrMnP1_XhoI_fw	GTAT*CTCGAG*AAAAGAGCTGTTCCACAAAGAGTTGC
MrMnP1_XbaI_rev	CA*TCTAGA*TTAAGATGGTGGAACAGCTG
MrMnP1_A172W_fw	CTCTAAGGAAGTTATCTGGTTGTTGGCTTCTCACTC
MrMnP1_A172W_rev	GAGTGAGAAGCCAACAACCAGATAACTTCCTTAGAG
MrMnP1_I171V_fw	GAAGTTGTCTGGTTGTTGGCTTCTCACTCTG
MrMnP1_I171V_rev	CAGAGTGAGAAGCCAACAACCAGACAACTTC
MrMnP1_K168V_fw	GCTGACGCTGGTTTCAACTCTGTGGAAGTT
MrMnP1_K168V_rev	AACTTCCACAGAGTTGAAACCAGCGTCAGC
MrMnP1_F259M_fw	GTGAATGGCAATCTATGGTTAACAACCAAGC
MrMnP1_F259M_rev	GCTTGGTTGTTAACCATAGATTGCCATTCAC
MrMnP1_A269R_fw	GCTAAGATGCAAACTAGATTCAAGGCT
MrMnP1_A269R_rev	AGCCTTGAATCTAGTTTGCATCTTAGC
MrMnP1_A273T_fw	TTCAAGGCTACTATGAACAAGTTGGCTG
MrMnP1_A273T_rev	CAGCCAACTTGTTCATAGTAGCCTTGAA

3.3 Insights into Laccase and Manganese Peroxidase Pretreatment of *Miscanthus*

3.3.1 Introduction

Biobased economy or bioeconomy is defined as the economy that "uses renewable biological resources from land and sea – such as crops, forests, fish, animals and micro-organisms – to produce food, materials and energy" [110]. Bioeconomy is highly desirable, as crude oil reserves are limited. Biorefineries are important integral parts of bioeconomy. They represent sustainable counterparts to oil refineries where the starting material crude oil is replaced by renewable biomass. Biomass can be regarded as a source of sugar or phenols that can be converted into raw materials and commodities. In first generation biorefineries mostly starch-containing edible plants as grains, corn, sugar beet, sugarcane and vegetable oil were used as carbon source. The use of edible plants, however, has fallen into disrepute because their cultivation is in competition with food supply [111-113]. Second generation biorefineries operate utilizing lignocellulosic biomass from fast growing plants like grasses as a source for fermentable sugars. Moreover, lignocellulosic plants can grow on less nutritious soils and therefore compete less with cultivation of edible plants.

In lignocellulosic biomass, the structural cellulose is crystalline [114] and more resistant to hydrolysis than energy-storing starch in edible plants. Additionally, the cellulose is protected of enzymatic degradation by the recalcitrant polymer lignin [115]. Although about 10-40 % of lignocellulose consists of lignin [116], it is rather considered as waste and most biorefineries focus on the liberation of fermentable sugars. Lignin is a hydrophobic three dimensional polymer produced in cell walls by radical polymerization of the phenylpropanoids *p*-coumaryl, coniferyl and sinapyl alcohol (**Figure 7**). Since these monolignols can polymerize randomly the lignin structure is very heterogenic and varies from plant to plant [117]. The bonds emerging through this polymerization are mainly β-O-4, β-5, β-β, 5-5, 5-O-4 and β-1 couplings (C-C and C-O bonds) [118]. To achieve efficient liberation of fermentable sugars, pretreatment of the

biomass is inevitable. Pretreatment methods can be of physical, chemical, physico-chemical or biological nature [97]. The goal of pretreatment is to remove lignin, reduce the crystallinity and increase porosity of cellulose in order to facilitate its accessibility for hydrolytic enzymes [97].

Physical pretreatment methods comprise milling, chipping, grinding, microwave treatment, ultrasonic treatment, high-energy electron radiation and high-temperature pyrolysis [119, 120]. These methods promote high energy consumption and/or are expensive. Chemical pretreatment methods involve concentrated or diluted acids, alkali, oxidizing agents, organic solvents or ionic liquids. These methods have high cost, can lead to equipment corrosion and/or to formation of inhibitors for cellulose hydrolysis [97, 119, 121]. Physicochemical pretreatment methods combine physical and chemical methods like steam explosion and electrical catalysis and have similar drawbacks as chemical or physical pretreatment methods.

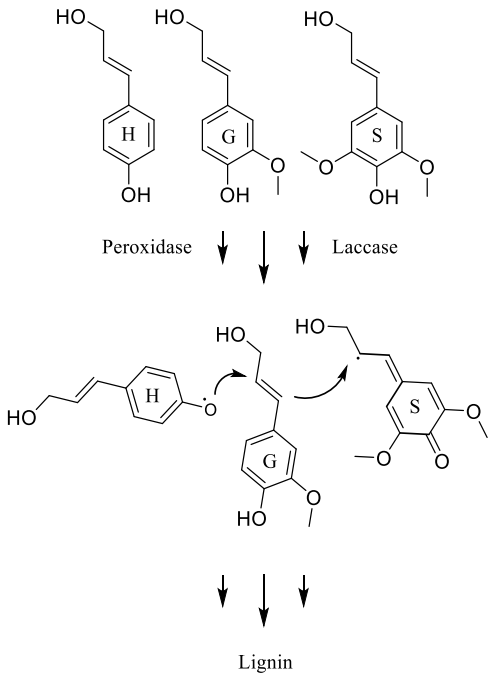

Figure 7 Scheme of monolignol polymerization. Lignin is mostly built of the monolignols p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol which form p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units in lignin, respectively [117].

