Production, purification and characterisation of *Lamprospora wrightii* and *Botrytis aclada* laccase

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Contents

Abstract

Extracellular laccases from the two fungal strains *Lamprospora wrightii* and *Botrytis aclada* were cultivated, purified and characterised. Additionally, a recombinant *B. aclada* laccase expressed in *Pichia pastoris* was examined. A tomato juice media containing fructose, soy peptone, copper sulphate and trace elements is appropriate for sufficient laccase production. Additionally, a high oxygen saturation is necessary for suitable growth. *L. wrightii* showed highest laccase concentrations in shaking flasks, whereas *B. aclada* showed best productivity during fermentation. Purification had to be done under various conditions for all laccases examined, indicating different structural properties. *L. wrightii* laccase was purified with a weak anion exchange chromatography, followed by hydrophobic interaction chromatography (HIC) and an ultrafiltration step, achieving a 26-fold increase in purity with a specific activity of 150 U/mg. *B. aclada* laccase from the wildtype strain was purified with a strong anion exchange chromatography, followed by HIC and an ultrafiltration step, achieving a 28-fold increase in purity with a specific activity of 120 U/mg. Problems in the reproducibility of *B. aclada* purification emerged from the excessive production of an extracellular polysaccharide during cultivation. The recombinant *B. aclada* laccase was purified with HIC followed by an ultrafiltration step, achieving a 4-fold increase in purity with a specific activity of 61 U/mg. Isoelectric focusing showed different pI values of 4.0 for *L. wrightii* and 5.2 for *B. aclada* laccase. No isoenzymes were detected in cultures of both strains. The molecular weight of all laccases examined by SDS-PAGE was found to be around 75 kDa. All laccases have typical pH optima of 3.0 and 6.0 for ABTS and syringaldazine, respectively, with low temperature optima of 35-45°C. The activation energy of 35 kJ/K mol for the *L. wrightii* laccase is two-fold higher than that of the *B. aclada* laccases. All enzymes show a sudden drop of the reaction rate in the Arrhenius plot, indicating enzyme inactivation at 40°C and 45°C for the laccases from *L. wrightii* and *B. aclada* wildtype. The recombinant enzyme is more heatresistant and inactivated at a temperature of 60°C. For all inhibitors and substrates tested, both *B. aclada* laccases show preferable kinetic constants to *L. wrightii* laccase. In particular, the recombinant enzyme with the lowest K_m values of about 5 μM for ABTS and syringaldazine has to be mentioned.

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Da steh ich nun, ich armer Tor!
Und bin so klug als wie zuvor.

Johann Wolfgang Goethe, Faust

Part I

Introduction

Chapter 1

Structure and Reactions of Laccase

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) was first described from the exudates of *Rhus vernicifera*, the japanese lacquer tree [49]. Interest was focussed on this enzyme when it was associated with the wood-decaying process of white-rot fungi. Laccase was found in many ascomycetes and is typical for the wood-rotting basidiomycetes causing white-rot. Laccases belong to a group of polyphenol oxidases containing copper atoms in the catalytic centre and are usually called multicopper oxidases. They catalyse the oxidation of a typically phenolic compound by reducing oxygen to water. Laccases are typically found in plants and fungi [3]. Whereas in plants the enzyme plays an important role in lignin polymer formation [44, 31, 6, 37, 22], in fungi its involvement in different mechanisms is considered, like plant pathogenity, stress defence and lignin degradation [46, 24]. Also occurrence of laccase-like multicopper oxidases (MCO) in prokaryotes was described [2, 32, 11, 17]. In natural environments laccase activity varies with time and reflects the decomposition of organic matter and the annual presence of fungal mycelia. Along with the vertical gradient of fungal distribution in soil profiles, laccase activity decreases with depth [3]. During the composting process laccase seems to participate in both degradation of lignin and humic substances and humic acid formation [7, 28, 29]. Laccase plays an important role in the carbon cycle and its activity is enhanced by phenolic compounds, e.g. after forest burning, and by oxygen [4]. Furthermore, the depletion of phenolic compounds enhances humification of organic matter as phenolic substances can inhibit oxidative and hydrolytic enzymes that are involved in the degradation process [15]. Some low molecular weight compounds that can be oxidised by laccase to stable radicals can act as redox mediators, oxidising other compounds that in principle are not substrates of laccase. These mediators can also diffuse far away from the mycelium to sites that are inaccessible to the enzyme itself. Syringaldehyde, vanillin and coumaric acid were found to be effective mediators for the natural degradation of lignin [3, 27].

Laccase is typically a monomer with three β-barrel domains and four copper atoms inside the molecule. However, some fungal laccases show a homodimeric structure, with two identical subunits with a molecular

weight typical for monomeric laccases. Some ascomycetes also produce oligomeric laccases [3].

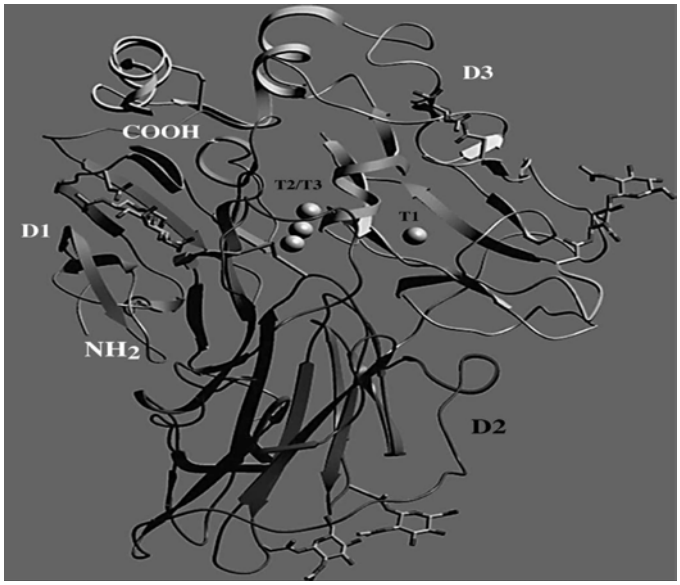

Figure 1.1: Crystal structure of *Trametes versicolor* laccase (figure taken from [41]).

Copper is a trace element which plays an important role in biological oxidation-reduction reactions and is also crucial for oxygen transport. There are three different types of copper atoms, which can be identified on their spectroscopic properties [10, 41]:

Type 1: This paramagnetic 'blue' copper is mononuclear and takes part in single electron transfer processes. It is located close to the substrate binding site and is responsible for substrate oxidation. The T1 is surrounded by four ligands which influence the oxidation potential in a range of 430-780 mV. There are three conserved ligands, two histidines and one cysteine. The covalent copper-cysteine bond shows an absorption at 610 nm and is responsible for the typical blue colour of this molecule. The variable axial ligand can be methionine (low redox potential), leucine (middle redox potential) or phenylalanine (high redox potential). This ligand strongly influences the oxidation potential E_0 and therefore regulates enzyme activity. According to Piontek *et al.*, laccases were found with manganese, zinc or iron atoms within the catalytic center, leading to different catalytic properties [36].

Type 2: This paramagnetic 'non-blue' copper is mononuclear and reveals redox activity, such as O_2 reduction to H_2O_2 via a two electron transfer.

