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Department of Food Sciences and Technology

Directed evolution of *Botrytis aclada* laccase for application in biofuel cells

Master Thesis

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submitted by

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Abstract

High redox potential laccases are versatile enzymes with several potential applications such as the use in enzymatic biofuel cells for supplying implantable bio devices with power. However, poor catalytic activity at pH 7.4, strong inhibition by chloride concentrations found in blood (140-150 mM) and the generally low expression levels limit their applicability.

Botrytis aclada, a plant pathogenic ascomycete, expresses a chloride tolerant high-redox potential laccase, which is a promising subject for enzyme engineering with the target to improve the catalytic activity and stability at physiological conditions.

The goal of this study is to identify amino acid residues influencing the behaviour of this particular laccase and explain the breakdown of catalytic activity at neutral pH observable in this type of laccases. For this purpose two different experiments, representing either a semi-rational or a random enzyme engineering approach, were carried out.

A library using the semi-rational site-saturation mutagenesis method was created based on mutations found during the directed evolution process of a laccase from the basidiomycete PM1. The random directed evolution library was based upon a thermostable *B. aclada* laccase variant that has been found earlier and was characterized during this work. Both libraries were analyzed with a two-step high-throughput screening which uses the yeast *Pichia pastoris* as expression host.

In the site-saturation experiment a variant showing a large substrate specific shift in the pH-profile was discovered and characterized. The directed evolution experiment yielded a variant with a significantly modified pH profile.

These variants prove the viability of the chosen enzyme engineering approach and simultaneously encourage further research in this field.

Kurzfassung

Laccasen mit hohem Redoxpotential sind vielseitige Enzyme mit verschiedensten Anwendungsmöglichkeiten, wie die Verwendung in enzymbasierten Biobrennstoffzellen, die Strom für implantierbare Biosensoren zur Verfügung zu stellen. Jedoch limitiert der Mangel an katalytischer Aktivität bei pH 7.4, die Inhibierung durch die hohe Chloridkonzentration im Blut (140-150 mM) sowie die niedrige Expressionsrate deren Einsatzfähigkeit.

Botrytis aclada, ein pflanzenpathogener Ascomycet, exprimiert eine chloridtolerante Laccase mit hohem Redoxpotential, die als ein vielversprechender Kandidat für die Enzymoptimierung, mit dem Ziel, Aktivität sowie Stabilität bei physiolgischen Bedingungen zu verbessern, gilt.

Ziel dieser Arbeit ist es, Mutationen zu finden, die das Verhalten dieser Laccase beeinflussen und damit den Zusammenbruch der Aktivität bei neutralen Bedingungen, den man bei derartigen Laccasen beobachtet, zu erklären. Zwei verschiedene Experimente, die entweder eine semi-rationale oder eine auf Zufall basierende Herangehensweise repräsentieren wurden hierfür durchgeführt.

Für den semi-rationalen site-saturation Mutagenese Ansatz wurde eine Bibliothek basierend auf Mutationen, die während der gerichteten Evolution einer Laccase vom Basidiomyceten PM1 gefunden wurden, verwendet. Die Enzymbibliothek, des auf Zufall beruhenden “directed evolution” Ansatzes basierte auf einer thermostabilen *B. aclada* Laccase Variante, die zuvor entdeckt und in dieser Arbeit charakterisiert wurde. Beide Bibliotheken wurden mittels eines effizienten zweistufigen Screenings, das *Pichia pastoris* als Expressionsplatform verwendet, untersucht.

Im “site-saturation” Experiment wurde eine Variante, die eine große substratspezifische Veränderung im pH-Profil aufweist, entdeckt und charakterisiert. Das “directed evolution” Experiment ergab eine Variante mit deutlich verändertem pH-Profil.

Diese Varianten beweisen die Viabilität dieses “enzyme engineering” Prozesses für dieses Protein und ermutigen so zu weiterer Forschung auf diesem Gebiet.

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1. Introduction

The implementation of enzyme-based biofuel cells has been a very active research topic during the last years. Possible applications are numerous, with the medicinal sector being probably the most interesting one. In this field the blood could serve as a virtually unlimited fuel source, providing fuel for powering pacemakers, biosensors and other implantable bio devices over a considerable period of time. This raises the need for cells capable of working under physiological conditions (pH 7.4, 37 °C and chloride concentrations around 100-140 mM).

Several different biocatalysts have been taken into consideration and analysed for their usefulness as part of such a cell. One group of enzymes known as laccases are especially interesting in this regard. These oxido-reductases are known for their broad spectrum of possible substrates and their capability to reduce oxygen to water, making them efficient and environmentally friendly catalysts.

The versatility and usefulness of laccases has been already demonstrated in several different applications. They are currently used for diverse tasks such as bioremediation, bleaching of textiles and process effluents in the paper industry, lignin degradation and synthesis of organic compounds. In regards to biofuel cells they are considered interesting due to their high redox potential, high operational stability and their capability of direct electron transfer (DET).

Nevertheless it is desirable to achieve improved knowledge of the reaction mechanism, structure-function relationship and other parameters influencing the functionality and stability before it is possible to understand the full potential of these enzymes.

1.1. The redox-active enzyme laccase

1.1.1. General characterisation

Laccases are a widely known group of redox-active enzymes that belong to the family of blue multicopper oxidases (MCOs) (benzenediol:oxygen oxidoreductases, EC 1.10.3.2).

The enzyme was first discovered in sap isolated from resin ducts of the Japanese lacquer tree *Rhus vernicifera* [Yoshida, 1883]. Today over 100 different types of laccases have been isolated from various organisms and thoroughly characterised. They are mostly found in fungi or plants but in recent years examples have also been observed in bacteria as well as insects [Clause H, 2003]. From this vast collection of variants those derived from ascomycetes and basidiomycetes are the most extensively examined subjects.

The enzyme is known for its broad range of possible physiological functions it is able to fulfil. Laccases found in white-rot fungi for example play, in combination with other oxidative enzymes, an important role in delignification of wood and herbaceous materials. Phytopathogenic fungi, however, use them as protection against phytoalexins, tannins and phytoanticipins, which are produced by the defensive system of their host plants [Mayer and Staples 2002]. In plants this redox enzyme is often involved in lignin biosynthesis while in insects it seems to have an important role in the cuticle sclerotization process. Lastly, it can be assumed that laccases are an important factor during sporulation in bacteria [Sharma et al. 2007].

On the genetic level multicopper oxidases are characterised by four ungapped sequence regions labelled as L1-L4 with the sequences L1 and L3 being distinctive to laccases [Kumar et al. 2003]. All these regions contain conserved residues that are generally associated with copper ligand binding.

Fungal laccases are mostly extracellular, glycosylated, monomeric enzymes with a molecular weight between 60-70 kDa and an isoelectric point around pH 4. Their structure is segmented into three similar cupredoxin-like domains with each domain presenting a so called greek-key β-barrel motif. This structural element is closely related to the small copper proteins plastocyanin and azurin [Giardina, 2010].

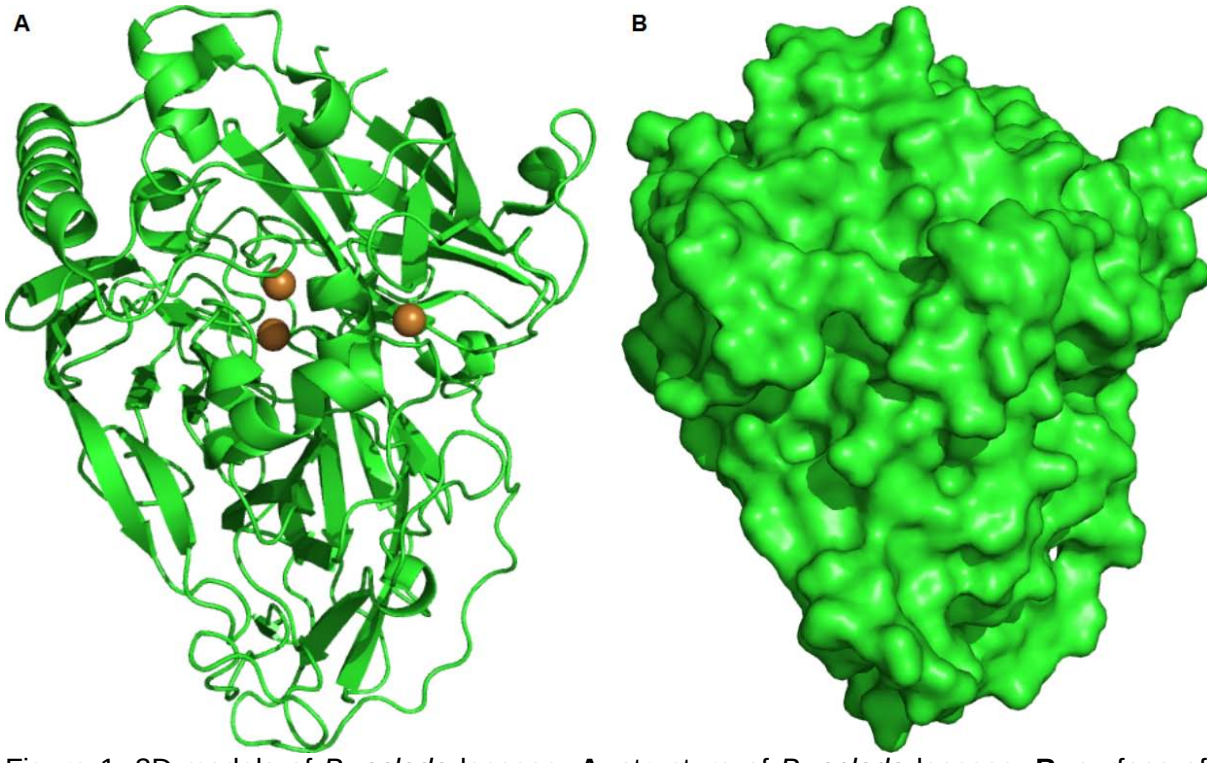

Figure 1: 3D-models of *B. aclada* laccase: **A**: structure of *B. aclada* laccase; **B**: surface of the protein; (images created with PyMol)

A characteristic feature of all MCOs are their four copper atoms, which can be divided into three different groups based on their observed EPR and optical absorption features [Solomon et al. 1996]. The single T1 copper is associated with the oxidative part of the redox-reaction and is coordinated in a planar triangular way by one cysteine and two histidine residues. The copper has an absorption maximum at 614 nm, which is responsible for the characteristic deep blue colour of this enzyme.

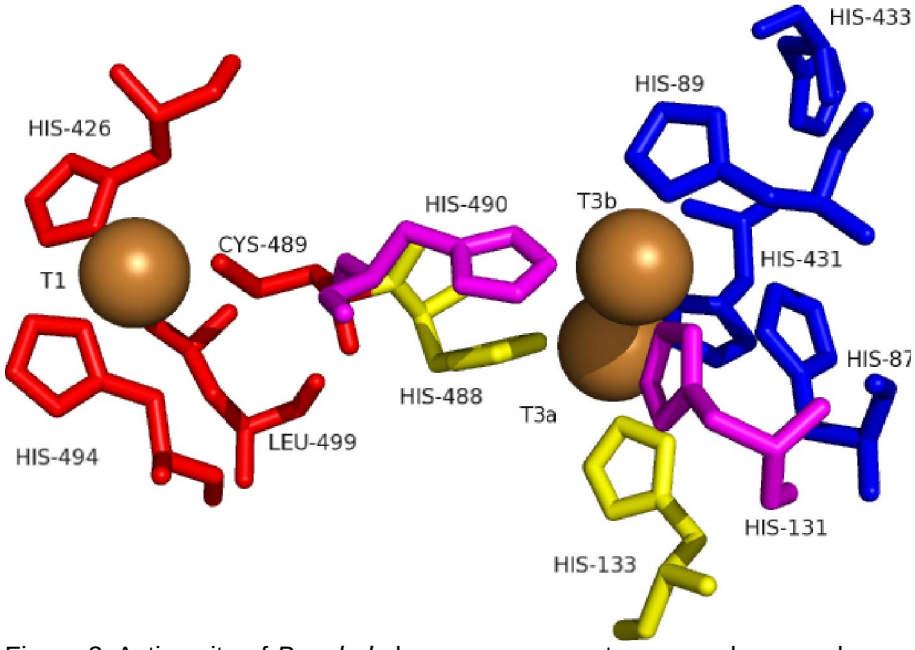

Figure 2: Active site of *B. aclada* laccase: copper atoms are shown as brown spheres. Coordinating ligands: red: T1, blue: T2 (missing from this structure), yellow: T3a, magenta: T3b

The other three copper atoms, consisting of one T2 and two T3 copper, form a trinuclear cluster (TNC) which is responsible for the reduction of O_2 to H_2O. They are coordinated by a strongly conserved pattern of four His-X-His motifs. The T2-copper, which is coordinated by two histidine residues, has no detectable optical absorption band but shows a distinct electron paramagnetic resonance spectrum. The two T3-coppers, commonly referred to as Cu3a and Cu3b, are coordinated by a total of six histidine residues and are coupled together through a hydroxide-bridge, which makes them undetectable in EPR spectroscopy. Their relatively weak optical absorption band is located at 330 nm.

1.1.2. Catalytic mechanism

Laccases are capable of oxidising a wide array of compounds, such as various phenolic substances including *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, aryl diamins and several inorganic ions [Solomon et al. 1996]. This broad substrate specificity can be partly attributed to the relatively large and easily accessible binding pocket, which is capable of accommodating a variety of chemically different compounds [Bertrand et al. 2002].

The T1-copper is located in close proximity to this pocket where it promotes the removal of one electron from the substrate, therefore oxidizing it. As a result of this oxidation process a radical is created which can react further and, depending on the conditions, either create dimers with itself or interact with molecules in its surroundings. This reaction can be used for the enzymatic production of polyphenols for various applications [Riva, 2006].

The electron is then transferred through a highly conserved His-Cys-His tripeptide bridge to the trinuclear copper cluster. There, the enzyme accepts up to four electrons until it reaches a completely reduced state. Consequently, it is able to catalyse the four electron reduction of molecular oxygen to water. This happens in two distinct steps where each time two electrons are transferred. As a result four substrate molecules need to be oxidised to fully reduce one molecule of oxygen [Rodgers, 2010].

Laccases can be, according to the reduction potential measured at the T1-copper, divided into two classes: the low redox potential (LRPL) and high redox potential laccases (HRPL). HRPLs show E_0 values up to 790 mV vs. standard hydrogen electrode (SHE) and are generally found only in fungi. They are generally considered as the most promising candidates for practical applications which can be partly attributed to the higher catalytic efficiency of these enzymes [Kunamneni et al., 2008].

An interesting feature of these catalysts is their ability to use a reaction mechanism based on mediating substances. This allows them to oxidise compounds which either have a particularly high redox potential or are otherwise sterically inaccessible, thereby further increasing their already wide substrate range [Call & Mücke, 1997].