In nature the main decomposers of lignocellulose are microorganisms and particularly fungi. White-rot fungi are the only known organisms that can completely degrade lignin with the help of secreted oxidoreductases leaving the white cellulose untouched [81]. The rate of biological pretreatment however, is too slow for industrial purposes, which make them less attractive at industrial scale. Instead, the use of multi-enzyme cocktails from white-rot fungi appears more attractive and has been in focus of research in recent years.

The ligninolytic machinery of white-rot fungi has extensively been investigated during the last decades. Peroxidases such as LiPs, MnPs and VPs produced by white-rot fungi can oxidize high redox potential non-phenolic aromatic substrates as lignin. In addition to peroxidases white-rot fungi secrete laccases but their function in lignin degradation has not been fully understood yet [122]. Peroxidases and laccases are considered the major enzymes involved in this process.

In 2004 Palonen and Viikari also showed that laccase and a laccase-mediator system (LMS) could be used to improve cellulase mediated hydrolysis of cellulose of steam pretreated softwood [123] and many others observed similar effects [124]. They speculated that this positive effect can correlate with reduced unproductive binding of cellulases to the lignin surface which has been modified during treatment. Jönsson and colleagues showed in 1998 that laccase and LiP from *Trametes versicolor* had a positive effect on ethanol production with *S. cerevisiae* [125]. The laccase treated hydrolysates were analyzed by gas chromatography–mass spectrometry (GC-MS) and gel-permeation chromatography (GPC). GC-MS analysis showed that laccase was able to decrease the concentration of various phenolic growth inhibitors [126] like 4-hydroxybenzoic acid, vanillin and vanillic acid which are liberated from the biomass. GPC has revealed that products of the enzymatic pretreatment had a higher molecular mass than the starting material, which might indicate that laccase does not depolymerize lignin but rather polymerize free phenol intermediates.

Here, we focused on enzymatic pretreatment of *Miscanthus x giganteus*. *Miscanthus* is a perennial grass, which has the potential to become an effective energy carrier due to its vigorous growth resulting in high yields and low fertilizer and pesticides requirement [127]. For pretreatment heterologously expressed laccase Mrl2 and manganese peroxidase MrMnP1 from the white-rot fungus *Moniliophthora roreri* were studied, which can be expressed in *Pichia pastoris* at high levels [128, 129]. Their application in biomass pretreatment was evaluated to gain insights into their mechanism of action and interaction.

3.3.2 Experimental Procedures

3.3.2.1 Protein Expression, Purification and Activity Measurements

The laccase Mrl2 and peroxidase MrMnP1 were purified and activities were determined as described before [128, 129]. The vanillyl alcohol oxidase PsVAO was cloned into the the pET-28a(+) vector and produced in the *E. coli* strain C41(DE3) in TB-medium (24 g l^{-1} yeast extract, 20 g l^{-1} tryptone, 4 ml l^{-1} glycerol, 50 µg ml^{-1} kanamycin, 1.7 mM KH_2PO_4 and 7.2 mM K_2HPO_4). 200 ml TB medium (37 °C, 180 rpm) were inoculated with an overnight culture and expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the culture reached OD_{600} = 0.6. After 24 h expression at 25 °C and 140 rpm the cells were harvested and stored at -20°C. Purification was based on a protocol by Benen and colleagues [130]. In brief, 50 mM potassium phosphate buffer pH 7.5 were added to 4 g cell pellet to a total volume of 15 ml and supplemented with 15 µl 0.1 M phenylmethane sulfonyl fluoride (PMSF) dissolved in isopropanol, 0.5 mM DTT, 0.2 mg DNaseI and 0.5 mM $MgSO_4$. Cells were disrupted by sonication on ice. After sonication another 0.2 mg DNaseI was added and cell debris was separated from the soluble protein by centrifugation (25 000 x g, 20 min, 4 °C). The supernatant was supplemented with ammonium sulfate to a concentration of 25 % and the precipitate was pelletized by centrifugation (15 000 x g, 15 min, 4 °C). To the supernatant ammonium sulfate was added again at a final concentration of 65 % and the precipitation was

pelletized again (15 000 x g, 15 min, 4 °C). The pellet, harboring the target enzyme, was resuspended in ca. 11 ml 50 mM potassium phosphate buffer pH 7 containing 0.5 M ammonium sulfate, and applied on a 20 ml HP Butyl Sepharose column connected to an Äkta FPLC (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) system. The applied protein was washed with 50 mM potassium phosphate buffer pH 7 containing 0.5 M ammonium sulfate, and eluted with potassium phosphate buffer pH 7 in 3 column volumes. Active fractions towards eugenol were pooled and applied on a size exclusion column Superdex200 Increase 10/300 GL (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) and eluted with 50 mM potassium phosphate buffer pH 7. Activity towards eugenol (ε_{300}= 7,400 M^{-1} cm^{-1}) and vanillyl alcohol (ε_{340}= 22,800 M^{-1} cm^{-1}) was measured photometrically in Britton-Robinson buffer pH 3-8 at room temperature. Cellulase (Cellulase from Trichoderma reesei ATCC 26921 (aqueous solution, ≥700 units/g) from Sigma Aldrich) activity was measured according to Adney and Baker [131]. The filter paper, however, was saturated in 50 mM sodium acetate buffer pH 5 and instead of the 3,5-dinitrosalicylic acid (DNS), the PAHBAH Reducing Sugar assay (3.3.2.4) was used to determine the liberated reducing sugars.