Type 3: This diamagnetic copper is binuclear and responsible for O_2 reduction.

T2/T3 cluster: The type 2 and type 3 copper atom accumulate in the enzyme to form a trinuclear cluster. This T2/T3 cluster is connected with the T1 by a conserved His-Cys-His tripeptide and leads to a O_2 reduction via a four electron transfer to release water. The cluster is surrounded by eight His-ligands. There are enzymes with similar substrate properties but lacking the catalytic copper atom, sometimes called 'yellow' or 'white' laccases [3].

Laccases are highly oxidising as with the monoelectronic oxidation at the T1 the substrate is transformed into a radical. As radicals are very reactive they can undergo non-enzymatic reactions, such as cross-linking of monomers, degradation of polymers and cleavage of aromatics. Oxygen as the co-substrate is reduced to water via the four electron transfer at the T2/T3 cluster. This co-substrate is easily available and converted into a non toxic side-product. In respect to substrate specificity laccases can oxidise various substances like phenols, amines, hetero- and alicyclic compounds, which overlaps with the group of monophenol mono-oxygenase tyrosinase (EC 1.14.18.1) and catechol oxidase (EC 1.10.3.1). This low substrate specifity makes it difficult to define laccases, but generally they have a high affinity for ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid), a non-natural test substrate, whereas the oxidation of guaiacol and 2,3-dimethoxyphenol is considerably slower. Syringaldazine is often considered to be a unique laccase substrate [3]. The pH optima for laccase activity are usually in the acidic pH range.

A lot of laccases studied so far were found both intra- and extracellularly. The localisation of laccase is connected with its physiological function and determines the substrate range [3]. Intracellular laccases can be involved in the transformation of phenolic compounds, whereas cell wall and spore-associated laccases may contribute to the formation of protective cell wall compounds [14, 16]. Like most fungal extracellular enzymes, laccases are glycoproteins with a sugar content between 15 and 80%. Glycosylation may protect the enzyme from high temperature and proteolysis [42]. This fact contributes to its resistance to denaturation and inhibition. However, glycosylation is one of the biggest problems for the heterologous production of this enzyme [3].

Many ligninolytic fungal species produce different laccase isoenzymes, which show different properties regarding subsrate affinity, pH and temperature optima and sometimes also different primary structures. This can be attributed to an optimal adaptation to different environmental conditions to assure optimal laccase function. It was shown, that the molecular basis for the production of different isoenzymes is the presence of multiple laccase genes in fungi [8]. These genes show differences in expression pattern and biochemical properties suggesting different physiological roles during the lifecycle of the organism [21]. As shown by Palmieri *et al.* on *Pleurotus ostreatus* the production of laccase isoenzymes is regulated by the presence of copper [35]. Anyway, the role of posttranslational modifications or proteolysis concerning the diversity of laccase isoenzymes is not absolutely clear yet.

Chapter 2

Applications

Due to the radical reaction mechanism of laccases they can be applied in the field of bioremediation, biopulping and -bleaching and they also have use in organic synthesis. Most laccases are produced extracellularly and show a considerable level of stability, with a generally low substrate specifity, which makes them useable for a lot of different processes [3].

2.1 Manufacturing Industries

Reactive phenolic radical intermediates that undergo laccase-catalysed coupling reactions, lead to the formation of dimers, oligomers and eventually polymers. This polymeric polyphenolic derivates are usually insoluble and can be separated easily by filtration or sedimentation. This selective removal of phenol derivatives is required for the stabilisation of beverages, such as fruit juices, wine and beer. But the main technological applications are in the textile, dye and paper industry, for processes related to decolourisation of dyes or delignification of woody fibres [38].

Wood is an agglomerisation of long, slim fibres mainly from celluloses and hemicelluloses. Furthermore it consists of lignin, a complex polymer of phenolic subunits (see figure 2.1), which is present in the middle lamella and in the secondary cell wall of the fibres. For the production of paper pulp the lignin must be eliminated, either by mechanical or chemical methods, using strong oxidising reagents. White-rot fungi are able to perform lignin degradation using a mixture of oxidative enzymes, including laccases, by selective oxidation of the benzylic hydroxyl groups. Additionally, redox mediators have to be used, such as 3-hydroxyanthranilic or the artificial mediator ABTS [38].

This degradation potential of laccases also constitute their use for bioremediation and wastewater treat-

ment. Laccases can immobilise soil pollutants, like phenolics or aromatic amines, by coupling them to humic substances, a process analogous to humic acid synthesis in soils [5]. This process is often accompanied by demethylations and dehalogenations. Industrial pollutants, such as synthetic dyes, pesticides, oil, polycyclic aromatic hydrocarbons (PAH) or polychlorinated biphenyls (PCB) can be effectively removed or immobilised, resulting in a decrease in toxicity [10].

Also non-phenolic substituents, such as cellulose and other polysaccharides, can be oxidised by using laccases. Selective oxidation of the primary hydroxyl groups of sugars can be done using a laccase-TEMPO system (2,2,6,6-tetramethylpiperidine-1-yloxy), which made it possible to develop different applications for the enzymatic production of novel sugars.

Laccases cannot only be used for degradation processes, but also for the synthesis of natural compounds, such as the dimers of penicillin X [1] and bisphenol A [47], as well as in peptide synthesis [39]. This refers to the environmentally friendly preparation of homo- and heterodimers of phenolic or amine derivatives.

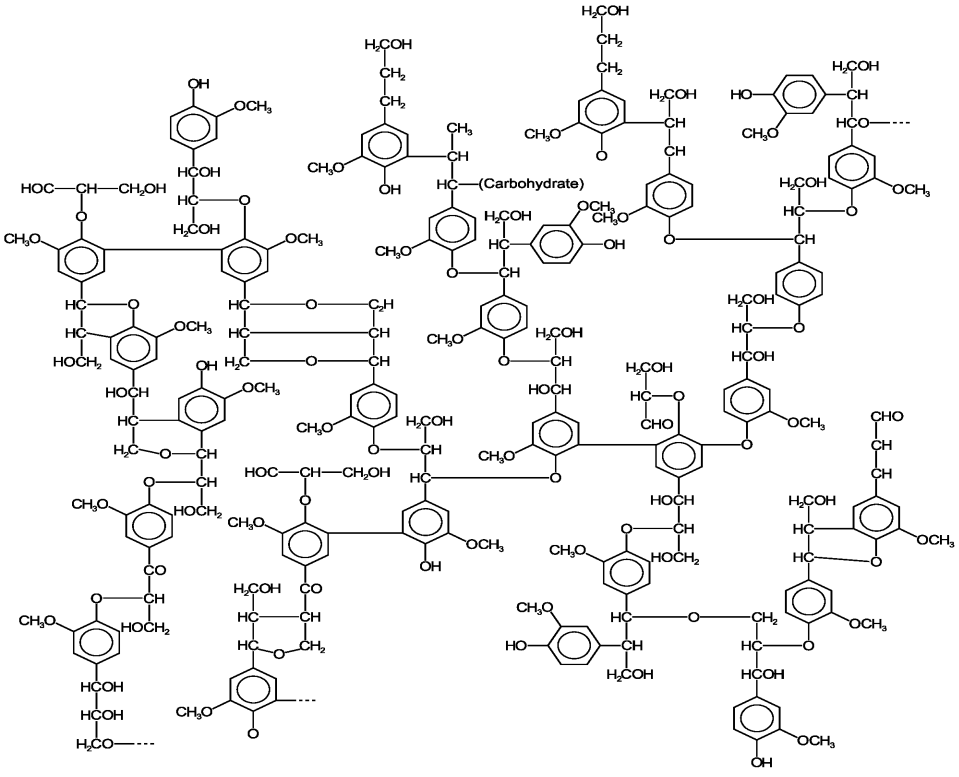

Figure 2.1: Structure of lignin (figure taken from [18]).