Mediators are various chemical compounds that serve as intermediates in an enzymatic reaction. They are oxidized directly by the enzyme and through diffusion they can reach and subsequently reduce a substrate that is generally not available to the protein. A prominent example for this mechanism can be seen during the lignin-degradation process. The bulky structure of this natural polymer prevents direct interaction with enzymes, therefore only small mediating molecules are able to enter this space and change the structure of the lignin molecule.

One of the first discovered and most used mediators for laccase is the synthetic compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), which also serves as activity indicator in biochemical studies. However, over 100 different substances, including 2,6-dimethoxyphenol (2,6-DMP) and 1-hydroxybenzotriazole (HBT) were tested for their possible use as mediator.

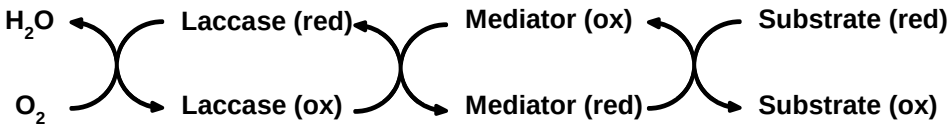

Figure 3: Overview of a typical mediator reaction. Laccase draws an electron from O_2 and transfers it via a mediator to the substrate

Several types of anions such as halides, pseudohalides, carbonates, sulfides and heavy metals are known to inhibit the enzyme. Especially smaller ions such as fluoride, azide and chloride are efficient in decreasing the catalytic activity. The inhibition mechanism is assumed to be based on the competitive binding of these ions to the T2 and T3 coppers which interrupts the electron transfer and therefore stops the oxidative reaction [Xu F., 2001].

1.1.3. Laccase from *Botrytis aclada*

Among the numerous different laccases one in particular has several interesting properties that make it worth investigating. It is produced by the ascomycete *Botrytis aclada,* a plant pathogen, which is known to cause characteristic neck rot diseases, especially on onion plants [Chilvers & du Tout, 2006].

The mature enzyme consists of 561 amino acids resulting in a molecular weight of 61565.1 Da. It has a calculated pI of 4.43 but the actual pI has been determined to be 2.4. Furthermore the 3-D structure of the crystallized enzyme has been resolved at a resolution of 1.7 Å and its redox potential measured at 720 mV [Osipov et al. 2014].

Furthermore, it shows remarkably high chloride tolerance with an I_{50} of 1.4 M for chloride, which is a necessary prerequisite for possible applications in the industrial or medicinal sector. Also, it possesses interesting kinetic properties including low K_M values for a variety of phenolic substrates as well as ABTS and generally high turnover rates. In the methylotrophic yeast *Pichia pastoris* it can be readily expressed in a recombinant way resulting in yields as high as 495 mg L^{-1} with 51000 U L^{-1} [Kittl et al. 2012].

Together with several other fungal laccases it has a low pH optimum for all its substrates which rapidly decreases with increase of pH and ends in complete loss of activity at neutral conditions. The stability of this enzyme can be considered as only moderate especially when compared to other laccases secreted by fungi.

1.2. Application of laccase in enzymatic biofuel cells

Enzymatic biofuel cells can be defined as devices capable of transforming chemical energy into electrical energy via electrochemical reactions using biocatalysts as fuel [Bullen et al. 2006]. In general every electric cell consists of two crucial parts: the anodic and the cathodic element.

At the anode an oxidation reaction occurs, while at the cathode reduction takes place. This process leads to the transportation of electrons from the anode to the cathode, which in turn results in electrical work and the produced energy can then be used for different applications.

Fuel cells are characterised by an external fuel supply in contrast to batteries, where all substances are contained in internal compartments. In comparison to conventional cells based on inorganic compounds such as various metals the amount of energy obtained from enzymatic systems is rather low. Nevertheless there are several unique advantages of these biofuel cells. They normally reach their optimal activity at mild conditions (neutral pH, low temperatures), they have a high chemical selectivity and they are potentially cheap to produce. As a result the use of this type of fuel cells is taken into consideration for various applications such as providing power for biosensors and other implantable devices.

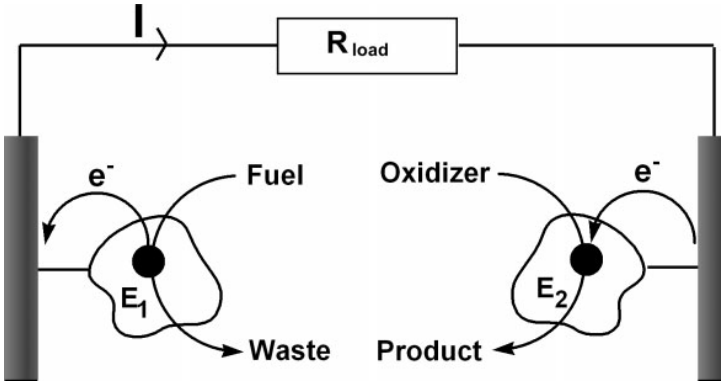

Figure 4: Schematics of an enzymatic biofuel cell: left: enzyme E1 as anodic element, right: enzyme E2 as cathodic element. The combination of both enyzmes in an electric cell creates power [Willner et al. 2008]

Laccases, together with other multicopper oxidases, are promising possible cathodic elements for the construction of biofuel cells. Their high redox potential makes them particularly efficient at reducing molecular oxygen to water and in combination with enzymes such as glucose oxidase or cellobiose dehydrogenase immobilized at the anode they are capable of producing a comparable high amount of energy.

Their substrates are readily available and since their reaction products are entirely non-toxic substances they are especially interesting for applications regarding human health. For implantable biofuel cells the nutrients and dissolved oxygen found in blood can serve as virtually unlimited fuel source, however a chloride concentration of 140 mM coupled with a pH of 7.4 has a noticeable negative impact on the activity and stability of laccase. This prevents their effective use and simultaneously raises the need to abolish these limitations by modifying the enzyme.

1.3. Enzyme engineering via directed evolution

Directed evolution is a powerful tool to study and improve different aspects of an enzyme and it even allows scientists to significantly change the function of a biocatalyst. As its name suggests this method is based on the evolutionary principle of *survival of the fittest*, which is influenced by using biochemical methods to introduce a high mutational load and create an environment that favours the previously defined selection goals.

The design of a directed evolution experiment is based on a simple cyclic layout. At first a suitable parent enzyme has to be chosen and a suitable engineering goal needs to be defined. Afterwards the genetic code of this protein is mutated using different methods such as DNA-shuffling and error-prone PCR. The modified enzyme is expressed and exposed to a defined selection pressure designed to test for the previously specified engineering goals. If a variant seems to possess improved functionality, its sequence is analysed and its enzymatic properties are further characterised. A variant carrying a beneficial mutation can then be used again as parent enzyme for the next round of directed evolution. If necessary the screening conditions can be optimized for this new round.

Laboratory evolution can help elucidating the influence of changes in the protein sequence on the function of the resulting enzyme. Its random nature often leads to unexpected beneficial mutations, which would not have been discovered with a rational approach otherwise.

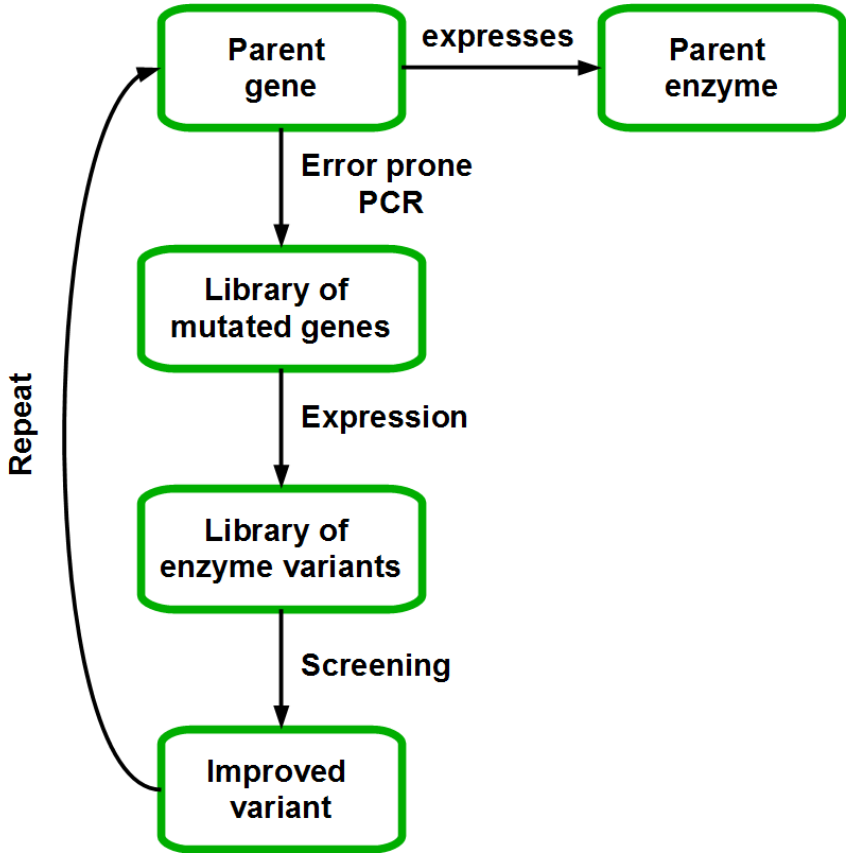

Figure 5: Overview of a simple directed evolution process

1.4. Aim of this work

The purpose of this work was to study the effect of different mutations on the behaviour of laccase isolated from *Botrytis aclada.* This should help to further elucidate structure-function relationships regarding thermal stability and pH behaviour. Furthermore this study could help in taking first steps to enable their effective use in biofuel cells.

In order to reach this goal a differential screening consisting of two selection criteria was defined. First, a necessary change would be to shift the pH-optimum towards higher pH-values in order to increase the enzyme activity at neutral conditions. Second, it is necessary to significantly increase the stability at elevated temperatures. The results of this enzyme optimisation strategy could help to reach a better understanding of how different amino acid positions influence the function of ascomycetous laccases.

Two different enzyme engineering approaches, a semi-rational one based on prior knowledge derived from similar studies and another one based on random mutations were employed.

The first experiment was based on existing information derived from the successful modification of a HRPL from the basidiomycete PM1 (Maté et al. 2013). The most beneficial mutations in this laccase variant were analysed and conferred to its ascomycetous counterpart. For the analysis whether a change of a residue at these positions improves the enzyme regarding the pre-defined evolution goal, random mutations were introduced using the site saturation technique. In this method the codon of an amino acid on a chosen position is exchanged in every other possible base triplet and the resulting enzyme variants are expressed and screened.

In the second experiment directed evolution was used and random mutations were introduced into the laccase gene via error prone PCR. This library of variants was based on a variant with increased thermostability, which was found in a previous experiment and characterized during this work. This second generation directed evolution library was analysed simultaneously for improved activity at higher pH values and increased stability after heat exposure.

For the reliable analysis of these libraries an elaborate high-throughput screening protocol was used. The high expression levels in *P. pastoris* combined with fast and sensitive activity assays for laccase allow the design of such a method thereby increasing the amount of clones that can be analysed per screening round.

2. Material and Methods

2.1. Genetics

2.1.1. Primers

For the site saturation experiment seven primer pairs were designed containing a random mutation at the corresponding base triplet in the forward primer. Both primers have an overlapping region of 15-20 bp, an extended region of about 10-15 bp length and a melting temperature $T_{M,}$ between 60-65 °C. For primer design the free DNA analysing tool GeneRunner was used. The primers were ordered and purchased from VBC Biotech (Vienna, Austria) and diluted with sterile HQ-H_2O to a concentration of 100 pg μL^{-1}.

Table 1: Primers designed for the site saturation experiment

notation	sequence
5D236sat	5'- CAATGTCGGAATT**NNK**AGTCACTTCGAATTCGCCATTG-3'
3D236sat	5'- AATTCCGACATTGATCAATCTC-3'
5L255sat	5'- CATTGCCAACGAT**NNK**GTTCCAATTGTACCCTACACTAC -3'
3L255sat	5'- ATCGTTGGCAATGACAGTAAGT-3'
5D313sat	5'- GTATCCTCCGATAC**NNK**AGCTCCAGCATCGCAAATCCTAC-3'
3D313sat	5'- GTATCGGAGGATACCTGTAGC-3'
5I428sat	5'- CATTTGGCATCCT**NNK**CATCTCCACGGCCACGATTTC-3'
3I428sat	5'- AGGATGCCAAATGCCGAAGCC-3'
5A466sat	5'- CGTGACGTCGCG**NNK**CTTCCCGGAAACGGTTATCTTG-3'
3A466sat	5'- CGCGACGTCACGACGTGGAGG-3'
5W493sat	5'- CATTGTCATATCGCA**NNK**CACGCATCTGAGGGGTTGGC-3'
3W493sat	5'- TGCGATATGACAATGAAGAAGC-3'
5N526sat	5´-GGATACTTGTGCA**NNK**TGGAATGCTTATACTCCTACTC-3´
3N526sat	5´-TGCACAAGTATCCTCAAATATAG-3´

Table 2: Standard primers used for cloning and sequencing

notation	sequence
3AOX	GCAAATGGCATTCTGACATCC
pGAPfw2	CCCAATTTTGGTTTCTCCTGAC
3ZLH	GGCGCTATTCAGACCTCTTC

The expression of functional *B. aclada* laccase for screening and fermentation purposes was done in the electro-competent *P. pastoris* strain X-33 (Invitrogen, Carlsbad, CA, USA).

For the creation of the error-prone as well as the site-saturation library the *Escherichia coli* strain NEB5-alpha (New England Biolabs, Ipswich, MA, USA) was used with the following genotype: *fhuA2_(argF-lacZ)U169 phoA glnV44 _80_(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.*

The *Ba*Lac variant BAGANSN consists of the wild type gene in a pGAPZA vector with restriction site for *Nde*I and *Not*I added and the site for *Sac*I deleted and was used as template for the site saturation library.

2.2. Enzymes

2.2.1.1. Restriction enzymes

All restriction endonucleases were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The digestion was carried out according to the protocol found in the manual provided with the enzyme if not noted otherwise.

Table 3: List of restriction enzymes and their restriction site

Enzyme	Restriction site	Buffer
DpnI	5´…G m6A ▼ T C…3	Tango
NotI	5´…C A ▼ T A T G…3´	O
NdeI	5´…G C ▼ G G C C G C…3´	O
PagI (BspHI)	5´…T ▼ C A T G A…3	O

2.2.1.2. Ligases and Kinases

FastAP (Thermo Scientific) is a thermo sensitive alkaline phosphatase which catalyses the removal of phosphate-groups from the 3' and 5' ends of DNA molecules in order to prevent spontaneous religation of a cut vector.

T4-DNA Ligase catalyses the formation of phosphodiester bonds between the 5' and 3' terminus of DNA molecules, thereby joining both strands together.

2.2.2. DNA polymerases

The Go*Taq*-Polymerase (Promega, Fitchburg, WI, USA) is derived from the thermophilic bacterium *Thermus aquaticus* and has no proof-reading activity, which makes it not suitable for exact DNA analysis and cloning purposes. It was used for the amplification of genes in *P. pastoris* with colony-PCR.