3.3.2.2 Miscanthus Pretreatment and Saccharification

2.5 or 5 g chopped *Miscanthus x giganteus* provided by Prof. Dr. Ralf Pude was weighed in 100 ml Erlenmeyer flasks and 50 ml 20 mM sterile sodium acetate buffer was added. To the samples 10 mU ml^{-1} (ABTS, pH 5) Mrl2, 10 mU ml^{-1} (ABTS, pH 3) MrMnP1, 150 mU ml^{-1} PsVAO (eugenol, pH 8) and 1 mM Mn^{2+} was added. H_2O_2 was added either at the beginning to a final concentration of 0.4 mM or step by step 6 times every 2 h to a final concentration of 0.1 mM each time. After pretreatment over night at 37 °C and 180 rpm, 1 filter paper unit (FPU) of commercial cellulases per gram biomass was added and the flasks were shaken for 24 h. After saccharification, solid/liquid separation was performed by centrifugation and the supernatant was sterile-filtered using a 0.45 µm membrane.

3.3.2.3 Growth Experiments with Saccharomyces cerevisiae

For growth experiments the *S. cerevisiae* strain INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52) was used. To 8.9 ml sterile-filtered supernatant after biomass saccharification 100 µl of 100 x amino acids solution (100 mg L-histidine, 600 mg L-leucine, 250 mg L-tryptophan and 100 mg uracil were dissolved in 50 ml water and sodium hydroxide was added until all solids were dissolved) and 1 ml of 10 x YNB (6.7 g l^{-1} yeast nitrogen base without amino acids) was added. A pre-culture of INVSc1 in YPD (20 g l^{-1} peptone, 10 g l^{-1} yeast extract and 20 g l^{-1} glucose) was used to inoculate the medium to an OD_{600} = 0.05. *S. cerevisiae* was grown at 30 °C and 180 rpm.

3.3.2.4 Sugar and Phenolic Content Analysis

Sugar and phenolic content analysis was performed after biomass treatment with ligninolytic enzymes and cellulases. The amount of reducing sugars was analyzed with the PAHBAH Reducing sugars assay based on Lever [132]. In brief, two solutions were used: Reagent A was prepared by dissolving 5 grams *p*-hydroxybenzoic acid hydrazide (PAHBAH) in 30 ml water under stirring. After addition of 5 ml concentrated HCl the volume was increased to 100 ml; and Reagent B contains 12.45 g l^{-1} trisodium citrate, 1.1 g l^{-1} calcium chloride, 20 g l^{-1} sodium hydroxide [133]. Before quantifying reducing sugars in a solution the working Reagent was prepared by mixing Reagent A and B in a ratio 1:9. 0.5 ml of unknown sugar solution are added to 1 ml of working Reagent. The mixture was heated in water (~95 °C) for 10 min, cooled down and the absorption was measured at 410 nm in a spectrophotometer. As a reference, solutions with different glucose concentrations were used. The glucose concentration after saccharification was determined with the Glucose HK assay (Sigma Aldrich, Schnelldorf, Germany) according to manufacturer's protocol.

The total phenolic content was determined based on a method published by Folin and Ciocalteau [134, 135]. To 20 µl sample 60 µl Folin-Ciocalteau reagent and 600 µl H_2O was

added and the solution was incubated for 5 min at room temperature. Then 120 µl 20 % $NaCO_3$ and 200 µl H_2O was added and incubated at room temperature at 800 rpm for 2 h. 200 µl were transferred to a 96 well plate and the absorbance was measured at 760 nm. As a reference vanillin was used at different concentrations. Additionally to total phenolic content the supernatant was analyzed by HPLC measurements. The aromatics were separated on a Chromolith® C18 100 mm, 4.6 mm reversed phase column (Merck, Darmstadt, Germany) on a Shimadzu HPLC (Shimadzu, Duisburg, Germany) and monitored in the UV-spectrum. The two mobile phases were water supplemented with 1 % formic acid and methanol. The phenols were eluted with a gradient beginning with 5 % methanol and going up to 55 % methanol within 32 minutes and another 30 s to 100 %. Then 3 minutes the column was washed with methanol and for 6.5 minutes re-equilibrated with 5 % methanol. 50 µl sample was applied on the column.

3.3.3 Results and Discussion

To evaluate the potential of the laccase Mrl2 and peroxidase MrMnP1 to detoxify *Miscanthus* slurries, chopped *Miscanthus* was pretreated with a solution containing either the laccase Mrl2 or peroxidase MrMnP1 or a combination of both. After 24 h pretreatment a commercial cellulase cocktail was added to hydrolyze the accessible cellulose. For evaluation of the enzymatic pretreatment, the amount of liberated sugars was measured using the PAHBAH assay and compared to the control without pretreatment. Furthermore, the obtained supernatant was applied as carbon source for culturing *Saccharomyces cerevisiae,* as a potential bioethanol producer.