2.2 Biofuel Cells

The idea of a biological fuel cell is the conversion of chemical energy (binding energy) to electricity through biocatalytic activity. Full glucose oxidation can generate 24 electrons used for producing electricity [13]. As some basidiomycete laccases have a redox potential close to that of O_2 | H_2O, they can also be used as a biocomponent for oxygen cathodes in biofuel cells. A NADH | laccase based biological fuel cell could theoretically span a potential range like a normal H_2 | O_2 fuel cell. This means that such biofuel cells can run with glucose and oxygen, substances that appear naturally inside our bodies. Such fuel cells enable implantable sensors that monitor blood glucose levels or other chemicals, or even pacemakers powered by the body itself.

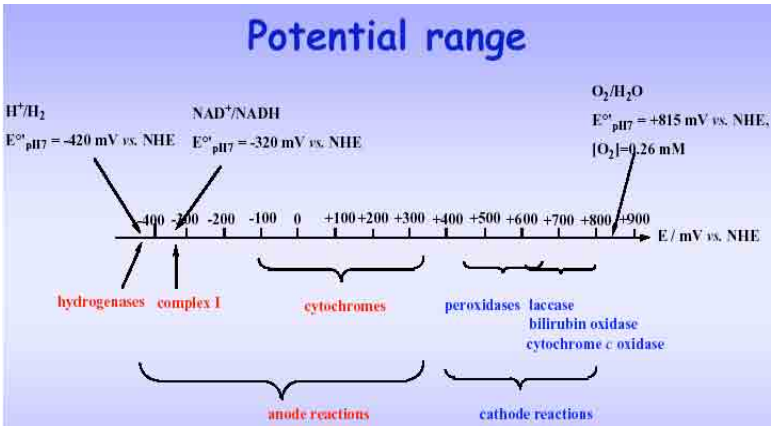

Figure 2.2: Potential range of different substrates for anode or cathode reactions.

Microbial fuel cells (MFCs) working with whole cells can either be used for ethanol and hydrogen production mainly from organic wastes like corn husks, whey or sewage. These secondary fuels can in turn run conventional H_2 | O_2 or ethanol | O_2 fuel cells [13]. A conventional fuel cell is shown in figure 2.3. Hydrogen is oxidised at the anode releasing protons and electrons. Both are transferred to the cathode, where they are used to reduce oxygen to water. The movement of the electrons from the anode to the cathode can be used to produce electricity. On the other hand MFCs can generate electricity directly from organic material. Two electrodes separated by a semi-permeable membrane are placed into a substrate solution. The microbial cells can either be in solution within the anodic compartment or can be immobilised at the electrode. The fuel becomes oxidised at the anode and the electrons released are transported to the cathode, where oxygen is reduced. For a complete glucose oxidation a range of enzymes would be necessary, which could facilitate a very efficient utilisation of the energy potential. MFCs have long runtimes and complex fuels as corn husks, whey and wastewater can be used. The main substrates of interest are carbohydrates, alcohols or hydrogen. But also cysteine, acetate and aromatic

compounds like benzoate and toluene were shown to be utilised. Nevertheless, the electrons generated inside the cell have to be transported across the cell membrane, what makes the use of mediators necessary. Furthermore microbial fuel cells can not be sterilised and therefore cause problems with implantation, biocompatibility and biofouling [13]. Until now the power density of MFCs is several orders of magnitude lower than that of chemical fuel cells, due to the high internal resistance and low catalyst density [26].

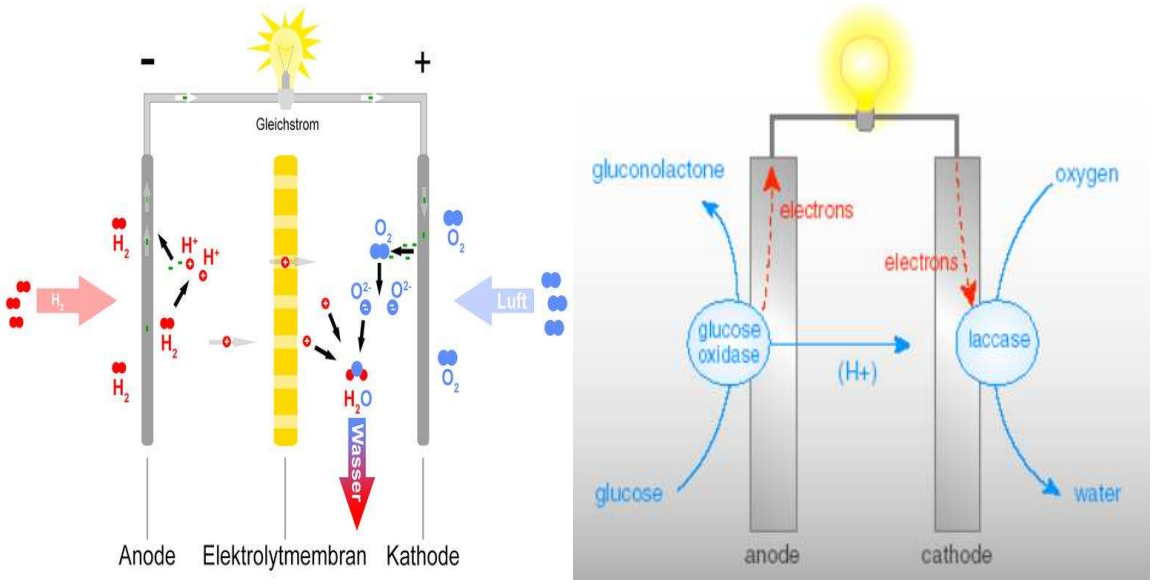

Figure 2.3: **Left figure** Conventional H_2 | O_2 fuel cell to produce electricity. **Right figure** Enzyme-based biofuel cell (EFC) using glucose oxidase and laccase immobilized on electrodes. The negatively charged anode strips electrons as glucose oxidase is oxidising glucose to gluconolactone. These electrons travel to the positively charged cathode and are transfered to oxygen producing water (figures taken from [40])

An often studied enzyme-based biofuel cell (EFC) is the glucose oxidase | laccase cell, which is shown in figures 2.3 and 2.4. For anode oxidation reactions enzymes like alcohol dehydrogenase, peroxidase, glucose oxidase, glucose dehydrogenase and cellobiose dehydrogenase can be used. The oxidation reaction at the anode produces electrons, which are transported to the cathode. There the laccase takes up the electrons and catalyses the reduction of oxygen to water. Very favourable is that both the glucose and oxygen required for operation can be taken from their immediate environment like the blood stream. Beneficial is also that no toxic by-products are produced with this system. Nevertheless, until now the use of enzyme-based biofuel cells is restricted by short lifetimes and that complex media, like body fluids, can deactivate the enzymes [13]. As laccase activity is strongly reduced at neutral pH and higher chloride concentrations, their use at physiological conditions (pH 7.4, 0.15 M NaCl, 37°C) is restricted. Therefore molecular biology techniques are in demand to reengineer the enzyme or screen for laccases in fungal

strains from extraordinary sources to find properties that meet the requirements [40]. This work was done with a focus on this purpose.