The enzyme Dream*Taq* (Thermo Scientific) is a genetically modified Taq-polymerase: It was used as an alternative for the Go*Taq*-Polymerase in colony-PCRs. Its biochemical properties and capabilities are comparable to the Go*Taq*-Polymerase.

Phusion High-Fidelity DNA-Polymerase (Thermo Scientific) is a genetically modified enzyme from the hyper thermophilic Archaea *Pyrococcus furiosus*. It possesses proof-reading activity and therefore it was used for creating the site saturation mutagenesis library and to prepare the parent gene for the directed evolution library.

2.2.3. Vectors

For the constitutive expression of a gene in *P. pastoris* the vector pGAPZA (Invitrogen) was used. The empty plasmid has a total length of 2884 bp and is derived from the methanol inducible pPICZ vector (Invitrogen). Its AOX1 promoter was replaced with the glyceraldehyde-3-phosphate dehydrogenase promoter allowing high protein expression. As a shuttle vector it contains a pUC origin of replication for efficient plasmid production in prokaryotes.

An additional feature of this vector is a *Sh ble* gene, which is under the control of another constitutive promoter. This allows the organism to express a protein capable of binding the antibiotic Zeocin (Invitrogen), therefore allowing it to be used as a selection marker.

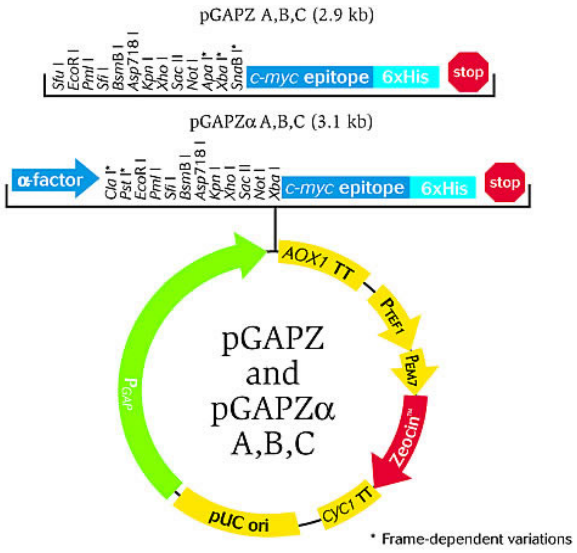

Figure 6: Map of pGAPZ and pGAPZα vector

2.3. Chemicals

All chemicals used during this work were of the highest purity available. Buffers and assay solutions were prepared with deionized reversed osmosis water (>16 MΩcm).

2.3.1. Media

Yeast Peptone Dextrose (YPD)

- 20 g Peptone from casein (Sigma-Aldrich, St. Louis, MO, USA)
- 10 g Yeast extract (Carl Roth, Karlsruhe, Germany)
- 4 g D-Glucose (Merck, Darmstadt, Germany)
- 1 L HQ-H_2O

For agar plates the following was added:

- 15 g Agar-Agar Kobe I (Carl Roth)

The medium was autoclaved at 121 °C for 15 min and afterwards cooled to 60 °C in a water bath. Zeocin (100 mg mL^{-1}) was then added to reach a concentration of 100 mg L^{-1}.

Buffered Yeast Peptone Dextrose (BYPD)

The BYPD-medium was prepared similarly to the YPD medium. The solid components were dissolved in 900 mL HQ-H_2O and autoclaved and afterwards 100 mL sterile 1 M KPP-buffer at the desired pH were added to the medium to reach a buffer concentration of 100 mM.

Lysogeny Broth (LB) low salt

- 10 g Peptone from casein
- 5 g Yeast extract
- 5 g Sodium chloride (Sigma-Aldrich)
- 1 L HQ-H_2O

For agar plates the following was added:

- 15 g Agar-Agar Kobe 1

The medium was autoclaved at 121 °C for 15 min and cooled to 60 °C in a water-bath. Zeocin (100 mg mL^{-1}) was then added to reach a concentration of 25 mg L^{-1}.

Buffered Minimal Glycerol (BMG) Media

- 15 g Agar-Agar Kobe 1
- 778 mL HQ-H_2O

The medium was autoclaved at 121 °C for 15 min and the following sterile solutions were added aseptically:

- 100 mL KPP
- 100 mL 10x GY
- 100 mL 10x YNB

The following solutions were added with a sterile filter:

- 10 mL ABTS-solution, 20 mM
- 10 mL Cu(II)SO_4-solution, 10 mM
- 2 mL 500x Biotin-solution

10x GY stock solution

- 100 mL Glycerol 99,5% (Carl Roth)
- 900 mL HQ-H_2O

The solution was autoclaved for 15 min at 121 °C and stored at 4 °C in the cold room.

1M KPP-buffer

see 2.3.2

10x YNB (Yeast Nitrogen Base)

- 35 g Yeast Nitrogen Base without amino acids and ammonium sulphate (AMRESCO LLC, Solon, OH, USA)
- 100 g Ammonium sulphate ($(NH_4)_2SO_4$) (Sigma-Aldrich)
- 1000 mL HQ-H_2O

The solution was sterile filtered and stored at 4 °C

500x Biotin stock solution

- 4 mg Biotin
- 20 mL HQ-H_2O

20 mM ABTS-solution

- 396 mg ABTS diammonium salt (AMRESCO)
- 36 mL HQ-H_2O

The solution was freshly prepared before addition to the agar.

10 mM $CuSO_4$-solution

- 99.87 mg Cu(II)SO_4*H_2O
- 40 mL HQ-H_2O

The solution was freshly prepared before addition to the agar.

2.3.2. Buffers

The following buffer systems were used during this work: NaOH/Citrate, KH_2PO_4/K_2HPO_4 and McIlvaine-Buffer (Citrate/Na_2HPO_4).

Most buffers were prepared with a concentration of 100 mM with the only exception being the 50 mM Citrate buffer used for concentrating and storing purified laccase.

Table 4: Buffer table of 100 mM KPP-buffer

pH	KH_2PO_4 [g L^{-1}]	K_2HPO_4 [g L^{-1}]
5	134.1	2.6
6	118.2	22.9
6.5	92.0	56.0
7	54.1	104.9
7.5	23.5	144.1

The pH was adjusted with phosphoric acid.

NaOH/Citrate Buffer, 100 mM:

- 19.21 g Citric acid monohydrate (Sigma-Aldrich)
- 1 L HQ-H_2O

The pH was adjusted to the desired value with 4 M NaOH.

The McIlvaine-Buffer system consists of citrate and Na_2HPO_4 and is applicable over a wide pH-range from 2.2 to 8.0. The buffer is made from a mixture of a 0.2 M Na_2HPO_4 and a 0.1 M citrate stock solution.

Table 5: Buffer table for mixing 100 mM McIlvaine buffer

pH	Citrate, 0.1 M, [mL]	Na_2HPO_4, 0.2 M, [mL]
3.0	79.4 mL	20.6 mL
3.5	69.7 mL	30.3 mL
4.0	61.4 mL	38.6 mL
4.5	54.6 mL	45.4 mL
5.0	48.5 mL	51.5 mL
5.5	43.1 mL	56.9 mL
6.0	36.8 mL	63.2 mL
6.5	29.0 mL	71.0 mL
7.0	17.6 mL	82.4 mL
7.5	7.9 mL	92.1 mL
8.0	2.3 mL	97.7 mL

50x TAE-Buffer:

- 242 g Tris base (Fluka)
- 100 mL 0.5 M EDTA stock solution (Sigma Aldrich)
- 57.1 mL Glacial acetic acid (Fluka)
- 842.9 mL HQ-H_2O

100 mL of the buffer were diluted to 1x working concentration by adding 900 mL HQ-H_2O.

2.4. Assays

2.4.1. Laccase activity detection

Laccase activity was measured by observing a shift in the absorbance maxima of the substrates 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,6-Dimethoxyphenol (2,6-DMP) over a time period of 180 s.

The standard assay for the detection of laccase activity is based on the O_2 dependent oxidation of ABTS. The oxidized ABTS cation radical has a changed optical absorption maximum at 420 nm and an absorption coefficient at 420 nm (ε_{420}) of 43.2 $mM^{-1}cm^{-1}$ which results in a deep green colour.

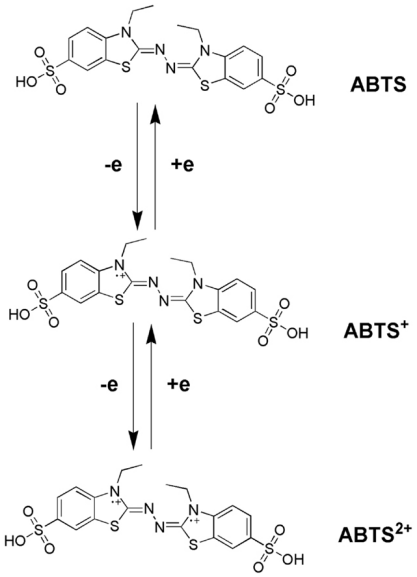

Figure 7: Oxidation reaction of ABTS [Christopher et al. 2014]

A similar assay was carried out by using 2,6-Dimethoxyphenol as substrate for oxidation. This phenolic substance has an absorption maximum at 469 nm in the oxidized state, which turns the solution from light orange to deep amber.

The protocols for both the photometer and the plate reader assay are nearly identical with the only difference being the measured wavelength.

<u>2,6-DMP-solution, 10 mM:</u>

- 7.7 mg 2,6-Dimethoxyphenol (Sigma-Aldrich)
- 5 mL HQ-H_2O

ABTS-solution, 10 mM:

- 10.3 mg ABTS diammonium salt
- 2 mL HQ-H_2O

Photometer assay:

100 µL freshly prepared 10 mM substrate solution were mixed together with 880 µL of 100 mM citrate or McIlvaine buffer in a cuvette and incubated for 10 min at 30 °C in a water bath. Afterwards the cuvettes were placed into the photometer. 20 µL of enzyme were added onto a comb, which was inserted into the cuvettes, stirred vigorously and afterwards the measurement was started immediately. After the measurement was done the volumetric activity, given in U mL^{-1}, was automatically calculated from the kinetic slope and the enzyme factor for this assay.

$$\frac{cuvette\ volume\ [\mu L] \times sample\ dilution\ \ factor}{sample\ volume\ [\mu L] \times \varepsilon} = enzyme\ \ factor$$

The assays were measured with the Perkin Elmer UV/Vis spectrophotometer Lambda 35 (Waltham, MA, USA).

Plate reader assay:

A master mix was prepared containing buffer and substrate in the same ratio used for the standard photometer assay. 20 µL enzyme dilution were pipetted into each well of a 96-well microtiter plate and 180 µL aliquots of the master mix were added with a multichannel pipette resulting in a total assay volume of 200 µL. After substrate addition the activity was immediately measured with the EnSpire Multimode plate reader (Perkin Elmer) using a predefined program.

2.4.2. Bradford-assay

The concentration of purified laccase was measured using the Bradford-method (Bradford, 1976). This assay is based on an absorbance maximum shift of Coomassie Brilliant Blue G-250 (Bradford reagent) from 465 to 595 nm after it has bound to the protein.

For the assay 15 µL enzyme sample were mixed together with 600 µL Bradford reagent in a cuvette and incubated for 15 min at room temperature. Afterwards the absorption was measured at 595 nm with a Beckman Coulter DU-800 Photometer (Brea, CA, USA). The calculation of protein concentration was based on a standard curve, which was created from a 1.0 mg mL^{-1} – 0.1 mg mL^{-1} dilution series of BSA in 0.15 M NaCl and 0.05 % NaN_3 (Protein Standard Sigma P-0914).

For the determination of the concentration a dilution series in a 100 mM Na-citrate buffer with pH 5.0 was measured beginning from 1:10 down to 1:10000 in duplicates.

Alternatively the absorption of an enzyme dilution at 280 nm was measured with a Lambda 35 UV/Vis-photometer (PerkinElmer). This assay is based on the high absorption of aromatic rings found in proteins at a wavelength of 280 nm [Layne, 1957].

An absorption coefficient of ε_{280} = 126.8 mM^{-1} cm^{-1} was used for determination of the concentration.

2.5. Library creation

2.5.1. Molecular Biology

2.5.1.1. Agarose Gel Electrophoresis:

A 0.8% agarose gel was mixed with either Ethidiumbromide (Carl Roth) or 20000x peq-GREEN (peqlab, Erlangen, Germany) as fluorescent DNA dye, cast into a form and left to polymerize for 30 min at room temperature. The gel was then put into an electrophoresis chamber and submerged in 1x TAE-buffer.

The sample was mixed with 6x Loading Dye and loaded into a pocket of the gel. Additionally a GeneRuler DNA-Ladder (Thermo Scientific) was added to a reserved pocket to allow size determination. The gel was then run at 90 V for about 30-40 min using the PowerPac 300 (Bio-Rad Laboratories, Hercules, CA, USA) as power supply. Afterwards the gel was taken out and analysed under UV-light with a Gel Doc 2000 (Bio-Rad Laboratories). For further analysis a band was cut out with a scalpel and transferred into a sterile tube.

2.5.1.2. DNA Gel Elution and purification

DNA was eluted from an agarose gel with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Uppsala, Sweden).

Before gel elution the weight of the gel was determined and 10 µL of Capture buffer type 3 were added for every 10 mg of gel. The mixture was incubated at 60 °C until the gel was dissolved completely and 600 µL transferred onto an illustra GFX Microspin column. After 1 min incubation at room temperature the column was centrifuged at 16000x g for 30 s and the flow-through discarded. Then, 500 µL of Wash buffer type 1 were pipetted onto the column and centrifuged for 30 s.

The column was then transferred to a fresh sterile tube and the sample was eluted by adding 20-50 µL of sterile, nuclease-free HQ-H_2O. After incubation for 1 min at room temperature the column was centrifuged for 1 min and the resulting flow-through stored at -20 °C.

2.5.1.3. Plasmid Isolation

E. coli cells carrying the target plasmid were inoculated in 3 mL LB low salt medium with Zeocin as selection marker and incubated overnight at 37 °C at a shaking speed of 125 rpm. The plasmid DNA was isolated with the PureYield Plasmid Miniprep System (Promega).

A modified version of the high-yield protocol described in the manual was used. 1.5 mL culture were centrifuged at maximum speed for 30 s with the centrifuge 5424 (Eppendorf AG, Hamburg, Germany), the supernatant was discarded and the pellet dissolved in 600 µL fresh culture.

Subsequently 100 µL Lysis Buffer were added and the tube was inverted six times. To stop the lysis reaction 350 µL of cold Neutralisation Solution were added and the tube was mixed gently by inversion. After 3 min of centrifugation at maximum speed 900 µL of the supernatant were transferred to a PureYield Minicolumn and centrifuged for 30 s.