First, the sample where cellulases were added to the non-treated *Miscanthus* biomass was compared to the sample with *Miscanthus* incubated in the same buffer but without addition of any enzyme. No increased sugar release was observed during incubation of *Miscanthus* with cellulases compared to *Miscanthus* alone (**Figure 8**). After *Miscanthus* pretreatment with laccase or peroxidase with the one-time addition of 0.4 mM H_2O_2 and Mn^{2+}, only in laccase-

containing samples higher sugar liberation and *S. cerevisiae* growth were observed. Around 0.2-0.25 g l^{-1} free sugar was achieved while in all samples without laccase the sugar amount was below 0.05 g l^{-1}. To confirm the results obtained with the PAHBAH assay, the samples were re-analyzed by the glucose-specific Glucose HK assay. Both assays gave similar results. Thus it was concluded that the PAHBAH assay is suitable for estimating sugar liberation. Pretreatment with the peroxidase alone did not result in increased sugar liberation and enhanced *S. cerevisiae* growth (**Figure 8**).

Phenolic content of the supernatant after enzymatic pretreatment determined by the Folin Ciocalteau method revealed decreased phenol concentration. Additionally, HPLC analysis revealed a decrease of peaks (especially between 17 and 35 min retention time) only in the samples containing laccase (**Figure 8**). These results suggest that the release of sugars and *S. cerevisiae* growth might be facilitated by the degradation of phenolic compounds by the laccase.

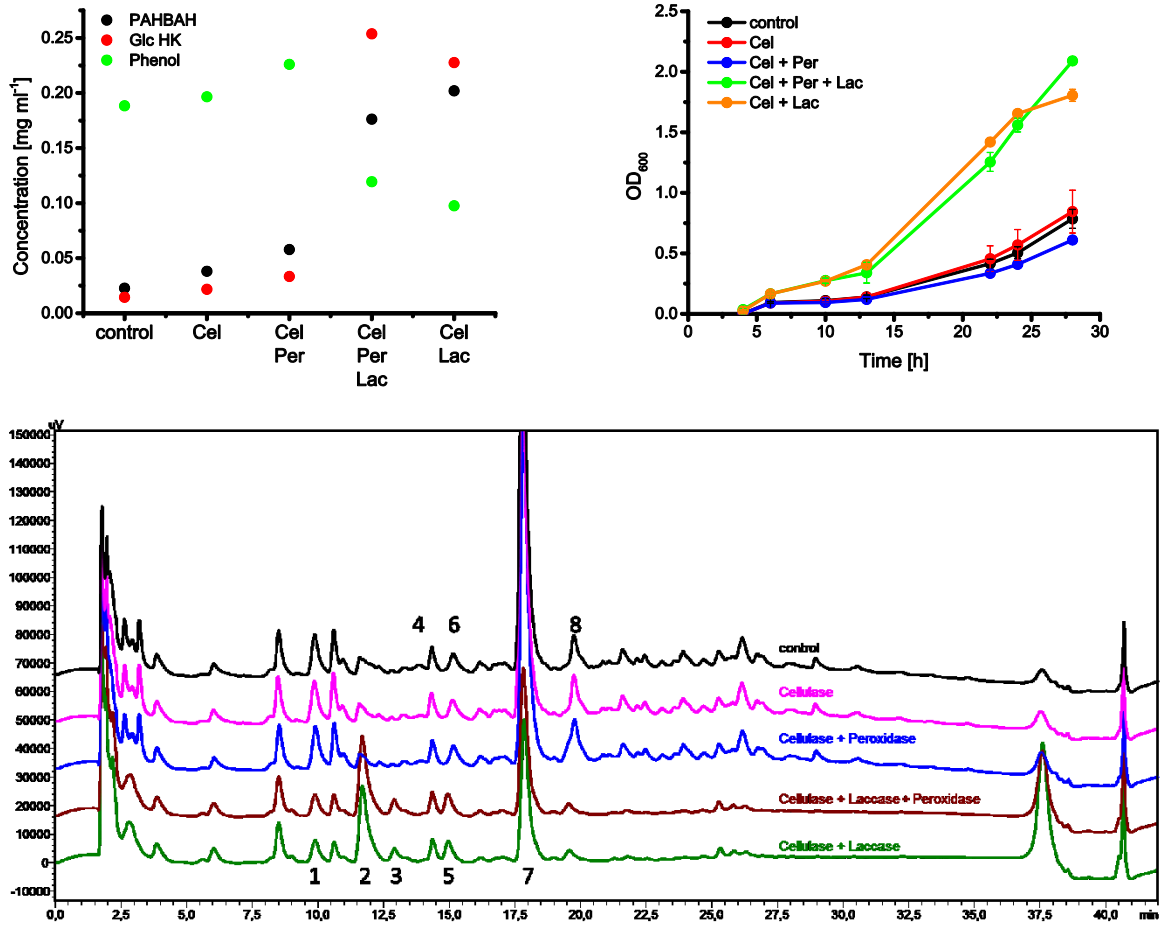

Figure 8 *Miscanthus* pretreatment by laccase and peroxidase, saccharification and *S. cerevisiae* growth. Top left: .PAHBAH Assay, Glucose HK Assay and total phenolic content after saccharification. Top right: *S. cerevisiae* growth curve on *Miscanthus* hydrolysate. Samples with MrMnP1 were supplemented with H_2O_2 once at the beginning to a final concentration of 0.4 mM. Bottom: HPLC analysis after pretreatment of *Miscanthus* hydrolysate (280 nm); Cel: cellulase; Lac: laccase Mrl2; Per: peroxidase MrMnP1; 1: 4-hydroxy benzoic acid; 2: 4-hydroxy benzaldehyde; 3: vanillic acid; 4: caffeic acid; 5: vanillin/isovanillin; 6: guaiacol; 7: p-coumaric acid; 8: ferulic acid.