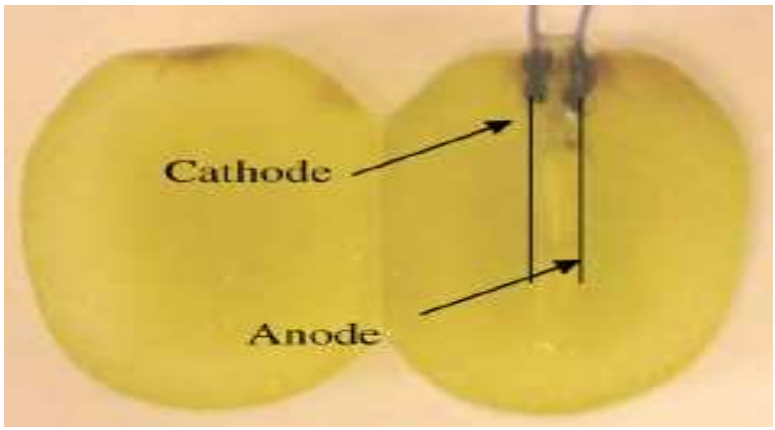

Figure 2.4: Glucose oxidase | laccase cell arrangement in a single grape producing a power output of 430 μW/cm^2 at an operating potential of 0.52 V (figure taken from [13]).

Immobilising enzymes on electrodes can often improve enzyme stability, lifetime and pH-dependency. For laccase it could be shown that the normal working pH of <5 could be increased to pH 7 by immobilisation and also the inhibition by chlorid ions was less distinct. As mentioned before a biofuel cell requires compartmentation of the anode and cathode to inhibit interference reactions in the solution. However, by immobilising enzymes on the electrode no semipermeable membrane is needed any longer, which also reduces mass-transfer restrictions [13].

2.2.1 Limiting factors

Various factors can influence the performance of a MFC as shown in figure 2.5. The rate of fuel oxidation and electron generation is dependent on the catalytic activity on the anode, fuel diffusion and the electron and proton transfer rates. The electron transfer to and from the electrodes depends on the mediator used or if a direct electron transfer is possible. The proton diffusion through the semi-permeable membrane is influenced as the proton consumption at the cathode is often higher than the transfer rate through the membrane. Typically, the cation concentration in a normal fuel medium, required for microbial metabolism, is higher than the proton concentration and therefore interferes with the proton transfer through the membrane. A low cation concentration or a single compartment assembly can improve the output. The oxygen diffusion and solubilisation is influenced as the oxygen consumption rate is very often higher than the low solubilisation rate. Immobilised laccase can face this problem due to its very high affinity to oxygen. In general, the proton mass transfer is the main constraint in a MFC, increasing the internal resistance [26].

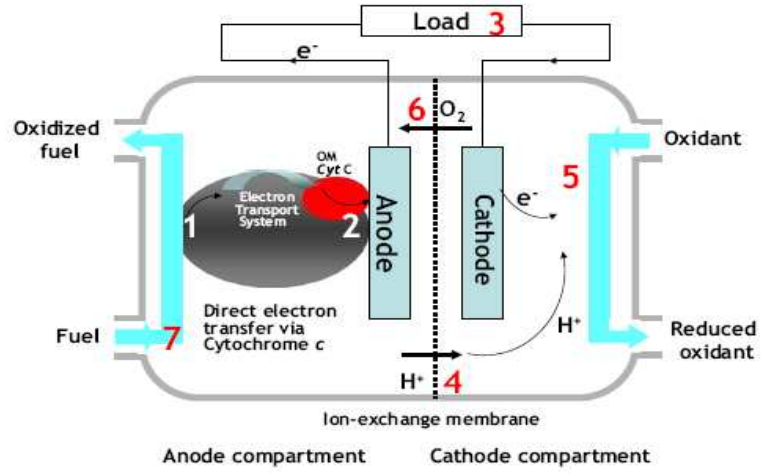

Figure 2.5: Limiting factors of a biofuel cell. **1** Fuel oxidation. **2** Electron transfer. **3** Internal resistance. **4** Proton diffusion. **5** Cathode reaction. **6** Oxygen diffusion. **7** Non-ideal fuel flow (figure taken from [26]).

2.2.2 Direct Electron Transfer (DET)

Compared to ligninolytic peroxidases (>1 V) the redox potential of laccases (450-800 mV vs. NHE) is quite low, which only allows the direct degradation of some wood compounds. Other compounds can only be transformed if the reaction product is subject to an immediately following reaction or when its redox potential is lowered, for example by chelation or redox mediators [3]. Therefore, to improve the electron transfer between microorganisms or enzymes and the electrode mediators such as ABTS, 2,6-dichloroindophenol (DCIP), methylene blue or ferrocene have to be used. These mediators have a big influence on the efficiency of a biofuel cell. For biological purposes it is very important that these mediators are non toxic. For many redox proteins, which have no intrinsic catalytic activity but act as electron transfer components in biochemical pathways (e.g ferredoxins, flavodoxins, cytochrome c and azurin), efficient DET reactions with electrodes have been demonstrated [41]. In contrast, only a restricted number of redox enzymes show efficient DET, most of them containing a metallocenter in their active site, e.g. heme, iron-sulphur cluster and copper. Some also have an organic cofactor like a flavin or a quinone [19]. Many of those redox enzymes that can communicate via DET are intracellular enzymes located in membranes, where they participate in biological electron transfer processes [41].

For non enzymatic oxygen reduction only platinum electrodes show reasonable performance, which in turn are expensive. By immobilising laccase on graphit electrodes it could be shown that the reduction of O_2 begins at the redox potential of T1 (about 780 mV vs. NHE). That leads to the assumption that the electrons are directly transferred from the electrode to the enzyme, without the need of mediators.