The column was washed in two steps, at first with 200 µL Endotoxin Removal Wash followed by 400 µL Column Wash Solution. In each step the column was centrifuged for 30 s and the flow-through discarded.

For elution 20-50 µL of sterile H_2O were added to the column, which has been placed on sterile tube, incubated for 1 min at room temperature and the column was then centrifuged for 1 min and the flow-through was kept and stored at -20 °C.

2.5.1.4. Colony-PCR

For the isolation and amplification of a gene from *P. pastoris* cultures a colony-PCR protocol was used. A large lump of cells was taken from a fresh agar plate with a sterile tooth pick, dispersed in 30 µL sterile HQ-H_2O and cooked at 95 °C for 10 min to break up the cells. After centrifugation at maximum speed for 5 min either 5 or 10 µL of the supernatant were used as template.

Reaction for colony-PCR

- 5 µL 5x GoTaq-Buffer
- 0.5 µL dNTPs, 10mM each
- 1 µL Primer pGAPfw2 (10 pg μL^{-1})
- 1 µL Primer 3AOX (10 pg μL^{-1})
- 0.5 µL GoTaq DNA polymerase
- 12/7 µL HQ-H_2O, nuclease-free
- 5/10 µL Template

Table 6: Standard program for colony-PCR

Temperature	Time	
95 °C	forever	
95 °C	120 s	
95 °C	30 s	Repeat 33 times = 34 cycles
60 °C	60 s	
72 °C	120 s	
72 °C	420 s	
12 °C	forever	

2.5.1.5. Transformation of chemically competent *Escherichia coli* strain NEB-5-alpha cells

An aliquot of frozen NEB-5-alpha cells was thawed on ice for 10 min and, if necessary, separated into 25 µL aliquots. Then up to 4 µL of purified plasmid DNA were added to 50 µL of cells, while gently stirring the suspension with the pipette tip. The mixture was cooled on ice for 30 min and then the tubes were placed in a thermo mixer set to 42 °C for 30 s to apply a heat shock. The transformed cells were cooled on ice for another 10 min and afterwards 500 µL of SOC-Medium at room temperature were added to the cell suspension. The cells were incubated for 1 h at 37 °C while shaking at 125 rpm.

The incubated cells were streaked out on LB low salt agar plates containing Zeocin. For the 200 mL agar plates 1 mL of cell suspension was used. For smaller agar plates the suspension was centrifuged, the pellet suspended in 300 µL SOC-Medium and plated out. All plates were incubated upside down over night at 37 °C.

2.5.1.6. Transformation of electroporation of competent *Pichia pastoris* strain X-33 cells

The plasmid was isolated from a corresponding *E. coli* culture and treated with the restriction enzyme *Pag* I to linearize the DNA molecules.

Pag I digest reaction:

- 10 µL Plasmid DNA
- 1.5 µL 10x Buffer O
- 0.5 µL *Pag* I
- 3 µL HQ-H_2O, nuclease-free

Prior to electroporation a MicroPulser cuvette (Bio-Rad Laboratories) with a 1 mm gap was washed with ethanol, dried at 50 °C and exposed to UV-light for 10 min for sterilization.

The frozen cells were thawed on ice for 10 min. After the plasmid was added, the solution was gently mixed with the pipette tip and then pipetted slowly onto the side of a chilled cuvette. The cuvette was placed in a MicroPulser electroporation system (Bio-Rad Laboratories) and a pulse with a voltage of 1.5 kV and 125 Ω resistance was applied for 3.0 ms. 500 µL cold 1 M sorbitol were added to the cells immediately afterwards, mixed vigorously by pipetting up and down and another 500 µL of YPD-Medium were added. The cells were transferred into a sterile 2 mL tube and incubated for 3-4 hours at 30 °C and horizontal shaking at 180 rpm.

After incubation 1 mL cell suspension were plated on 200 mL agar plates containing YPD-agar supplemented with Zeocin. The plates were incubated for two days at 30 °C.

2.5.1.7. Preparation of a cryo stock culture

For the conservation of an *E. coli* culture either a single colony from an agar plate was inoculated in 3 mL liquid LB low salt medium and grown at 37 °C overnight. Then 3 mL sterile 30% glycerol were added to the culture and 1 mL of this culture were distributed to three sterile cryo-tubes. The tubes were then submerged in liquid nitrogen and stored at -80 °C.

P. pastoris cells were conserved using a similar protocol with the only difference being that the cells are grown in YPD medium at 30 °C.

Alternatively to this standard protocol stock cultures were also prepared from liquid cultures by adding in a 2 mL tube 500 µL of 30 % glycerol to 500 µL of the culture, submerging it in liquid N_2 and storing it at -80 °C.

2.5.2. Site-saturation library

Seven mutation sites were chosen based on the blood tolerant laccase variant Chu-B and its predecessor OB-1. The sequences of these enzymes and the *B. aclada* laccase were aligned using a BLAST-like algorithm incorporated into the molecular modelling software PyMol (Schrödinger, LLC, Portland, OR, USA). The corresponding position on the *Ba*Lac gene was determined with the free software GeneRunner.

Table 7: Variants chosen from the directed evolution process of variant Chu-B; underlined positions are found in the variant

Mutation in Chu-B	Position in BaLac	Comment
D205N	Asp236	Increase in activity at higher pH, not found in Chu-B variant
F396I	Ile428	Showed strongest increase in activity at neutral pH
F454E	Trp493	Resulted in a pH-optimum shift from pH 4 to 5
T487S	Asn526	Increased the relative activity at pH 7, not used in variant Chu-B

Table 8: Variants chosen from laccase variant OB-1

Mutation in OB-1	Position in BaLac	Comment
S224G	Leu255	Inserted by site directed mutagenesis to increase stability
D281E	Asp313	Increased enzyme stability
S426N	Ala466	Mutated to Asp in Chu-B, resulted in a shift in the pH-optimum

The site saturation mutations at these positions were introduced with the Phusion High-Fidelity PCR system using the wild type laccase gene (BAGANSN) as template. The forward primer contains the base triplet NNK to ensure that all variants are possible at the designated position.

Phusion-PCR reaction:

- 12.5 µL Phusion Master mix
- 1 µL Primer, forward (=pGAPfw2, 10 pg/µL)
- 1 µL Primer, reverse (=3AOX, 10 pg/µL)
- 10.5 µL sterile HQ-H_2O
- 1 µL Template dilution 1:10

Table 9: Standard protocol for Phusion-PCR reaction: For annealing a temperature gradient was inserted to find the optimal annealing conditions

Temperature	Time	
98 °C	forever	
98 °C	120 s	
98 °C	10 s	Repeat 29 times = 30 cycles
58-68 °C	20 s	
72 °C	120 s	
72 °C	300 s	
15 °C	forever	

The PCR-product was run on an agarose gel and subsequently eluted in 30 µL HQ-H_2O. 3 µL Tango-Buffer and 1 µL *Dpn* I were added to the eluted plasmid and digested for 1 hour at 37 °C to remove template DNA. *Dpn* I recognizes and selectively cuts methylated DNA sequences found only in the plasmid isolated from *E. coli*. The reaction was heat-inactivated at 65 °C for 10 min.

4 µL of digested plasmid were used for the transformation into chemically competent *E.coli* cells (see 2.5.1.5). The transformed cells were streaked on LB low salt agar plates containing Zeocin and incubated at 37 °C overnight. If the number of colony-forming units (cfu) was over 300, the probability all possible mutations are found was deemed to be high enough and the cells were washed off with 2 mL LB low salt medium. The cells were incubated for 1 hour at 37 °C and afterwards 500 µL and 600 µL culture were used for a cryo stock and for plasmid preparation, respectively.

The isolated plasmids were sequenced from two directions using the primers pGAPfw2 in the 5'- and 3AOX in the 3' direction to check if the chosen position is mutated to an acceptable extent. This was indicated by an approximately equal concentration of all four bases at the first two positions of the triplet and an equal distribution of guanosine and thymine at the third position. The plasmid DNA was subsequently digested with *Pag* I (*BspH* I) and transformed into electro competent *P. pastoris* X-33 cells.

2.5.3. Error-prone library

The library was created using the thermostable T383I *Ba*Lac variant as parent enzyme. The plasmid encoding this protein was isolated from an *E. coli* culture, subsequently purified and quantified by measuring the absorption at 260 nm with the Beckman DU-800 spectrophotometer. For the measurement 15 µL of plasmid preparation were mixed together with 85 µL HQ-H_2O in a quartz micro cuvette with 10 mm optical path length.

The following formula was used for the calculation of DNA concentration:

$$Concentration\ of\ \ DNA\ in\ \mu g/mL = A_{260} \times 50\ \mu g/mL \times dilution\ \ factor$$

Random mutations into the BaLac gene were introduced with error prone PCR using the GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA).

Several different template plasmid concentrations were tested together with a varying amount of cycle numbers in the PCR program to find out the optimal conditions for reaching the desired mutation rate of 2-3 mutations per gene.

Standard reaction for error prone PCR with Mutazyme II-Kit

- 2.5 µL 10x Mutazyme II reaction buffer
- 0.5 µL dNTPs, 10 mM each
- 0.5 µL Primer pGAP fw2
- 0.5 µL Primer 3ZLH gap 1
- 0.5 µL Mutazyme II DNA polymerase
- x µL Template plasmid
- fill with nuclease-free HQ-H_2O to 25 µL

Table 10: Standard protocol for error prone PCR with Mutazyme II-Kit

Temperature	Time	
95 °C	forever	
95 °C	120 s	
95 °C	30 s	Repeat x times
56 °C	30 s	
72 °C	120	
72 °C	600 s	
4 °C	forever	

The PCR product was separated from the template with gel electrophoresis, eluted and digested with the restriction enzymes *Not* I and *Nde* I followed by another gel electrophoresis and elution of the corresponding band. Finally the resulting DNA fragment was ligated with the backbone DNA.

The backbone was created by digesting a pGAPZA vector with both *Not* I and *Nde* I, and separating it with gel electrophoresis. The corresponding larger band was cut out, eluted and purified. Afterwards the DNA was dephosphorylated with FastAP to prevent spontaneous re-ligation. After heat inactivating the reaction at 75 °C for 5 min the backbone was stored at -20 °C.

In the ligation reaction an approximate insert to backbone ratio of 4:1 was used. This ratio was determined from the intensity of the band after gel electrophoresis. The ligation was done overnight using the C1000 Thermal Cycler (Bio-Rad Laboratories). Ligation was carried out by increasing the temperature every 30 min by 0.5 °C from 10 to 16 °C and a last step holding 4 °C until the samples are taken out.

<u>Ligation Reaction:</u>

- 20 µL Template (=*Not* I + *Nde* I-Digest)
- 20 µL Backbone
- 12 µL T4 Ligase buffer
- 4 µL T4 Ligase
- 64 µL H_2O

The ligation reaction was purified and concentrated with the illustra GFX PCR DNA and Gel Band Purification Kit using a modified protocol. 500 µL Capture Buffer Type 3 were added to 120 µL enzyme reaction, loaded onto an illustra GFX Microspin column and incubated for 1 min at room temperature. The column was centrifuged for 30 s at 16000 rcf, the flow-through discarded and 500 µL Wash Buffer Type 1 added. After another 30 s of centrifugation 25 µL HQ-H_2O were pipetted onto the column, incubated for 1 min at room temperature and centrifuged for 1 min.

4 µL of the eluted plasmid were transformed into chemically competent *E. coli* cells (see 2.5.1.5) and streaked out on 200 mL LB low salt agar plates supplemented with Zeocin. Also a dilution series of the cells from 1:10 to 1:1000 created in SOC-Medium and streaked out. All plates were incubated upside down at 37 °C overnight. After the amount of colonies was determined with the dilution series they were washed off from the 200 mL plate with liquid LB medium, the slurry collected, transferred into sterile glass tubes and incubated in the shaker for 1 hour at 37 °C. From this liquid culture a plasmid preparation was done (see 2.5.1.3) and a cryo stock culture created.

The purified plasmid was transformed into cells of the electro-competent *P. pastoris* strain X33 via electroporation (see 2.5.1.6) and streaked out onto 200 mL plates containing YPD-agar with Zeocin as selection marker.

2.6. Screening

The screening process for variants *Ba*Lac variants carrying beneficial mutations was divided into two main steps: the pre-screening and the differential screening.

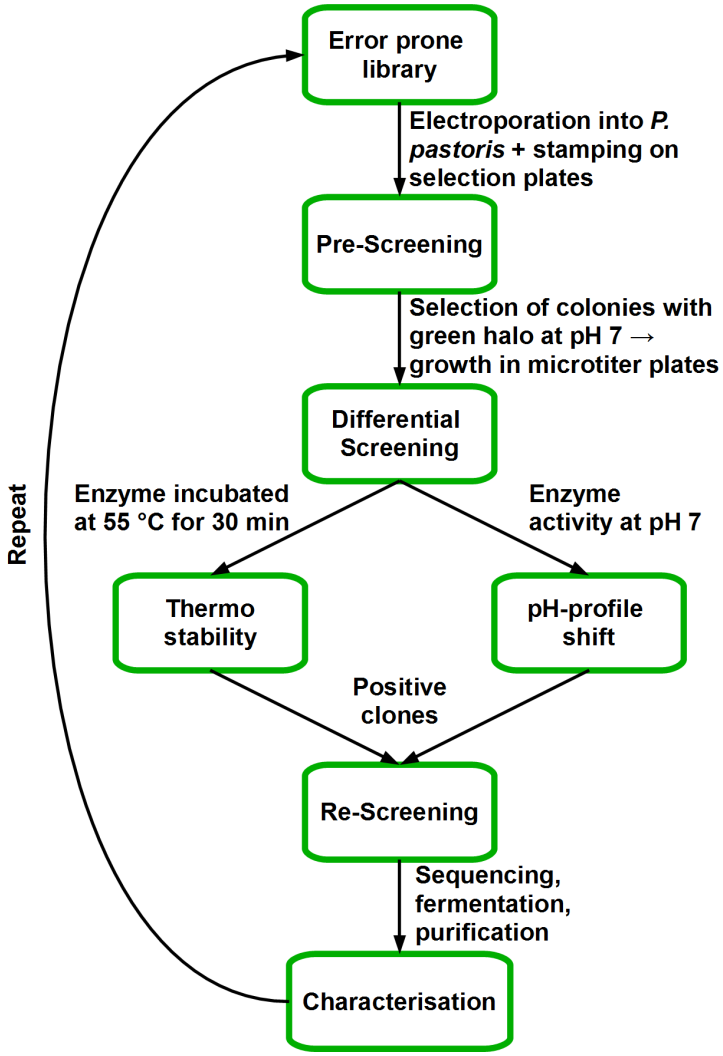

Figure 8: Flow diagram of important steps in the screening process

2.6.1. Pre-Screening

The incubated 200 mL YPD Zeo agar plates, which resulted from the electroporation of plasmid from either the site-saturation mutagenesis or directed evolution library, were stamped onto BMG-agar plates (see p. 14) with different pH-values. The plate was pressed onto a sterile cloth that was draped over a flat sterile surface. Afterwards the indicator agar plates were pressed onto the inoculated stamp and the plates were incubated for 48 hours at 30 °C.