Laccase depends on oxygen while peroxidase rely on H_2O_2 to be able to oxidize substrates. Lower glucose concentration and growth of *S. cerevisiae* in samples treated with peroxidase alone might be due to the inactivation of the enzyme by H_2O_2 or insufficient H_2O_2 supply. A feed of low amounts of H_2O_2 or the use of the H_2O_2-producing accessory enzyme vanillyl alcohol oxidase from *P. simplicissimum* PsVAO was investigated. While ligninolytic enzymes secreted by white rot fungi are active at rather acidic pH values [128, 129], the H_2O_2-producing vanillyl alcohol oxidase PsVAO is an intracellular enzyme with a basic pH optimum [136]. We measured the activity of the vanillyl alcohol oxidase towards vanillyl alcohol and eugenol at

pH 3-8 (**Figure 9**). For both substrates the highest activity was measured at pH 8. At pH 5, where MrMnP1 shows higher activity for Mn^{2+}, ABTS and 2,6-DMP when compared to pH 8, while being very stable [129], the vanillyl alcohol oxidase showed around 0.5 % and 22 % residual activity for vanillyl alcohol and eugenol, respectively, when compared to pH 8 (**Figure 9**). Since both, peroxidases and laccases, are hardly active at basic pH due to loss of structural Ca^{2+} ions and OH^- inhibition, respectively, pH 5 was used for pretreatment experiments in order to achieve the greatest benefit from all enzymes.

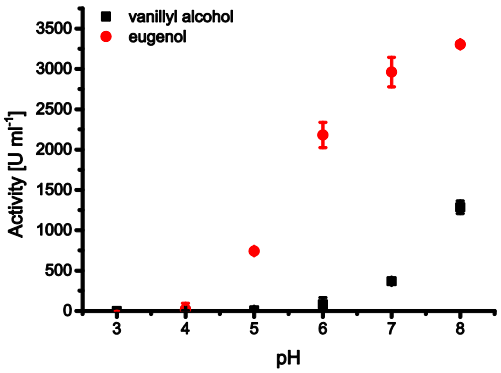

Figure 9 PsVAO activity. Activity of vanillyl alcohol oxidase PsVAO towards vanillyl alcohol and eugenol in Britton-Robinson buffer at pH 3-8.

Indeed, *Miscanthus* samples treated with the peroxidase supplemented with H_2O_2 in a step by step manner or generated by the vanillyl alcohol oxidase, also showed increased sugar liberation and similar *S. cerevisiae* growth to the samples treated with laccase (**Figure 10**). However, unlike in laccase treated samples the phenolic concentration was not lower than in the negative control, neither when determined by the Folin Ciocalteau assay nor by HPLC (**Figure 10**). This contradicts the hypothesis that soluble phenols were responsible for reduced sugar release and lower *S. cerevisiae* growth.

It was shown before that cellulases may adhere with their hydrophobic parts to the hydrophobic lignin which hinders saccharification [123]. It can be assumed that lignin in *Miscanthus* was

altered by laccase or peroxidase + Mn^{2+} in a manner that the cellulases no longer adhere to lignin. Most experiments on enzyme pretreatment described in the literature were performed with (steam-) pretreated biomass. Rajak and Banerjee, however, also showed a positive effect of a laccase from *Lentinus squarrosulus* MR13 on pulverized Kans grass (*Saccharum spontaneum*) which was not chemically or steam-pretreated. After analyzing the biomass they concluded, that the accessible surface of the cellulose for cellulases was increased due to laccase pretreatment [137]. This could be another explanation for increased sugar release also in this study.