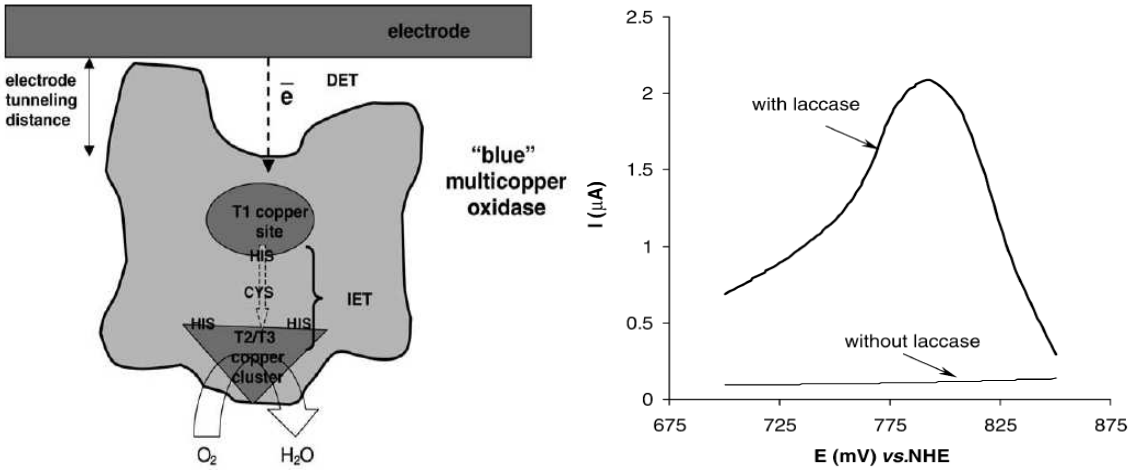

Figure 2.6: **Left figure** Scheme of the direct electron transfer. **Right figure** Voltammogram recorded with and without the *T. hirsuta* laccase (both figures taken from [41]).

The limiting step is the electron transfer to O_2 at the T2/T3 cluster. In contrast, the immobilisation of laccase on gold electrodes shows two electron transfer processes at 370 mV and 870 mV. This leads to the assumption that the reduction of O_2 begins at lower potential (400 mV) and DET can also be achieved by interaction with the T2 copper [41]. In general, laccase can be used to be immobilised on cheap graphite electrodes and dramatically enhances their performance as their high affinity to oxygen needs less O_2-concentrations for good response.

The power output of MFCs is too small for reasonable usage so far, but a very interesting and feasible procedure is the combination of bioremediation and energy recovery. In aerobic wastewater treatment processes microorganisms utilize all energy provided by the organic contaminants for biomass generation and therefore producing a lot of sludge. But if the electrons are transported from the cell surface to electrodes and further to oxygen, only $\frac{1}{3}$ of the energy is available for the microorganisms and $\frac{2}{3}$ is converted to electricity. Therefore less excess sludge would be produced, since their disposal is very expensive. At the moment the combination of bioremediation and energy conversion leads to an output of 0.6 V, which theoretically means that a town of 150.000 people could produce 2.3 MW of power [26].

Even though the performance of MFCs is too poor for a broad commercial usage nowadays and their efficiency and lifetime have to be improved, it can be shown that the future prospects pave the right way. There is no possibility of ignoring this development considering the discussion of sustainable usage of energy.

Part II

Materials and Methods

Chapter 3

Microorganisms

The examined organisms comprise fungal strains from the existing strain collection of the Department of Food Sciences and Technology at the University of Natural Resources and Applied Life Sciences, Vienna (see table [**?**]). For vegetative reproduction agar plates were inoculated aseptically with plugs of hyphae from slant agar tubes, which had been stored covered by paraffin oil. Three plugs of each strain were distributed on the plate, while trying to separate the hyphae from the paraffin oil. The plates were sealed with Parafilm and incubated upside down at 25°C, until the mycelium covered the agar surface.

For further subculturing agar pieces with a size of about 5 x 5 mm were cut from the well-grown mycelium and applied with the surface downwards on fresh agar plates. The plates were sealed with Parafilm and incubated upside down at 25°C, until satisfactory growth could be observed. Then the plates were stored at 4°C and subcultured every four to six weeks.

Four different strains were screened for laccase activity.

	Botrytis aclada	*Lamprospora wrightii*	*Glomerella cingulata*	*Phoma destructiva*
Kingdom	Fungi	Fungi	Fungi	Fungi
Phylum	Ascomycota	Ascomycota	Ascomycota	Ascomycota
Class	Leotiomycetes	Pezizomycetes	Sordariomycetes	Dothideomycetes
Order	Helotiales	Pezizales	-	Pleosporales
Family	Sclerotiniaceae	Pyronemataceae	Glomerellaceae	-
Genus	Botrytis	Lamprospora	Glomerella	Phoma

Table 3.1: Classification of different fungi used for screening (taken from the *Catalogue of Life*).

Table 3.2 shows the media used for subcultivation. The media compositions are based on the diploma thesis of Andrea Graf [20]. All components were dissolved in deionised water according to the manufacturer's instructions and autoclaved at 121°C for 20 min. As an inducer for laccase production $CuSO_4$ was used, which was sterile filtered and added separately under sterile conditions to the cooled media.

Components	**Basal** Concentration	**PDA** Concentration	**Malt extract** Concentration	**Fructose** Concentration
Fructose	10 g/l	-	-	5 g/l
Glucose	5 g/l	20 g/l	-	-
Potato extract	-	4 g/l	-	-
Malt extract	-	-	30 g/l	-
Soy Peptone	1 g/l	-	3 g/l	5 g/l
TES	0.3 ml/l	-	-	-
Agar	15 g/l	15 g/l	15 g/l	15 g/l

Table 3.2: Media compositions used for subculturing.

Not immediately used media were stored at 4°C.

For the cultivation experiments a trace element solution (TES) was used, containing following components: 1 g/l $ZnSO_4 \cdot 7H_2O$, 0.3 g/l $MnCl_2 \cdot 4H_2O$, 3 g/l H_3BO_3, 2 g/l $CoCl_2 \cdot 6H_2O$, 0.1 g/l $CuSO_4 \cdot 5H_2O$, 0.2 g/l $NiCl_2 \cdot 6H_2O$, 4 ml/l H_2SO_4 conc.

Chapter 4

Enzyme Assays and Kinetics

Different activity assays were used to characterise the enzyme using a Beckman coulter DU800 UV/Vis spectrophotometer equipped with a 6-fold sample holder connected to a water bath. The standard ABTS assay was used to determine laccase activity in crude extracts or partially purified preparations by the oxidation reaction of ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid), which leads to a colour change into green. The increase of absorbance is directly proportional to the reaction rate of laccase. The amount of enzyme that oxidises 1 μmol of ABTS per minute is defined as one unit (1 U) of enzyme activity.

Standard ABTS-assay: This assay was performed in semi-micro plastic cuvettes. Eight hundred eighty μl of 100 mM sodium-acetate buffer pH 4 and 100 μl 10 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

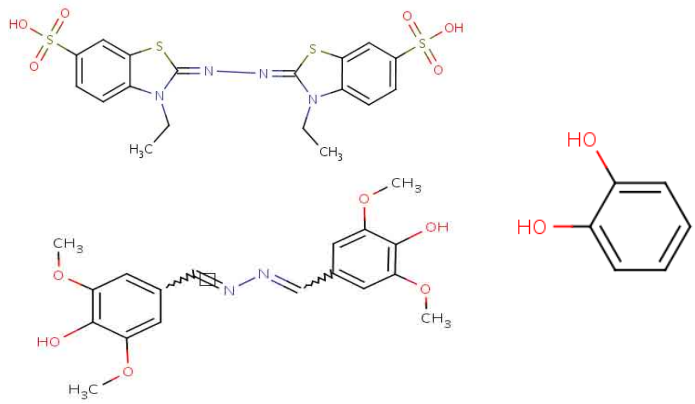

Figure 4.1: Structures for ABTS (top), Syringaldazine (bottom) and Catechol (right). Figures were taken from *BRENDA The Comprehensive Enzyme Information System*.