The positive control for this experiment were BMG indicator agar plates with pH 5. The plates with pH-values of either 6 or 7 were used to identify positive variants. After incubation active clones showed a distinct green halo and they were gathered on fresh YPD agar plates containing Zeocin using sterile tooth picks. The plates were incubated at 30 °C for 48 h. The resulting colonies were further screened with the differential screening.

In the directed evolution experiment the pre-screening protocol was altered to only test activity at pH 5 due to the high mutational load.

2.6.2. Differential Screening

The differential screening was carried out in a 96-well microtiter plate format. The colonies which have been selected beforehand in the pre-screening step were inoculated in sterile 96-well microtiter plates, each well filled with 200 µL BYPD medium at a pH of 5.0. Five colonies expressing the corresponding parent laccase (BAGANSN or T383I) were inoculated on reserved positions as control for each plate. The plates were then sealed with a Breathe-Easy sealing membrane (Sigma-Aldrich) to prevent evaporation losses and incubated for 48 hours at 30 °C, 90% air humidity while shaking at 320 rpm.

After incubation the plates were centrifuged for 10 min at 3000 rpm with the 5804 swing-bucket centrifuge (Eppendorf). 20 µL of the supernatant were transferred to clean microtiter plates with the use of the JANUS automated workstation (PerkinElmer).

Immediately after the pipetting robot was finished the plates were taken out and 100 µL of a 200 mM citrate or phosphate buffer were added to the supernatant followed by 80 µL of a freshly prepared aqueous 2.5 mM ABTS or 2,6-DMP solution, resulting in final concentrations of 100 mM buffer and 1 mM ABTS or 2,6-DMP. Subsequently the plate was measured in the EnSpire plate reader to determine the enzymatic activity over time.

In this experiment two different properties were screened for at different conditions:

- Activity at increased pH: The enzyme was exposed to buffer with different pH-values. The enzymatic activity was initially measured at pH 7.0. This value was lowered to 6.5 since no activity could be detected at neutral conditions. Both ABTS and DMP were used as substrate.
- Thermostability: The microtiter plate containing the enzyme was incubated together with 100 µL of 200 mM citrate buffer pH 5.0 at 55 °C for 30 min in a water bath. Only ABTS was used as substrate for this assay.

The activity at pH 5.0 served as positive control and baseline for catalytic activity.

In data evaluation the relative activities of both testing conditions were determined with the following formula:

$$\frac{Activity\ at\ testing\ condition}{Activity\ at\ \ pH\ 5} = Relative\ activity$$

The resulting values were then compared to the mean of the relative activities of the wild type, which was measured on the same plate. Colonies with an activity higher than the threefold standard deviation of the parent enzyme were considered for re-screening. Furthermore the R^2 values of each kinetic slope were taken into consideration to filter out false positive results. If the R^2 was below 0.7 the result was immediately disregarded and if it was higher the shape of the slope was analysed visually to determine whether a discernible slope can be seen.

2.6.3. Re-Screening and analysis of positive clones

Clones which showed improved activity compared to the wild type during the differential screening were chosen for a re-screening. The colonies were inoculated in duplicates on a fresh YPD agar plate supplemented with Zeocin and incubated for 48 hours at 30°C. These were then tested again at the conditions at which they showed increased activity. The measurement was done in microtiter plates using the same protocol as in the differential screening. If the results are comparable to the previous ones this colony was streaked out onto an YPD-agar plate supplemented with Zeocin and incubated for 48 hours at 30°C. From these colonies a colony-PCR was done to isolate and amplify the gene. The mutations were then analysed via sequencing from at least two directions.

2.7. Enzyme characterisation

2.7.1. Measurement of activity

The activity of the purified enzyme on both ABTS and 2,6-DMP was measured and related to the protein concentration in order to obtain the specific activity in U mg^{-1}. Three 10x dilutions from 1:100 to 1:10000 were measured in duplicates using the standard assay for the corresponding substrate to get a reliable result in the optimal activity range.

2.7.2. Measurement of pH-profile

The activity in dependence of the pH of laccase variants was measured using a 100 mM McIlvaine buffer system in the range of 2.5 to 8.0 in increments of 0.5, if not stated otherwise. The assays were done with either the EnSpire plate reader for the BaLac variant T383I or with the PerkinElmer Lambda 35 UV-VIS-photometer for D236E using both ABTS and 2,6-DMP as substrate. Measurements were carried out in triplicates.

2.7.2.1. pH-profile measurement of purified laccase

The purified enzyme was diluted with 100 mM pH 5.0 citrate buffer to a concentration where the kinetic slope reached a value between 0.5 and 1. This dilution together with the wild type laccase was measured in triplicates at different pH values.

For the characterisation of the thermostable variant T383I as well as the wild type a dilution of 1:5000 was used for both substrates.

For the characterisation of the variant D236E a 1:500 dilution was used, while the pH-profile of the wild type was measured with a 1:1000 dilution.

2.7.2.2. pH-profile measurement of laccase in culture supernatant

Variants from the library with improved activity after the differential screening were grown in 3 mL BYPD liquid medium with a pH of 5.0 for 2-3 days at 180 rpm and 30 °C. The grown cultures were centrifuged at 14000 rpm (18407 rcf) for 10 min and the supernatant was transferred to a fresh tube. 20 µL from the supernatant were transferred to a microtiter plate. After adding 180 µL of a 100 mM buffer with the corresponding pH value the activity was measured using the plate reader.

For the analysis of the variants D236E and A466S, the two cultures with the highest relative activity at pH 7 were chosen, inoculated and grown over night.

pH-profiles were determined for both ABTS and 2,6-DMP and subsequently compared to a profile of a purified wild type enzyme measured during the characterisation of variant T383I.

For the analysis of a variant from the error prone library the four highest active variants were chosen and incubated together with the T383I variant in three separate tubes each.

2.7.3. Kinetic properties

For the analysis of the catalytic efficiency of the *Ba*Lac variant as well as the wildtype the enzyme was diluted to 1:100 for 2,6-DMP and 1:500 for ABTS as substrate in a 100 mM McIlvaine buffer with pH 3.

Table 11: Dilutions and the corresponding concentrations used for the determination of K_M, V_{max} and k_{cat} of T383I and wild type

Sample	Substrate	Dilution	Concentration [µM]
T383I	ABTS	1:100	0.249
Wild type	ABTS	1:100	0.264
T383I	2,6-DMP	1:500	0.050
Wild type	2,6-DMP	1:500	0,053

The values for V_{max} , K_M and k_{cat} of laccase were determined by measuring the activity of a known constant enzyme amount of over varying substrate concentrations. In a preliminary step a wide range of substrate concentrations from 1000 µM to 1 µM was tested in logarithmic steps to find the optimal range.

Table 12: Substrate concentrations measured for the determination of K_M-values

Substrate	Concentration [µM]							
ABTS	100	70	40	20	10	5	2	1
DMP	100	70	40	20	10	5	2	1

For the concentrations ranging from 100 µM to 40 µM of the substrate ABTS assays were carried out with sequential mode, which allows the measurement of up to eight samples in one run by moving the cuvette holder.

For the substrate 2,6-DMP only the two highest concentrations 100 µM and 70 µM were measured in one run.

The values for both ABTS and 2,6-DMP were measured in duplicates using the single point measurement mode at the photometer for the lower concentrations to obtain a linear slope. In this mode only a single cuvette is measured continuously, which allows the detection of fast reactions.

All measurements were done at a pH of 3.0 using a 100 mM McIlvaine buffer. The results were analysed and fitted to the Michaelis-Menten equation with the program SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA).

2.7.4. Thermostability

The thermostability of laccase variants was characterised with two different experimental setups. In the first experiment the enzyme was exposed to a high temperature for different time periods to obtain an inactivation curve. In the second experiment the catalytic activity was measured after incubating the enzyme for a constant time of 10 min while varying the temperature.

2.7.4.1. Temperature stability over time

The laccase was diluted in 100 mM citrate buffer with a pH of 5 and a 100 µL aliquot of this dilution was pipetted into PCR tubes. The enzyme was incubated at 55 °C in the MyIQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories) for 0, 1, 5, 10, 15, 30, 45 and 60 min. After heat exposure for the chosen duration the sample tubes were taken out and stored on ice for at least 10 min to ensure all samples have the same temperature. Afterwards the activity was measured in triplicates with the EnSpire plate reader with ABTS as substrate using the standard assay conditions.

2.7.4.2. Stability over a temperature gradient

For both the variant T383I and the wild type a 1:2000 dilution of the enzyme in 100 mM citrate buffer pH 5 was aliquoted to 100 µL in PCR tubes. The tubes were incubated at different temperatures for 10 min and stored on ice for another 10 min to ensure that every sample has cooled down to the same temperature before measuring.

Table 13: Gradient of incubation temperatures used for thermostability experiment

Temperature (°C)	25	37	40	45	50	55	60	65
Incubation Method	RT	Incubator	Thermocycler					

For the temperature range from 40-65 °C the MyIQ Single Color Real-Time PCR Detection System was used. For the exposure to 37 °C the sample was put in an incubator and lastly one PCR tube was kept at room temperature.

From each tube 20 µL of enzyme were taken out and the activity was measured in the EnSpire plate reader using the standard protocol for ABTS (see 2.4). All conditions were measured in triplicates for each sample and in every experiment the corresponding parent enzyme served as positive control and comparison.

3. Results

3.1. Site saturated mutagenesis library

3.1.1. Library screening

A total of 58220 colonies were analysed during the site saturation mutagenesis experiment. All seven positions were analysed using the substrates ABTS and 2,6-DMP as substrate. With the latter substrate two positions, D236 and A466 showed a measurable increase in activity at pH 7.

At the position D236 five clones showed increased activity at pH 7 and from these three colonies underwent a colony PCR and their isolated genes were sequenced. All genes were found to contain a glutamic instead of an aspartic acid at this position.

Table 14: Activity of saturation variant D236 at pH 6.5 on 2,6-DMP in relation to pH 5; underlined variants were chosen for sequencing

Position	relative activity [%]	Position	relative activity [%]
B7	5.91	A2	2.77
D2	3.54	F12	2.49
E2	3.29	D12	2.47

At the position A466 a total of 24 clones showed increased activity at pH 7 and from these five colonies underwent a colony PCR and their isolated genes were sequenced. All genes were found to contain a serine instead of an alanine at this position.

Table 15: Activity of saturation variant A66 at pH 6.5 on 2,6-DMP in relation to pH 5; underlined variants were chosen for sequencing

Position	relative activity [%]	Position	relative activity [%]	Position	relative activity [%]
C10	5.46	A2_2	4.40	G6	3.11
A3	5.38	D1	4.22	B1_2	3.08
A1	5.19	B11_2	3.97	B4	3.00
A3_2	5.12	F1	3.94	B8_2	2.94
B9	5.08	F10	3.84	E1	2.90
E3	5.00	E6	3.60	A1_2	2.51
G9	4.98	D12	3.48	F9	2.05
B6	4.64	B3	3.42	B1	1.44

3.1.2. BaLac variant D236E

3.1.2.1. pH-profile of culture supernatant

The pH-profiles of two variants found during the differential screening of the site saturation library were measured from the supernatant of a *P. pastoris* culture on the substrates ABTS and 2,6-DMP. This should verify whether the activity at neutral conditions is increased.

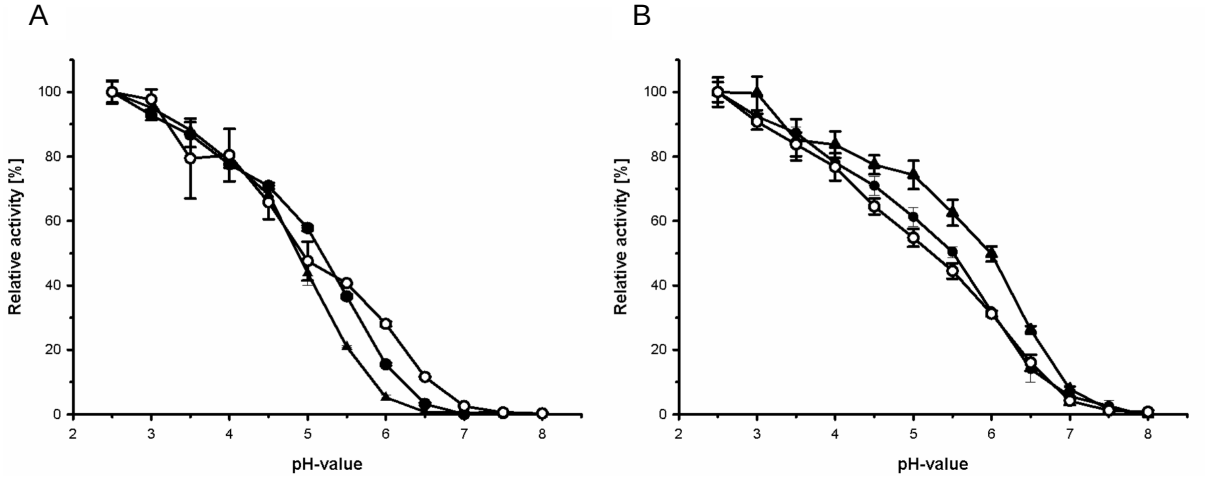

Figure 9: pH profile of two *Ba*Lac variants with mutation D236E: Culture supernatant of (●) D236E_D2 and (▲)D236E_B7) compared to the purified wild type (○). (A): relative activity on ABTS from pH 2.5 to 8.0, (B): relative activity on 2,6-DMP from pH 2.5 to 8.0

Figure 9 (A) shows the relative activity of two laccase variants carrying the mutation D236E and the wild type BAGANSN over a pH-range from 2.5 to 8 on ABTS. The activity is decreasing monotonically until pH 7, where it reaches zero. At pH 6 the wild type is about 4 times more active.

Figure 9 (B) shows the pH-profile of the two aforementioned D236E variants which was measured from the culture supernatant with 2,6-DMP as substrate. As a comparison the profile of a purified wild type enzyme is again shown. Both graphs have 100% of their activity at pH 2.5 from where it decreases monotonically until it reaches zero at pH 7.5. Subsequently this variant was chosen for further characterisation.

3.1.2.2. pH-profile of purified enzyme

For the verification of the shift in the pH-profile of the variant D236E observed in Figure 9 and to obtain exact results, the pH-profile on ABTS of the purified *Ba*Lac variant D236E_B7 was compared to the wild type laccase BAGANSN.