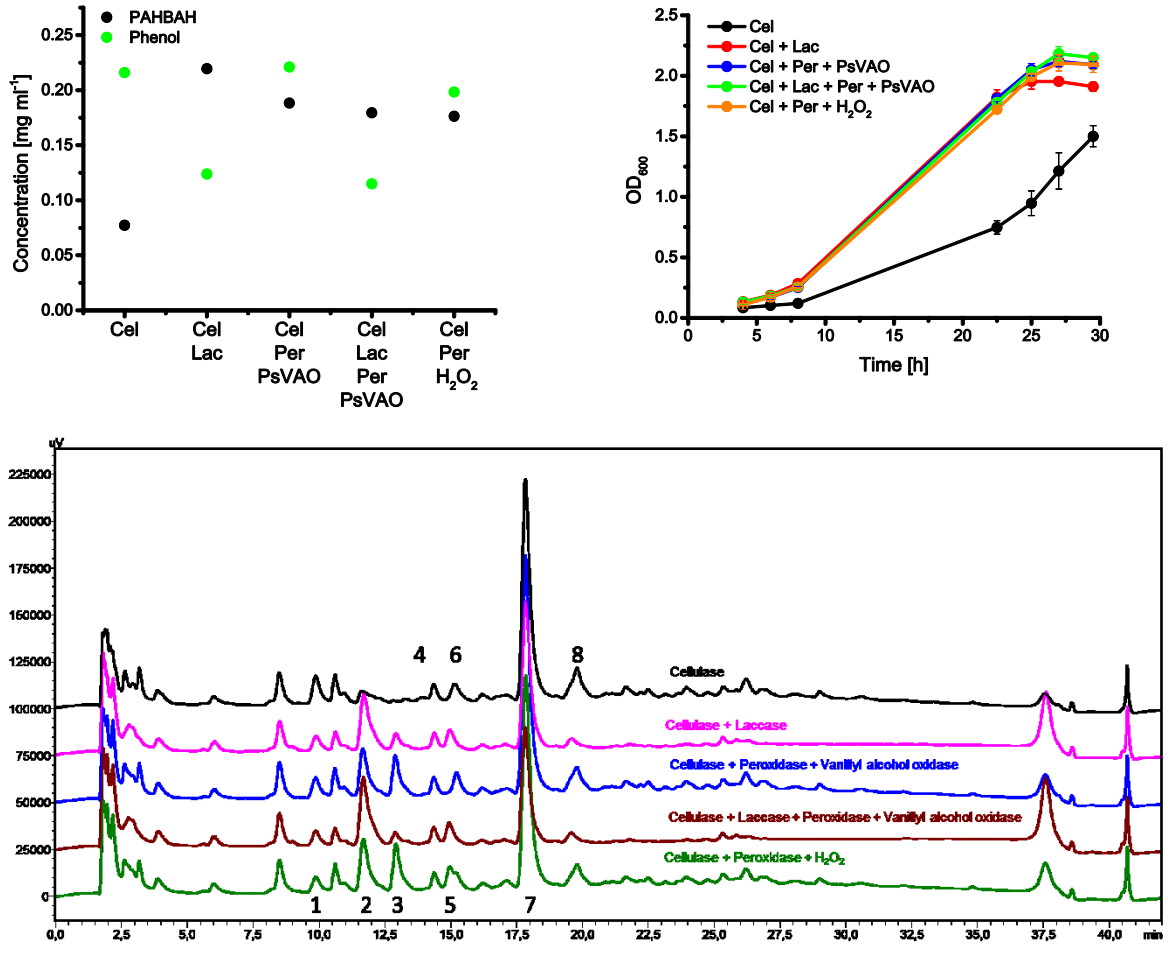

Figure 10 *Miscanthus* pretreatment by laccase and peroxidase + vanillyl alcohol oxidase, saccharification and *S. cerevisiae* growth. Top left: PAHBAH Assay and total phenolic content after saccharification. Top right: *S. cerevisiae* growth curve on *Miscanthus* hydrolysate. Samples with MrMnP1 were supplemented with H_2O_2 step by step 6 times every 2 h to a final concentration of 0.1 mM each. Bottom) HPLC analysis (280 nm) of *Miscanthus* supernatant after pretreatment and saccharification; abbreviations and numbers are explained in **Figure 8**.

It is suggested that higher biomass loadings are necessary to achieve sufficient glucose concentrations for biorefineries to be profitable [138]. In this study, at a substrate load of 10 % instead of 5 %, approximately twice the sugar concentration in all samples was achieved (**Figure 11**). At 5 % substrate load treated with either laccase alone or peroxidase with vanillyl alcohol oxidase, *S. cerevisiae* growth reached the highest OD_{600} value after ca. 25 h. In comparison, in the untreated samples even though hardly any sugar was measured, after a lag phase, *S. cerevisiae* could also grow and reached similar OD_{600} values after 45 h as in the pretreated sample. This lag phase was also observed in the pretreated samples, but was shorter (**Figure 8** and **Figure 10**). It is possible that the yeast adapted to other possible carbon sources such as phenols released from the biomass. This effect, however, was not observed in the control sample with 10% substrate load. It is suggested that (phenolic) inhibitors which are present in higher concentrations at higher substrate loads might have inhibited the growth. A clear lower phenolic content was only observed in samples treated with the laccase Mrl2 but not with the peroxidase MrMnP1 in combination with the vanillyl alcohol oxidase PsVAO or H_2O_2. Although the total phenolic content was not lowered in the peroxidase samples with 10 % substrate load, growth of *S. cerevisiae* was still possible presumably due to the higher resistance of the cells in the presence of sugar, since faster initial cell growth leads to more biomass, which may be able to degrade inhibitory phenols faster than slow-growing cells.