4.1 Determination of Protein Concentration

The protein concentration was determined using a Beckman Coulter DU800 UV/Vis spectrophotometer at a wavelength of 595 nm with the dye-binding method of Bradford (1976). This method is based on binding of Coomassie brilliant blue primarily to basic and aromatic amino acid residues, whereupon the colour changes from brown to bright blue. The BioRad protein assay dye reagent (BioRad, Hercules, CA, USA) was used and prepared according to the manufacturer's instructions.

Protein assay: Twenty μl of the sample was added to 1 ml of Bradford solution in a semi-micro plastic cuvette. The assay was mixed by stirring with a plastic spatula and incubated at room temperature for 15 min. Then the absorbance was measured at 595 nm. The protein concentration was calculated by using a bovine serum albumin (BSA) standard curve in the range of 0.1 to 1.0 mg/ml, prepared as described above.

4.2 Determination of pH Optima

The effect of pH on laccase activity was measured over the range of pH 3 to 8 in 50 mM Na_2HPO_4/citrate buffer using ABTS, Syringaldazine, Catechol and TEMPO as substrates. To obtain the pH optima, the absorbance rates were recorded at different pH values at the wavelength of the absorbance maximum of each substrate. Therefore a Perkin Elmer Lambda 2 spectrophotometer equipped with a 6-fold sample holder connected to a water bath was used. Buffer preparation was done by mixing a 50 mM sodium citrate buffer and a 50 mM Na_2HPO_4 buffer to the desired pH in a range of 3-8 in steps of 0.5 units. The experiments were done with purified enzyme.

The substrates used can be seen in table 4.1. Some stock solutions of the substrate had to be prepared in ethanol or methanol before they could be mixed with buffer. This also influenced the assay preparations, as higher alcohol concentrations can affect enzyme activity. For each assay the absorbance was also measured without addition of laccase to determine the stability on auto-oxidation of the substrate.

ABTS Assay: This assay was measured in a semi-micro plastic cuvette. Eight hundred eighty μl buffer and 100 μl of 10 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

Syringaldazine Assay: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer was prewarmed in a waterbath at 30°C. Afterwards 20 μl of sample was added and the reaction started with 20 μl of 1 mM syringaldazine. The absorbance was recorded at 530 nm (ε_{530} = 64

Substrate	Concentration [mM]	Molar Weight [g/mol]	Solvent
ABTS*	1	548.0	water
Syringaldazine⋆	0.02	360.4	methanol
Catechol°	2	110.1	ethanol
TEMPO•	0.2	156.3	ethanol

Table 4.1: Substrates for the determination of pH optima. *2,2'-azinobis 3-ethylbenzthiazolinesulfonic acid. ⋆N,N'-bis 3,5-dimethoxy-4-hydroxybenzylidenehydrazine. °1,2-benzenediol. •2,2,6,6-tetramethylpiperidin-N-oxide radical

$mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 0.781.

Catechol Assay: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer was prewarmed in a waterbath at 30°C. Afterwards 20 μl of sample was added and the reaction started with 20 μl of 100 mM catechol. The absorbance was recorded at 390 nm (ε_{390} = 8.738 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 5.722.

TEMPO Assay: This assay was measured in a 1 mm path length quartz cuvette. Nine hundred seventy μl buffer and 10 μl of 20 mM TEMPO were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 245 nm (ε_{245} = 0.25 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 200.

4.3 Determination of Temperature Optima

The effects of temperature on enzyme activity and stability were measured with syringaldazine at pH 6 in a range of 30 to 65°C using a 50 mM Na_2HPO_4/citrate buffer. A Perkin Elmer Lambda 2 spectrophotometer equipped with a 6-fold sample holder connected to a water bath was used. Buffer preparation was done by mixing a 50 mM sodium citrate buffer and a 50 mM Na_2HPO_4 buffer to the desired pH of 6. The buffers were prepared in steps of 0.5 units and temperatured in a cuvette in the waterbath. The temperature was controlled with an electronic thermometer in the cuvettes. The experiments were done with purified enzyme.

Temperature Assay: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer was brought to the right temperature. Afterwards 20 μl of 1 mM syringaldazine was added and the reaction started with 20 μl of sample. The absorbance was recorded at 530 nm for exactly 5 min and the resulting graph was integrated over the whole period.

To determine the activation energy E_a of the enzyme only the initial straight slope (steady-state conditions) from the temperature assay was taken and plotted to the Arrhenius equation. The reaction rate was measured at different temperatures and plotted as log k versus 1/T. By using equation 4.2 the activation energy can be calculated with 4.3.

$$k = A \cdot e^{\frac{-E_a}{RT}} \tag{4.1}$$

$$logk = -\frac{E_a}{2.3R} \cdot \frac{1}{T} + logA \tag{4.2}$$

$$slope = -\frac{E_a}{2.3R} \tag{4.3}$$

4.4 Inhibition

The effect of various inhibitors on laccase activity was determined by measuring the enzyme reaction rates at the presence of different salts (see table 4.2). Buffers were prepared by mixing a 50 mM sodium citrate buffer and a 50 mM Na_2HPO_4 buffer to obtain the desired pH. Stock solutions of the inhibitors were prepared and diluted to the desired final concentrations in the particular buffer. A Perkin Elmer Lambda 2 spectrophotometer equipped with a 6-fold sample holder connected to a water bath was used for measurement. The experiments were done with purified enzyme.

Inhibition by NaCl at pH 3.0: This assay was measured in a semi-micro plastic cuvette. Nine hundred seventy μl buffer (pH 3) and 10 μl of 100 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

Inhibition by NaCl at pH 6.0: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer (pH 6) was prewarmed in a waterbath at 30°C. Afterwards 20 μl of sample was added and the reaction started with 20 μl of 1 mM syringaldazine. The absorbance was recorded at 530 nm (ε_{530} = 64 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 0.781.

Inhibition by NaF at pH 3.0: This assay was measured in a semi-micro plastic cuvette. Nine hundred seventy μl buffer (pH 3) and 10 μl of 100 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

Salt	pH	Conc. Range [mM]	Molar Weight [g/mol]	Solvent
NaCl	3.0	1 - 500	58.4	phosphate/citrate buffer
NaCl	6.0	1 - 500	58.4	phosphate/citrate buffer
NaF	3.0	0.01 - 5	42	phosphate/citrate buffer
NaN_3	3.0	0.001 - 2	65	phosphate/citrate buffer

Table 4.2: Salts used for inhibition studies on laccase.

Inhibition by NaN_3 at pH 3.0: This assay was measured in a semi-micro plastic cuvette. Nine hundred seventy μl buffer (pH 3) and 10 μl of 100 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

4.5 Exchange of Metal Ions

Two experiments were performed to determine the effects of different metal ions inside the enzyme. It was tried to exchange the active atom with either cobalt, zinc or copper. 100 mM metal stock solutions of $CoSO_4$, $ZnSO_4$ or $CuSO_4$ were prepared in water. Buffer preparation was done by mixing a 50 mM sodium citrate buffer and a 50 mM Na_2HPO_4 buffer to the desired pH of 3. For each assay the absorbance was also measured without addition of laccase to determine the stability on auto-oxidation of the substrate. A Perkin Elmer Lambda 2 spectrophotometer equipped with a 6-fold sample holder connected to a water bath was used for measurement.