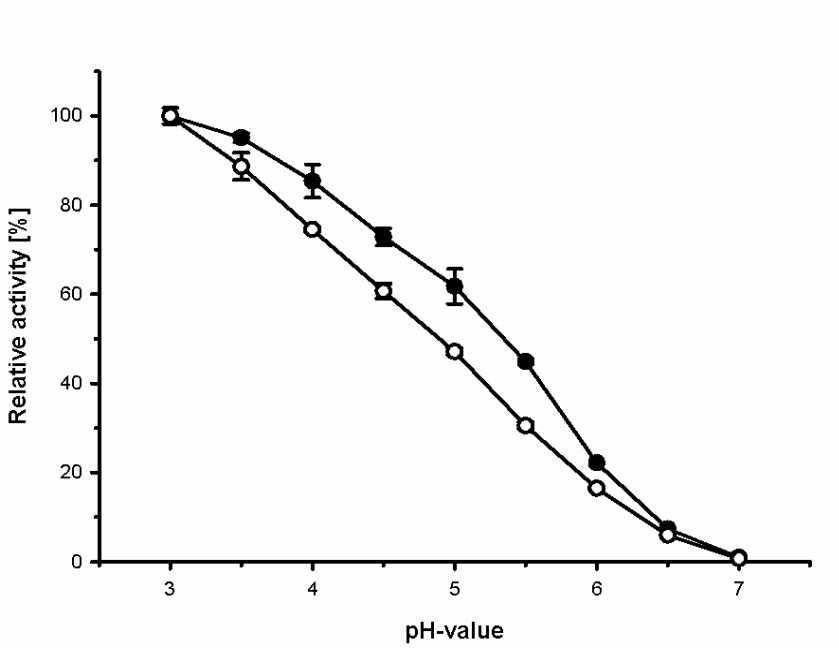

Figure 10: pH-profile of *Ba*Lac variant D236E on ABTS: relative activity from pH 3 to 7 of purified mutant D236E (●) compared to the wild type BAGANSN (○)

Figure 10 shows the pH profile of the purified D236E variant on the substrate ABTS compared to the wild type BAGANSN. Both graphs have their respective maximum at pH 3 from where the relative activity is decreasing over the whole pH-range. At pH 5 the variant retains a relative activity of 61.77% and the wild type has an activity 47.14%. At pH 6 the profiles are beginning to converge and at pH 6.5 both have similar activity which reaches near zero at pH 7.

In addition to the pH-profile of the purified enzyme on ABTS another one was measured on 2,6-DMP within the same pH-range using the plate reader assay.

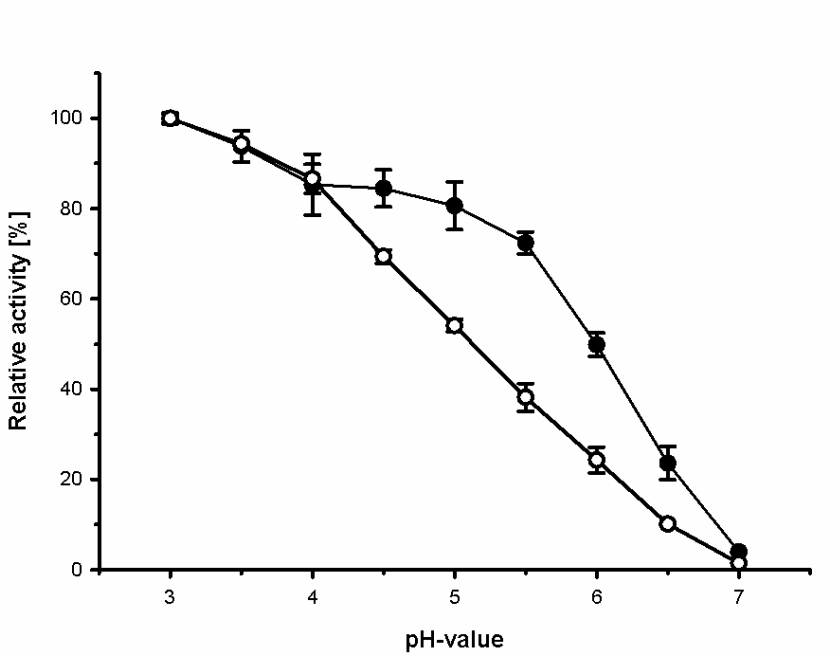

Figure 11: pH-profile of *Ba*Lac variant D236E on 2,6-DMP: relative activity from pH 3 to 7 of purified mutant D236E (●) compared to the wild type BAGANSN (○)

Figure 11 shows the pH profile of the *Ba*Lac variant D236E on the phenolic substrate 2,6-DMP in comparison with the wild type (BAGANSN). The relative activity of the laccase variants is plotted against a pH ranging from 3 to 7. From pH 4 to 5.5 the slope of the variant decreases by 12.92% from 85.32% to 72.40%. The wild type BAGANSN drops from 86.60% to 38.19% resulting in an approximately two-fold reduction in activity.

3.1.2.3. Kinetic properties

The analysis of the kinetic properties is necessary to determine, which influence this mutation has on the catalytic properties of this enzyme.

Table 16: K_M and k_{cat} values of D236E compared to the wild type (Data provided by Regina Paukner)

Sample	Substrate	Method	pH	K_M (µM)	k_{cat} (s^{-1})
Wild type	ABTS	Plate reader	4	2.9±0,6	50.8
Wild type	2.6-DMP	Plate reader	4	3.7±0,9	30
D236E	ABTS	Plate reader	4	5.7±0,4	10.3
D236E	2.6-DMP	Plate reader	4	2.2±0,3	1.9

The K_M-value for the substrate ABTS is in comparison to the wild type about two-fold higher in the variant D236E. The k_{cat} is with 10.3 s^{-1} compared to 50.8 s^{-1} five-fold lower in the wild type. For the substrate 2,6-DMP a K_M- value of 2.2 µM for D236E and a K_M of 2.9 µM for the wild type. The k_{cat} of D236E for 2,6-DMP has a value of 1.9 s^{-1} and is about 15-fold lower than the wild type.

3.1.2.4. Thermostability

Figure 12 shows the relative activity of the D236E laccase variant and the wild type as positive control measured after 10 min incubation at different temperatures. D236E reaches 50 % relative activity (T_{50}) at 50.62 °C while the wild type reaches this point at a temperature of 51.36 °C. Above 37 °C the relative activity steadily declines and above 60 °C no activity could be detected reliably indicating a total inactivation of the enzyme.

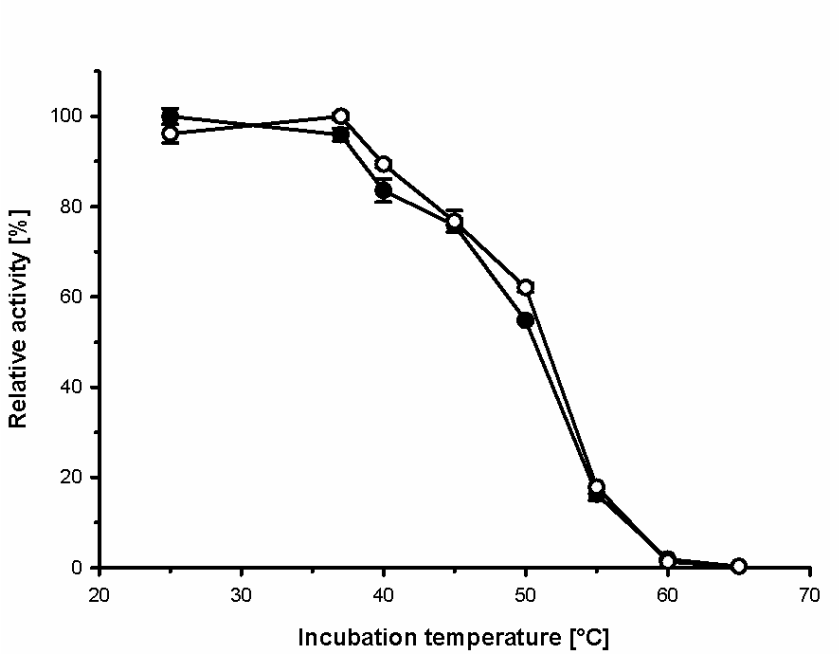

Figure 12: Thermostability over temperature of *Ba*Lac variant D236E: Relative activity of the purified variant D236E (●) compared to wild type (○) after 10 min incubation at 25-65 °C

In figure 13 the relative activity of the enzymes are plotted against the incubation time. Both graphs feature the same general shape. Using linear interpolation the t_{50} (= incubation time where relative activity reaches 50%) was calculated to be 2.26 min for the variant D236E and 3.42 min for the wild type.

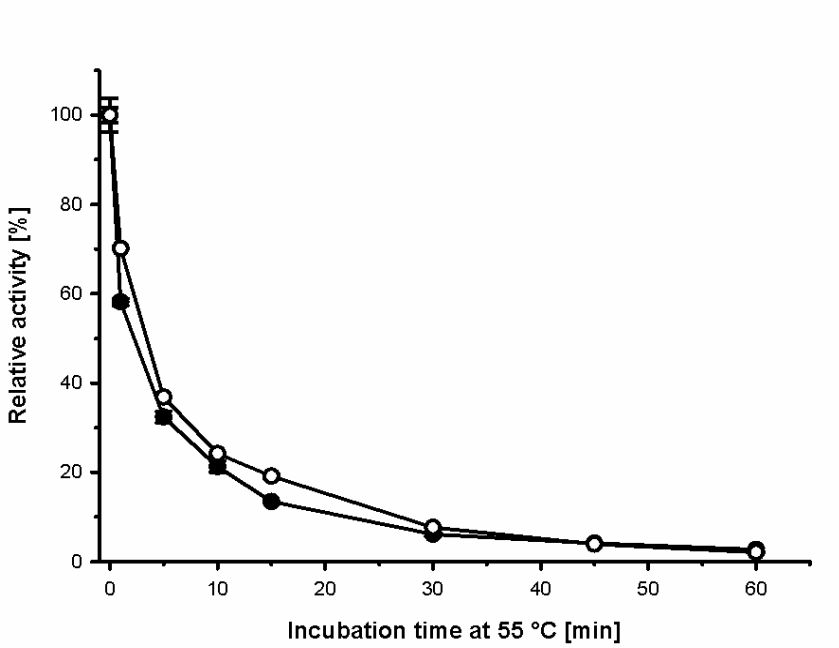

Figure 13: Thermostability over time of *Ba*Lac variant D236E: Relative activity of purified variant D236E (●) compared to wild type (○) after 0-60 min incubation at 55 °C

3.1.3. *Ba*Lac variant A466S

3.1.3.1. pH-profile from culture supernatant

Figure 14 (A) shows the pH profile of the culture supernatant of *Ba*Lac variant A466S and the corresponding purified wild type laccase. Both graphs exhibit the same behaviour over the whole range with only small differences. The relative activity of both A466S and the wild type has its maximum at pH 2.5 and reaches zero at pH 7.

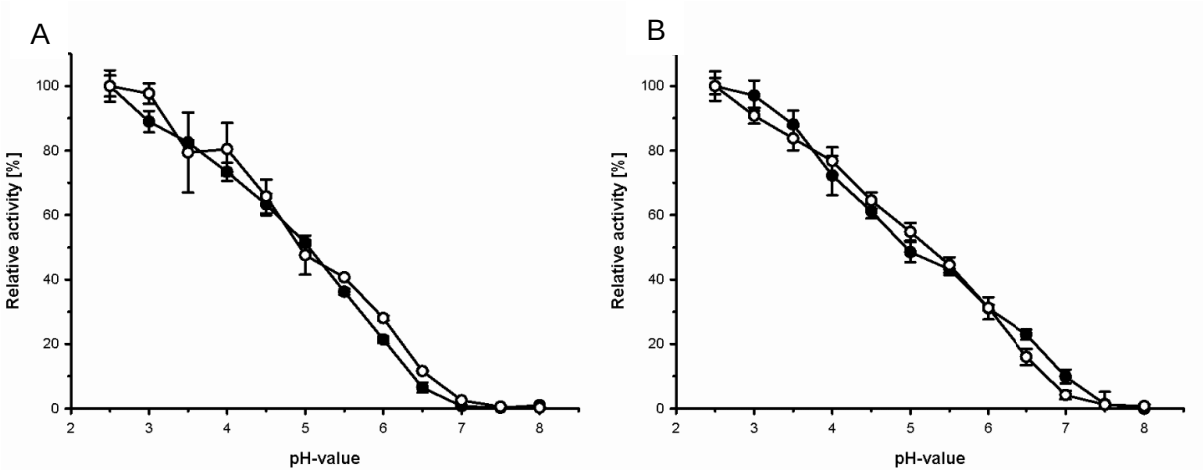

Figure 14: pH-profile of *Ba*Lac variant A466S culture supernatant: (●) A466S, (○) purified wild type; left: relative activity from pH 2.5 to 8.0 on ABTS, right: relative activity from pH 2.5 to 8.0 on 2,6-DMP

Figure 14 (B) shows the pH profile on 2,6-DMP of A466S culture supernatant together with the pH profile of the purified wild type enzyme. Both graphs decrease monotonically from pH 2.5 to 8. At pH 7.5 and 8 no activity could be detected in both cases.

3.2. Directed evolution library

3.2.1. Library screening

Prior to the creation of the library the DNA concentration of the template was determined and the optical absorption at 260 nm (= A_{260}) was determined to be 2.82 which corresponds to a DNA concentration of 141.0 µg mL^{-1}.

For the error-prone library a PCR-reaction with 9 cycles and 1.13 µg template DNA were used. The library consisted of a total of 25850 variants, which were obtained via the *E.coli* transformation described in 2.5.1.5. From these variants 14.8 % showed activity at pH 5 during the pre-screening resulting in 3826 colonies. From these 1092 clones with the highest activity were chosen for further re-screening due to time constraints and limited throughput.

3.2.2. *BaL*ac variant A180D

This variant was obtained from a colony found in the error-prone library from where it was re-screened in duplicates. One clone (C1) showed a 2.4 – fold and the other one (G1) a 1.7 – fold increase in activity when compared to the parent enzyme *Ba*Lac T383I. These two clones were streaked out again on fresh plates and twelve colonies of each clone were tested again.

Table 17: Ratio of activity at pH 6.5 to pH 5.0 measured from the culture supernatant of a variant found during directed evolution compared to the ratio of T383I. The underlined values indicate colonies chosen for further characterisation

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.96	-	1.73	-	1.89	-	-	2.09	1,95	-	1,73	2.21
B	-	-	-	-	-	2.40	-	-	-	2.23	-	-

The four colonies with the highest activity were taken and incubated in BYPD-Medium with a pH of 5.0 to measure a pH profile from the supernatant.