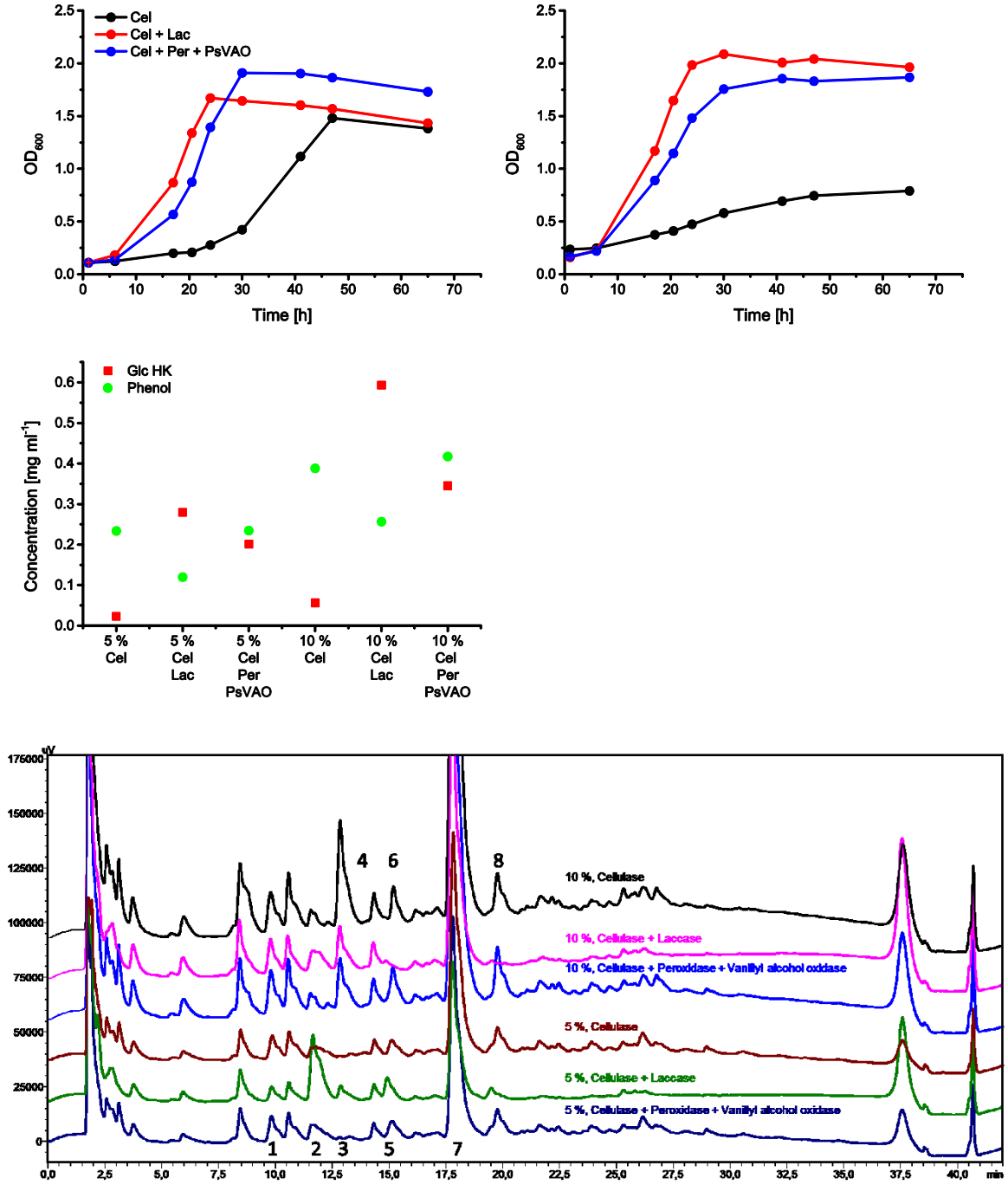

Figure 11 Comparison of **5 % and 10 % substrate loading.** Top: *S. cerevisiae* growth curve on *Miscanthus* hydrolysate with 5 % (left) and 10 % (right) substrate loading. Middle; Glucose HK Assay and total phenolic content after saccharification. Bottom: HPLC analysis (280 nm) of *Miscanthus* supernatant after pretreatment and saccharification; abbreviations and numbers are explained in **Figure 8**.

The experiments using laccase and peroxidase from *M. roreri* for pretreatment of *Miscanthus* suggest that these oxidative enzymes indeed can improve enzymatic hydrolysis of lignocellulose by cellulases. Improved hydrolysis of *Miscanthus*, however, was not attributed

to reduced phenolic content, as only laccase was able to reduce those but not the peroxidase, but probably rather due to modifications in the lignin structure.

3.3.4 Conclusion

Lignocellulose is considered as an important feedstock for biorefineries in a bio-based economy. High crystallinity of cellulose and high resistance of hydrophobic lignin, which contribute to the structure of plants, make lignocellulose very resistant to saccharification by industrial cellulases. Therefore lignocellulose must be pretreated before hydrolysis.

The laccase Mrl2 and manganese peroxidase MrMnP1 from *Moniliophthora roreri*, which were recombinantly expressed in *Pichia pastoris* in high titers, were evaluated in lignocellulosic biomass pretreatment. The laccase alone or peroxidase supplemented with H_2O_2 or the H_2O_2 producing vanillyl alcohol oxidase PsVAO from *Penicillium simplicissimum* was able to increase sugar liberation by cellulases as well as *S. cerevisiae* growth. Oliva-Taravilla and co-workers showed that laccase had a negative effect on hydrolysis due to grafting (binding of small, soluble molecules to lignin) [139-141]. Moreover, Jurado and colleagues even suggested that laccase pretreatment has to be performed after hydrolysis, since laccase pretreatment prior to hydrolysis decreased sugar release in their experiments [142]. Our findings, however, indicate that phenols do not inhibit cellulases. Furthermore, we hypothesize that lignin was modified by laccase or peroxidase in a way that cellulases were not absorbed any longer. This, however, must still be clarified in further experiments. *S. cerevisiae* was also able to grow in supernatants where very little reducing sugars or glucose was detected which suggests other C-sources as for examples aromatic compounds. When the substrate load was increased from 5% to 10%, cell growth was almost completely inhibited, probably due to high concentration of toxic phenol compounds. Biological pretreatment with enzymes seemed to be even more necessary with higher substrate loadings. Even though *S. cerevisiae* growth was not inhibited by phenols in samples with 10 % substrate loading when peroxidase and vanillyl alcohol

oxidase were added, probably due to microbial inactivation of these inhibitors, higher substrate loadings (15 or 20 %) might lead to growth inhibition and laccase may be superior to peroxidase.