Cofactor Exchange Assay: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer, 10 μl metal and 10 μl of 100 mM ABTS were prewarmed in a waterbath at 30°C. The assay was started with 20 μl of sample and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

Extended Cofactor Exchange Assay: This assay was measured in a semi-micro plastic cuvette. Nine hundred sixty μl buffer, 10 μl metal and 20 μl of sample were prewarmed in a waterbath at 30°C and incubated for 15 min. The assay was started with 10 μl of 100 mM ABTS and the absorbance was recorded at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$). The linear slope of enzyme activity at the beginning of the reaction was multiplied with an enzyme factor of 1.182.

Substrate	pH	Conc. Range [mM]	Molar Weight [g/mol]	Solvent
ABTS	3.0	0.0002 - 0.2	548.0	water
ABTS	5.0	0.0002 - 10	548.0	water
Syringaldazine	6.0	0.0006 - 0.06	360.4	methanol
Catechol	5.0	0.02 - 40	110.1	ethanol

Table 4.3: Substrates for determination of steady state kinetic constants.

4.6 Michaelis-Menten Kinetics

To obtain steady-state catalytic constants of laccase, several kinetic experiments were performed. The steady-state phase of an enzyme reaction, obeying the Michaelis-Menten kinetics, can be described by two parameters. The K_m value (Michaelis-Menten constant) is equal to the substrate concentration that yields a half-maximal reaction rate and gives an appropriate measure of the affinity of an enzyme for its substrate. v_{max} is the maximum velocity of the reaction at saturated substrate conditions. Both values can be determined from experimental data of reaction velocity versus substrate concentration.

$$v = v_{max}\frac{[S]}{[S] + K_m} \tag{4.4}$$

The Michaelis-Menten constant K_m and maximum reaction velocity v_{max} were calculated by nonlinear least-square regression, by fitting the observed data to the Michaelis-Menten equation. The program used for the calculation was Sigma Plot 2000. The experiments were done with purified enzyme and different substrates at various pH values, which can be seen in table 4.3. Some stock solutions of the substrate had to be prepared in ethanol or methanol before they could be mixed with buffer. This also influenced the assay preparations, as higher alcohol concentrations can affect enzyme activity. Buffer preparation was done by mixing a 50 mM sodium citrate buffer and a 50 mM Na_2HPO_4 buffer to the desired pH. Stock solutions for the substrates were prepared and diluted to the desired concentrations in the particular buffer. The different assays were prepared equally as described in section “Determination of pH Optima”, using a Perkin Elmer Lambda 2 spectrophotometer equipped with a 6-fold sample holder connected to a water bath.

Chapter 5

Cultivation Experiments

Four different fungal strains (*Lamprospora wrightii* CBS 600.69, *Botrytis aclada* DSMZ 62081, *Glomerella cingulata* DSMZ 62728 and *Phoma destructiva* CBS 162.78) were subcultured in different media and under different conditions to examine growth and laccase production. All components for the cultivation media were dissolved in deionised water according to the manufacturer's instructions and autoclaved at 121°C for 20 min. As an inducer for laccase production $CuSO_4$ was used, which was sterile filtered and added separately under sterile conditions to the cooled media. Not immediately used media were stored at 4°C.

The cultivation experiments were performed in shaking flasks or agar plates using liquid media. For inoculation a piece of about 1 x 1 cm was cut from well-grown mycelium of the subcultivated fungi on agar plates, divided into 3 pieces and put aseptically into the medium. Cultures were incubated at 25°C at different shaking conditions on a rotary shaker with an excentricity of 2.5 cm. All experiments were carried out in duplicates.

For sampling, 1 ml of culture broth was taken aseptically and transferred into an Eppendorf microcentrifugation tube. To avoid perturbations during the photometrical measurements (i.e. light scattering), the mycellium was removed by centrifugation at 13000 rpm for 5-10 min with an Eppendorf centrifuge 5415R. The clarified supernatant was used to determine laccase activity, protein concentration and pH.

5.1 Determination of optimum Shaking Flask Conditions

To determine the effects of different shaking conditions the fungi *L. wrightii* was cultivated in Erlenmeyer flasks at 0 and 90 rpm and in baffled flasks at 90 rpm, respectively. All experiments were done in 1 l shaking flasks, each containing 100 ml media. The media contained 10 g/l fructose, 5 g/l glucose, 1 g/l soy peptone, 0.3 ml/l TES and 0.1 mM $CuSO_4$. All cultures were incubated at 25°C.

Name	**F10P1**	**F10P5**	**F5P1**	**F5P5**	**F10P5-T**
Components	Concentration	Concentration	Concentration	Concentration	Concentration
Fructose	10 g/l	10 g/l	5 g/l	5 g/l	10 g/l
Glucose	-	-	-	-	-
Glycerine	-	-	-	-	-
Soy Peptone	1 g/l	5 g/l	1 g/l	5 g/l	5 g/l
TES	0.3 ml/l	0.3 ml/l	0.3 ml/l	0.3 ml/l	-
$CuSO_4$	0.1 mM	0.1 mM	0.1 mM	0.1 mM	0.1 mM

Name	**G10P5**	**Gly10P5**	**F5G5P5**	**F10G5P1-Cu**
Components	Concentration	Concentration	Concentration	Concentration
Fructose	-	-	5 g/l	10 g/l
Glucose	10 g/l	-	5 g/l	5 g/l
Glycerine	-	10 g/l	-	-
Soy Peptone	5 g/l	5 g/l	5 g/l	1 g/l
TES	0.3 ml/l	0.3 ml/l	0.3 ml/l	0.3 ml/l
$CuSO_4$	0.1 mM	0.1 mM	0.1 mM	-

Table 5.1: Composition of basal media for cultivation of *L. wrightii*.

5.2 Cultivation of *Lamprospora wrightii* in Basal Media

All experiments were done in 300 ml shaking flasks, each containing 100 ml media. All cultures were incubated at 25°C and 130 rpm on a rotary shaker. The media used can be seen in table 5.1.

5.3 Cultivation in Vegetable Media

These experiments comprised all fungal strains (*L. wrightii*, *B. aclada*, *G. cingulata* and *P. destructiva*). Shaking flasks (250 ml) were used, each containing 100 ml media. All fungi were incubated at 25°C and 130 rpm on a rotary shaker. The media used can be seen in table 5.2.

Potato infusion according to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH): Old potatos (200 g) were peeled, cut and cooked in deionised water by autoclaving at 121°C for 30 min. Afterwards the potato infusion was homogenised with a Ultraturrax.