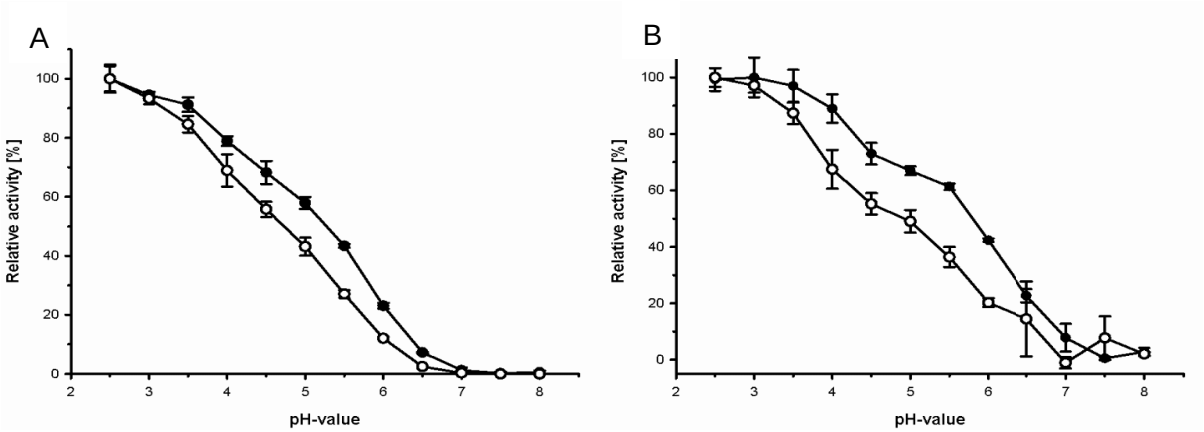

Figure 15: pH-profile of pH-variant B10_1 culture supernatant: (●) B10_1, (○) T383I; (A): relative activity from pH 2.5 to 8.0 on ABTS; (B): relative activity from pH 2.5 to 8.0 on 2,6-DMP

The plot in figure 15 (A) shows the pH profile on the substrate ABTS of a variant found in the screening of the directed evolution library compared to the parent enzyme. Figure 15 (B) shows the pH-profile on 2,6-DMP of the same cultures. The highest difference between the variant and the wild type for the substrate ABTS can be seen at pH 5.5 where the variant retained a relative activity of 43.38 % while the wild type has a relative activity of 27.06 %. For the substrate 2,6-DMP the variant has at pH 5.5 a relative activity of 61.31 % compared to 36.38 % observed with the *Ba*Lac variant T383I.

A colony PCR of this variant followed by sequencing showed a total of 5 mutations, with one being silent. The mutation A180D is near the T1 copper while three others are either located at the beginning or the end of the sequence.

3.3. Characterisation of *Ba*Lac variant T383I

The enzyme has been produced in *P. pastoris* cells and purified via two chromatography steps by Regina Paukner.

3.3.1. Protein concentration and activity

Table 18: Protein concentration of T383I and wild type determined with Bradford assay and the absorption at 280 nm

Sample	Method	
	Bradford assay	A_{280}
T383I	7.16 mg mL^{-1}	15.29 mg mL^{-1}
Wild type	9.24 mg mL^{-1}	16.24 mg mL^{-1}

The enzyme concentration of the variant T383I is for both measuring methods lower than the wild type. The optical measurement resulted in an approximately two – fold higher enzyme concentration compared to the measurement with the Bradford-method and was used as basis to calculate the concentration.

Table 19: Concentration of *Ba*Lac variant T383I and the wild type

Sample	Concentration
T383I	248.76 µM
Wild type	264.21 µM

Table 20: Catalytic activity in relation to enzyme concentration of T383I and wild type on ABTS and 2,6-DMP

Sample	Specific activity	
Substrate	ABTS	2,6-DMP
T383I	41.82 U/mg	4.70 U/mg
Wild type	26.74 U/mg	3.03 U/mg

The specific activity of the wild type laccase is consistently lower than the variant. The relative activity with 2,6-DMP is for both enzymes approximately 10 times lower than the activity measured with ABTS.

3.3.2. pH-profile of purified enzyme

Figure 16 indicates that both the *Ba*Lac variant T383I and the wild type show the same characteristic steadily decreasing behaviour from pH 2.5 until pH 7.5 observed in this type of laccase for the substrate ABTS. At 8.0 no activity could be measured reliably.

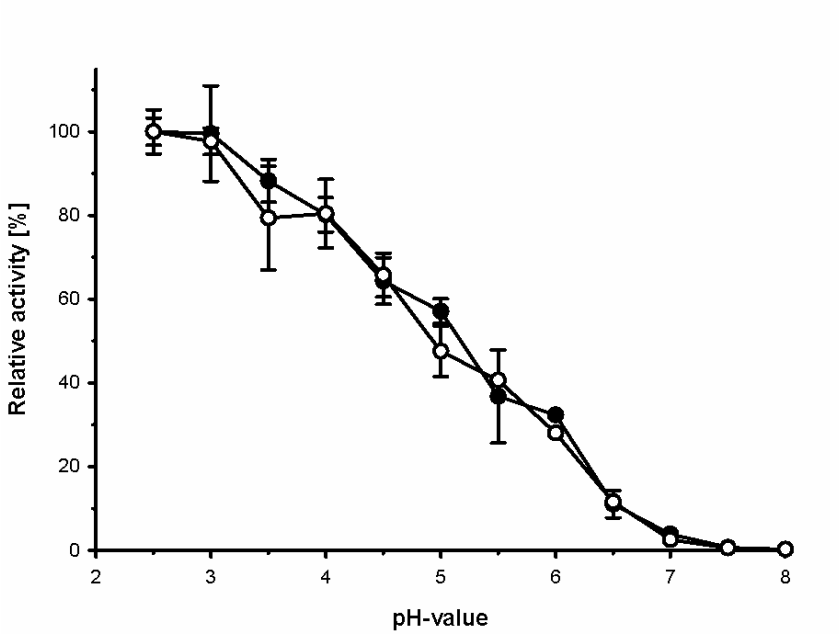

Figure 16: pH-profile of *Ba*Lac variant T383I on ABTS: Relative activity measured from pH 2.5 to 8 of purified mutant T383I (●) compared to wild type (○)

Figure 17 shows the pH-profile on the phenolic substrate 2,6-DMP of the variant T383I compared to the wild type enzyme it was derived from. Both graphs exhibit the same monophasic profile over the whole pH-range.

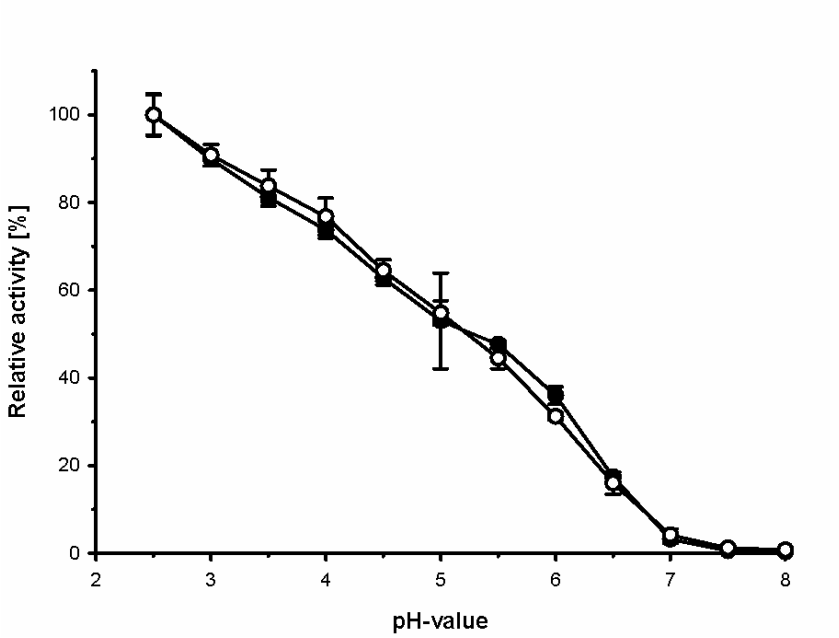

Figure 17: pH-profile of *Ba*Lac variant T383I on 2,6-DMP: Relative activity measured from pH 2.5 to 8 of purified mutant T383I (●) compared to wild type (○)

3.3.3. Kinetic properties

The basic kinetic values K_M, V_{max} and k_{cat} were measured from the variant T383I and compared to the wild type BAGANSN. The kinetic properties were determined by measuring the activity of a known amount of enzyme over varying concentrations of either ABTS or 2,6-DMP.

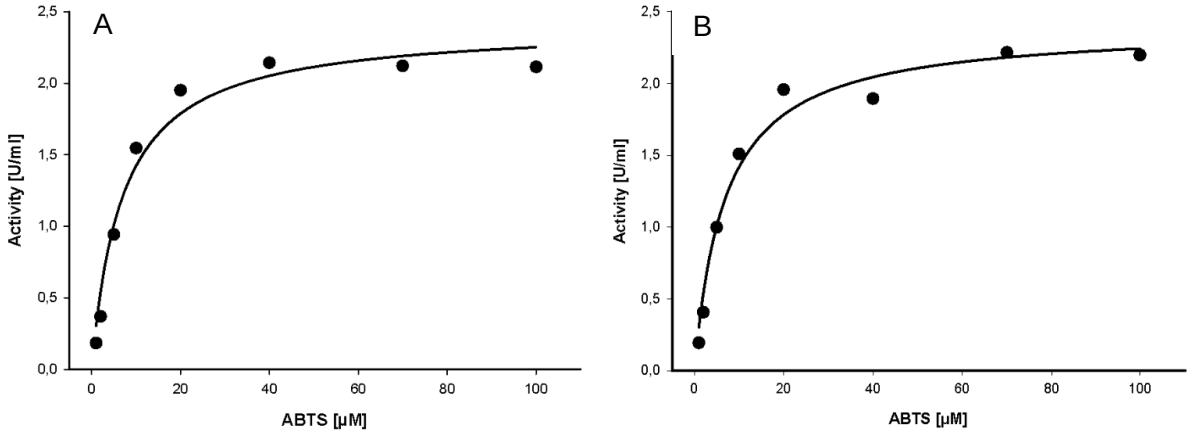

Figure 18: K_M determination of T383I with ABTS as substrate: The data points were obtained by measuring catalytic activity at substrate concentrations from 1-100 µM and are fitted using the Michaelis-Menten equation; (A): *Ba*Lac variant T383I, (B): wild type

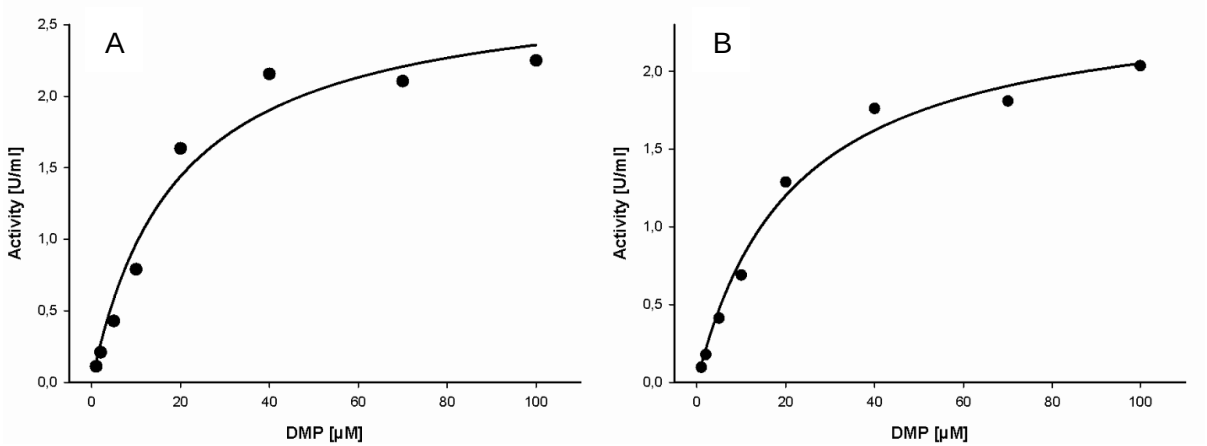

Figure 19: K_M determination of T383I with 2,6-DMP as substrate: The data points were obtained by measuring catalytic activity at substrate concentrations from 1-100 µM and are fitted using the Michaelis-Menten equation; (A): *Ba*Lac variant T383I, (B): wild type

From the graphs in figure 18 and 19 the values for K_M and V_{max} were calculated with the software SigmaPlot 11.0. From the known enzyme concentration and the obtained V_{max} the turnover number k_{cat} was calculated for both substrates.

As shown in table 22 the V_{max} of T383I and the wild type for the substrate ABTS are with 2.40 U mL^{-1} and 2.41 U mL^{-1} comparable while the KM values on 2,6-DMP differs by 0.32 U mL^{-1}. The k_{cat} value of T383I is 5.03 s^{-1} higher than the wild type for ABTS and 3.12 s^{-1} for 2,6-DMP.

Table 21: Summary of K_M, V_{max} and k_{cat} values of wild type and T383I

	ABTS			2,6-DMP		
	K_M [µM]	V_{max} [U/mL]	k_{cat} [s^{-1}]	K_M [µM]	V_{max} [U/mL]	k_{cat} [s^{-1}]
Wild type	6.91 ± 1.17	2.40 ± 0.10	75.70	21.51 ± 3.51	2.49 ± 0.14	15.71
T383I	6.94 ± 1.34	2.41 ± 0.12	80.73	18.97 ± 4.94	2.81 ± 0.24	18.83

3.3.4. Thermostability

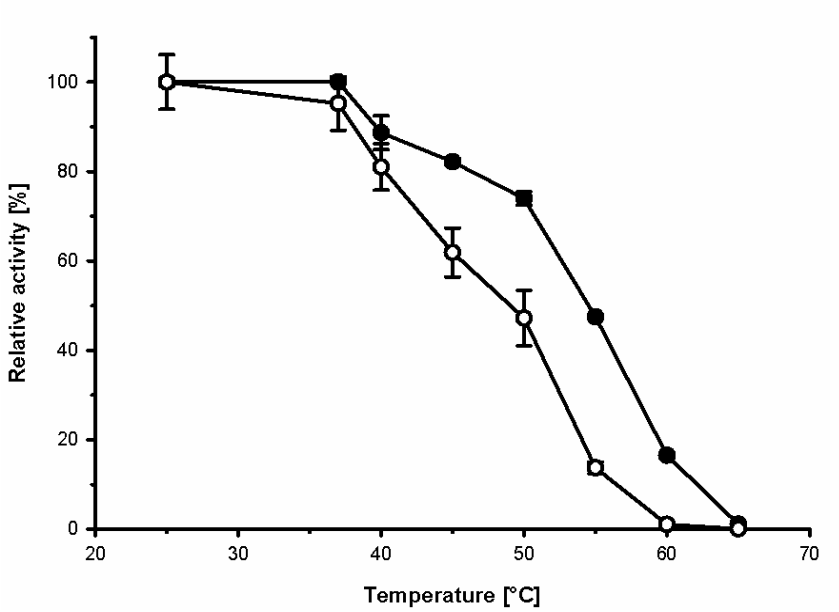

Figure 20: Thermostability of T383I over temperature: relative activity of the purified mutant T383I (●) compared to wild type (○) after 10 min incubation at 25-65 °C

Figure 20 shows the relative activity of the laccase variant T383I in comparison to the wild type enzyme after 10 min exposure to temperatures ranging from 25 to 65 °C. The T_{50} values, which are defined as the temperature where relative activity reaches 50% after 10 min of incubation, were calculated by using linear interpolation. The variant T383I has a T_{50} of 54.43 °C while the wild type reaches this value at 49.05 °C. At an incubation temperature of 60 °C the variant retains 16.55% activity while the wild type has a remaining activity of 1.32%.