4 CONCLUSION AND OUTLOOK

In this thesis, two ligninolytic genes for the two enzymes, laccase Mrl2 and peroxidase MrMnP1, originating from *Moniliophthora roeri*, were expressed in *P. pastoris* X 33 at high titers. In a 7.5 L bioreactor, approximately 1 g laccase and 130 mg peroxidase per L medium culture were achieved, which are among the highest concentrations reported so far [98]. Especially for peroxidases this concentration of active enzyme is a big step forward towards biotechnological applications since most reports are of peroxidases that are expressed as inclusion bodies in *E. coli*. In contrast to *P. pastoris*, where the peroxidase is secreted into the medium as fully active glycosylated enzyme, in *E. coli* the protein accumulates in inclusion bodies and tedious refolding is inevitable. Yields that are achieved during this process are around 20 mg L^{-1} at the highest. In future, the production of the enzymes might be optimized to achieve even higher yields. Here, a standard fermentation protocol for *P. pastoris* on methanol to express the peroxidase was used and for laccase production the pH was shifted from 5 to 6, which probably provided higher stability to Mrl2. Other fermentation parameters as different temperatures, continuous methanol addition, other promoters, other strains or mixed feeding strategies (e.g. methanol + polyol) can be investigated to obtain even higher yields.

The enzymes were purified and characterized and their biotechnological application was evaluated. Micropollutants are an emerging problem in the environment and wastewaters as they can harm various living beings. The laccase Mrl2 was able to degrade some micropullutants including endocrine disruptors and non-steroidal anti-inflammatory drugs faster than the high-redox potential laccase TvL from *Trametes versicolor*, making it an excellent candidate for the use in wastewater treatment. Since the reusability of enzymes in wastewater treatment is important, the immobilization of Mrl2 on different supports would further increase the usefulness of this enzyme for biotechnological applications.

In addition, the MnP MrMnP1 was tested for dye degradation and lignin modification. The textile industry pollutes wastewater with dyes that have to be neutralized before entering rivers and lignin modification is important in biomass pretreatment for bioethanol production for example. The MnP MrMnP1 alone was not able to oxidize non-phenolic lignin derivatives and recalcitrant dyes. The introduction of a catalytically active tryptophan on the surface of MrMnP1 extended the substrate spectrum from MrMnP1 to that of a VP. The mutant enzyme was able to oxidize all tested lignin derivatives and recalcitrant dyes. In the case of dyes, however, pH, time and manganese supplementation were key to the degree of decolorization. For example it was observed that manganese could be inhibitory to dye degradation at pH 5 but not at pH 3. These results show that MrMnP1 might be a suitable enzyme for use in dye degradation and lignin modification. Further experiments are necessary to explain this behavior. The introduced mutations into MrMnP1, however, led to reduced expression levels. DNA and RNA analysis did not allow to elucidate the reason for this observation. Apparently, gene expression until transcription is not impaired by the mutations, which means that the problem is probably in translation and/or processing of the protein. To visually monitor expression by microscopy the green fluorescent protein (GFP) could be fused to MrMnP1 and its mutants. In case of overexpression of secreted proteins the homeostasis of the endoplasmic reticulum (ER) can be disturbed due to misfolded proteins. This issue has been addressed by Guerfal and colleagues who co-expressed the HAC1-gene which encodes for a transcription factor that activates the unfolded protein response (UPR) [143]. UPR is a signaling pathway that counteracts the accumulation of misfolded and unfolded proteins by increasing the expression of genes that support the folding ability of proteins secreted into the ER [144].

Finally, Mrl2 and MrMnP1 (in presence of Mn^{2+} and H_2O_2 supply) were able to increase saccharification of chopped *Miscanthus* and subsequent *S. cerevisiae* growth. Although PsVAO was able to deliver H_2O_2 to MrMnP1, a fungal aryl alcohol oxidase (AAO) might be more

suitable for this process. In contrast to PsVAO, which is an intracellular enzyme and has a very high pH optimum (pH 10), fungal aryl alcohol oxidases (AAOs) are secreted together with peroxidases and laccases and have a rather similar pH optimum (pH 5-6) [145]. It is suggested that together with laccases and peroxidases AAOs help in lignin degradation via the “upper” or “funneling” degradation pathway (depolymerization of lignin), as they provide H_2O_2 for the peroxidase and are supposed to prevent small molecular lignin derivatives to be oxidized and repolymerized again [146-148]. In contrast, PsVAO is an intracellular enzyme and its physiological role might be rather in the “lower” pathway of lignin degradation like ring fission although little is known yet [149, 150].

The hypothesis suggested on the basis of the experiments carried out in this thesis was that lignin was modified in chopped *Miscanthus* resulting in less unproductive binding of cellulases and more sugars being released which were used by the yeast for growth. To confirm that, closer analysis of the lignin composition by NMR or Py-GC for example are needed. Further, cellulase binding onto lignin could be studied. In further experiments the production of ethanol by a suitable yeast under anaerobic conditions will have to be measured. Finally, the two ligninolytic as well as other cellulolytic enzymes could be integrated into a suitable microorganism that would be able to degrade both lignin and cellulose and produce ethanol (or other commodities) simultaneously in a process called consolidated bio-processing [151, 152]. In the best case, the organism in this process would not only be able to use sugar, but also to "funnel" various aromatics derived from lignin via the β-ketoadipate pathway to produce catechol and protocatechuate, which in turn can be converted into target compounds [147, 153].

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