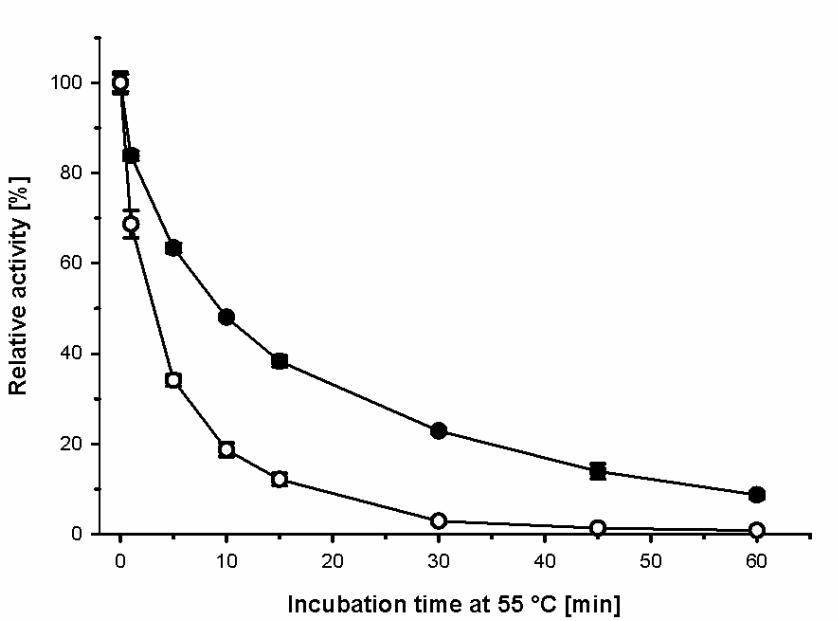

Figure 21: Thermostability of T383I over time: Relative activity of purified mutant T383I (●) compared to wild type (○) after 0-60 min incubation at 55 °C

Both graphs in figure 21 feature the same exponentially decreasing shape that is characteristic for heat inactivation of enzymes. From these results a t_{50} value, which is defined as the time where the activity reaches 50% after incubation at 55 °C, was calculated via linear interpolation. The variant T383I reaches this point after 9.36 min while the wild type has a nearly threefold lower t_{50} of 3.16 min. The variant T383I has a relative activity of 8.68% after 60 min of heat inactivation and no activity could be detected with the control at this point.

4. Discussion

In the course of this work two interesting mutations have been found with the rational site saturation approach. One of these, the variant D236E, has been characterised and proven to possess a shifted pH-profile on 2,6-Dimethoxyphenol. The other variant, A466S, only showed improved activity during the differential screening, which could not be verified in further analysis.

In order to create the error-prone library for the directed evolution the thermostable variant T383I has been characterised and successfully established as parent enzyme for further error-prone libraries.

The random directed evolution method yielded the promising variant A180D, which shows increased activity at higher pH-values for the substrates ABTS and 2,6-DMP it was tested for.

4.1. Site saturation experiment

The site saturation experiment yielded two interesting mutations, with increased activity at pH 7. At position 236, which has been derived from the directed evolution process of the blood tolerant Chu-B variant, a change from aspartate to glutamate could be observed.

The second mutation was found on position 466 and was initially derived from the thermostable variant OB-1, which is the parent enzyme of Chu-B. Here a change from the non-polar amino acid alanine to the polar serine could be observed.

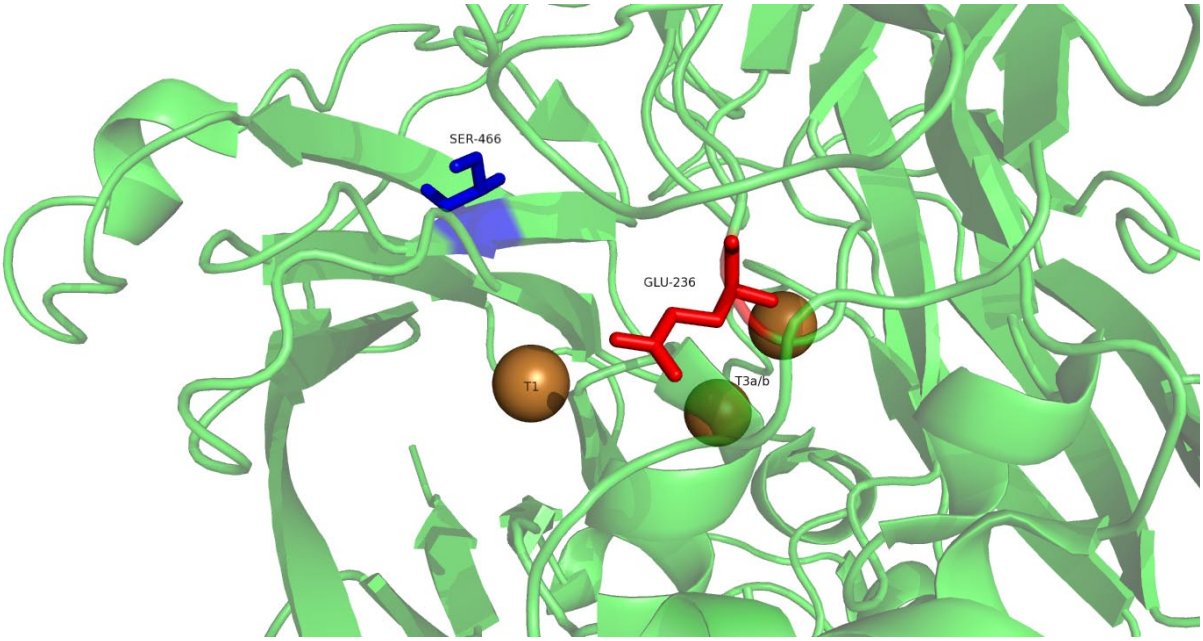

Figure 22: Mutations discovered in the site saturated mutagenesis experiment located in vicinity to the T1-copper red: D236E, blue: A466S

At first glance, the variant D236E (see Figure 10 & Figure 11) exhibits the same characteristic monophasic curve for both ABTS and 2,6-DMP normally observed in *B. aclada* laccase. A closer analysis showed a significant shift towards more alkaline conditions in the pH-profile of the variant with the phenolic substrate 2,6-DMP. For ABTS however only a slight increase in relative activity could be detected.

A possible reason for this substrate-dependent behaviour may be that this position is found in the substrate-binding pocket. In this structure it makes up the only exposed hydrophilic residue therefore making it an important part of the substrate binding mechanism. Another study done with a similar laccase from *Melanocarpus albomyces,* another ascomycete, showed a similar behaviour after mutating this position [Kallio et al. 2009]. Although in this case the mutation introduced was a change from glutamate to aspartate.

In contrast to the results which have been reported in the aforementioned publication the K_M-value for ABTS was increased nearly twofold, whereas it remains unchanged in *M. albomyces*. Furthermore the k_{cat} of this variant is decreased compared to the wild type which together with the change in the K_M suggests a decreased efficiency in catalysing the oxidation of ABTS. On 2,6-DMP the K_M as well as the k_{cat} is lowered in comparison to the wild type.

The proximity of this mutation to the active centre of the T1 copper centre might negatively influence the reactivity and stability of the laccase. However, experiments (see 3.1.2.5) show that at least the thermostability is only affected to a small degree. As shown in figure 12 and 13 both the variant D236E and the control enzyme BAGANSN have moderate thermostability, with the variant being only slightly less stable. This result indicates that the structural stability of the enzyme is largely conserved.

The variant A484S could not be further characterised due to the non-conclusive pH-profiles measured in the supernatant and the loss of activity during small-scale fermentation. However the results of the preceding differential screening with 2,6-DMP as substrate showed a consistently high relative activity when compared to the wild type enzyme. It is interesting to note that this position was initially chosen for site saturation because it increased the thermostability of a basidiomycetous laccase. However there seems to be no major influence on the stability of *Ba*Lac, although further characterisation of the purified enzyme would be necessary to verify this claim. Furthermore this mutation could be interesting since alanine is considered to fulfil a neutral role with low impact on enzymatic activity whereas serine contains an additional OH-group.

The other positions tested, especially those actually used in the blood tolerant variant Chu-B showed no improvement in activity at neutral conditions. This can probably be attributed to the fact that these organisms belong to different phyla. One laccase was derived from the basidiomycete PM1 and the other from the ascomycete *B. aclada*. It is well known that these two types of fungi express laccases with different structural features, which limits the comparability between these enzymes regarding structure-function relationships.

The mutation with the highest increase in activity in the variant Chu-B was F396I. The site saturation mutagenesis of the corresponding position I446 yielded no positive result in *Ba*Lac. However it is interesting to note that for this enzyme the amino acid isoleucine is already present.

4.2. Directed evolution experiment

In the random directed evolution approach one promising variant was found after extensive screening of the error prone library. After sequencing the gene of this laccase five mutations were found. From these the mutation A180D seems to be the most likely candidate for the visible shift towards higher relative activity in the pH-profile on ABTS and 2,6-DMP as seen in Figure 15.

The amino acid A180 is 13.1 Å away from the T1 copper and is located in an alpha helix which is relatively close to the active centre. The other mutations however are either at terminal parts of the peptide chain and are therefore far away from the catalytic copper atoms or silent.

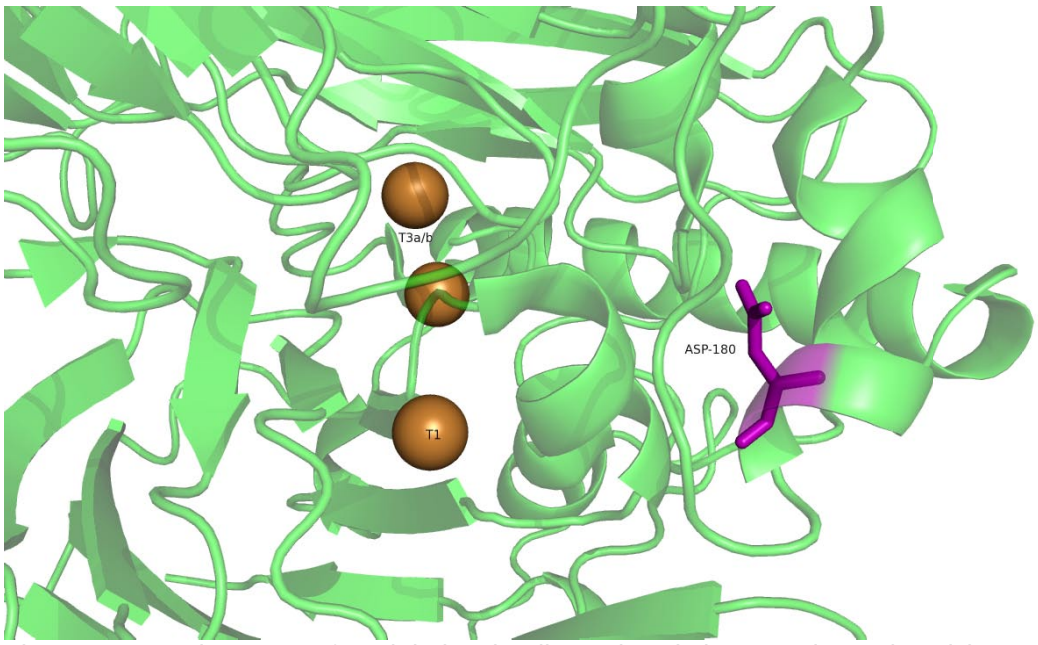

Figure 23: Mutation A180D found during the directed evolution experiment (purple)

The change from the uncharged alanine to the charged aspartate could have a significant impact on the structure and properties of the enzyme, which may influence the geometrical parameters of the active site. The T1-copper is responsible for deducing electrons from the different substrates and transferring them to the trinuclear cluster TNC. If the charge distribution in the vicinity around this metal ion is changed the redox potential and the interaction between the copper and these substrates for oxidation might be significantly affected.

However it remains largely unknown which effect this mutation causes on the properties of the enzyme besides the pH-profile. Further characterisation especially regarding thermostability and determination of the redox potential would be necessary to gain better insight.

4.3. Characterisation of T383I

The thermostable variant T383I was identified during the screening of a previous error prone library of this *B. aclada* laccase. In this work it was further characterised and proven to possess significantly increased thermostability as shown in figure 20 and 21. The experiment testing the stability over a temperature gradient showed that T_{50} is 5.38°C higher. The second experiment, where the thermostability over time was tested showed that the t_{50} of the variant is approximately threefold and that furthermore the enzyme retains activity after incubating it for 60 min at 55 °C.

Regarding the kinetic properties no significant changes in comparison to the wild type laccase it was initially based on can be seen (see table 22). The K_M and v_{max} values for ABTS are basically identical indicating no influence of the mutation on the oxidation of this substrate. The k_{cat} is slightly higher however the difference is not large enough to conclude that the mutation influences the turnover number. For the substrate 2,6-DMP the measured K_M and v_{max} values were also within a comparable range especially after taking the standard deviation into consideration. As with ABTS the k_{cat} value of the variant T383I is slightly higher.

The increased thermostability of *Ba*Lac variant T383I makes it an ideal parent enzyme for a directed evolution library. Furthermore it proves that it is possible to increase stability of this laccase without sacrificing its catalytic efficiency, which is a necessary prerequisite for its use in practical applications such as biofuel cells.

4.4. Conclusion and Outlook

During the site-saturation experiment it could be shown that it is possible to shift the pH-profile in a substrate dependent manner. However the catalytic properties of the enzyme are also influenced. Further characterisation of the *Ba*Lac variant D236E and a determination of its redox potential would be necessary to explain these effects.

The successful discovery of beneficial mutations proves that the directed evolution approach is viable. It has been shown that it is possible to use this random approach to generate variants with modified characteristics such as increased thermostability and a shifted pH-profile. A new directed evolution library based on the thermostable variant T383I was successfully created and screened. However it might be necessary to adapt the screening protocol regarding the testing conditions for increased thermostability.

Nevertheless there is still a lot of research to be done to better understand the special properties of this laccase from *B. aclada* and elucidate the different factors influencing the catalytic activity of high redox potential laccase in general.

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8. List of abbreviations

2,6-DMP	2,6-dimethoxyphenol
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BaLac	Laccase from *Botrytis aclada*
dNTP	Desoxynucleotidtriphosphate
EPR	Electron paramagnetic resonance
HRPL	High redox potential laccase
I_{50}	Inhibitor concentration where the relative activity reaches 50 %
KPP	Potassium phosphate buffer
LRPL	Low redox potential laccase
MCO	Multicopper-oxidase
PCR	Polymerase chain reaction
SOC	Super optimal broth with catabolite repression
TNC	Trinuclear cluster
T_{50}	Temperature where relative activity reaches 50 % after 10 min incubation
t_{50}	Time when relative activity reaches 50 % after incubation at 55 °C