Tomato and vegetable juice: purchased at Spar, label "Natur pur", pH 4.2.
Apple juice: purchased at Hofer, label "Gold Land", cloudy, directly squeezed, pH 3.4.
Grape juice: purchased at Hofer, label "Sweet Valley", directly squeezed, pH 3.4

5.4 Fermentation Experiments

All fermentation experiments were done with *L. wrightii* and *B. aclada* in 20 l scale with tomato juice as cultivation broth. The fermenter was heated up to 95°C and held at this temperature for about 15 min, as tomato juice shows a tendency of excessive foaming at higher temperatures. Additionally 2 ml

Name	**PotF10**	**PotF10Cu0.01**	**PotF10-Cu**	**PotF10Cu5***
Components	Conc.	Conc.	Conc.	Conc.
Fructose	10 g/l	10 g/l	10 g/l	10 g/l
Glucose	-	-	-	-
Potato infusion	100 ml	100 ml	100 ml	100 ml
TES	-	-	-	-
$CuSO_4$	0.1 mM	0.01 mM	-	0.1 mM

Name	**PotF10T**	**PotF10Petri•**	**PotG10**	**PotF5G5**
Components	Conc.	Conc.	Conc.	Conc.
Fructose	10 g/l	10 g/l	-	5 g/l
Glucose	-	-	10 g/l	5 g/l
Potato infusion	100 ml	100 ml	100 ml	100 ml
TES	0.3 ml/l	-	-	-
$CuSO_4$	0.1 mM	0.1 mM	0.1 mM	0.1 mM

Name	**TomF10**	**Tom1:2F10P5**	**Tom1:5F10P5**	**Tom1:10F10**
Components	Conc.	Conc.	Conc.	Conc.
Fructose	10 g/l	10 g/l	10 g/l	10 g/l
Soy Peptone	-	5 g/l	5 g/l	-
Tomato juice	100 ml	50 ml	20 ml	10 ml
$CuSO_4$	0.1 mM	0.1 mM	0.1 mM	0.1 mM

Name	**VeggyF10**	**AppleF10**	**GrapeF10**	**Maltose**
Components	Conc.	Conc.	Conc.	Conc.
Fructose	10 g/l	10 g/l	10 g/l	-
Malt extract	-	-	-	30 g/l
Soy Peptone	-	-	-	3 g/l
Vegetable juice	100 ml	-	-	-
Apple juice	-	100 ml	-	-
Grape juice	-	-	100 ml	-
$CuSO_4$	0.1 mM	0.1 mM	0.1 mM	0.1 mM

Table 5.2: Composition of vegetable media. *copper induction was started after 5 days of incubation. •incubation in a petridish (⌀ 10 cm).

Substrate	Concentration	Concentration
Fructose	10 g/l	10 g/l
Soy Peptone	5 g/l	1 g/l
Tomato juice	0.5 l/l	0.5 l/l
$CuSO_4$	-	0.1 mM

Table 5.3: Media for fermentation experiments. **Left** First fermentation of *L. wrightii*. **Right** Second fermentation of *L. wrightii* and fermentation of *B. aclada*.

Antifoam was added and the stirrer set to 400 rpm. Aferwards the temperature was increased to 121°C and held for 15 min. The fermentation conditions were set to 25°C with a stirrer speed of 200 rpm and an air flow rate of 20 l/min.

The medium for the first fermentation of *L. wrightii* is given in table 5.3. The inoculum was prepared in 1 l shaking flasks, each containing 300 ml media, with an overall volume of 2.1 l. For inoculation a piece of about 2 x 2 cm was cut from well-grown mycelium of the agar plates, divided into 3 pieces and put aseptically into the flask. Incubation was done at 25°C and 120 rpm for 2 days before inoculating the fermenter. It turned out to be practicable to use baffled flasks, as the mycelium will grow in small aggregates, which facilitates the inoculation of the fermenter.

The media for the second fermentation of *L. wrightii* and first fermentation of *B. aclada* are shown on table 5.3. The inoculum was prepared in 1 l baffled flasks, each containing 300 ml media, with a overall volume of 2.1 l. For inoculation a piece of about 2 x 2 cm was cut from well-grown mycelium of the agar plates, divided into 3 pieces and put aseptically into the flask. Incubation was done at 25°C and 120 rpm for 3 days before inoculating the fermenter.

Chapter 6

Purification

Sufficient laccase activity for further studies was only produced by *L. wrightii* and *B. aclada*. Therefore, purification was only done for laccase from these two strains. As laccase is an extracellular protein the culture broth was first centrifuged for 15 min at 6000 rpm and 4°C with a Sorvall centrifuge and then filtrated through a filter paper (Rotilab). For further downstream processing a cross-flow filtration module (Pall, Microza) equipped with a 10 kDa molecular weight cut-off hollow fiber unit was used to concentrate the supernatant and to get rid of small molecular contaminants.

B. aclada showed an excessive tendency to produce an extracellular polysaccharide, which led to a very high viscosity of the broth and to problems while loading the sample onto the column. Therefore, the sample was mixed with an Ultraturrax to break the polysaccharides and reduce viscosity.

The *B. aclada* laccase was also produced recombinant using *Pichia pastoris* as host. This work was done by R. Kittl and K. Mueangtoom and provided for purification purposes and determination of kinetic properties.

6.1 Fractionated Precipitation

For exploring the salt stability of laccase and finding the right conditions for purification a fractionated precipitation was done with $(NH_4)_2SO_4$. The salt was added in small portions to the ultrafiltrated fermentation broth to a final saturation of 20 to 80%, incubated for 15 min at room temperature and afterwards centrifuged for 15 min at 13200 rpm. Laccase activity and protein content were measured in the supernatant. Afterwards the samples were stored at 4°C over night and laccase activity was measured again, to determine effects on enzyme activity due to the presence of $(NH_4)_2SO_4$.

Laccase activity and protein content were also determined in the pellet, by dissolving the pellet in 10 mM sodium-acetate buffer pH 4 and incubating at room temperature for 30 min. This turned out to be

problematic for *B. aclada*, as the polysaccharides in the sample started to precipitate with salt addition and the resulting pellet could not be dissolved again. Furthermore, the reversibility of inhibitory effects of $(NH_4)_2SO_4$ was studied, by diluting the supernatant 1:50 in 100 mM sodium-acetate buffer pH 4 and following laccase activity over time.

6.2 Preliminary Chromatographic Experiments

All experiments were done with unpurified enzyme from the fermentation broth after cross-flow filtration. The stability of laccase in various buffers with different strength and pH was determined to find optimal conditions for each purification step. The aim was to obtain good purification rates combined with best recovery. Therefore 500 μl buffer and 250 μl sample were incubated in an Eppendorf tube at room temperature for 30 min. Afterwards the remaining enzyme activity was measured with the standard ABTS assay (see chapter "Enzyme Assays and Kinetics"). If the ionic strength of the buffer was very low, it was necessary to adjust the pH in the sample first to assure right pH values in the preparations.

Binding and elution experiments for different chromatography matrices were carried out at small scale in Eppendorf tubes. Therefore, 250 μl gel was washed 6 times with 1 ml of the desired buffer. Afterwards, 500 μl buffer and 250 μl sample were added and incubated for 5 to 10 min in a thermomixer (Eppendorf comfort) at 23°C and 1100 rpm. It was waited until the beads were settled and the supernatant was measured with the standard ABTS assay.

Another approach was done with a ready-to-use 1 ml cation exchange column S HyperD F from PALL. Sample and buffers were applied by hand with a syringe. The following conditions were used (table 9.16):
Purification 1: The sample was adjusted to pH 3 with citric acid and diluted to 3.5 mS/cm with water. Binding buffer: 20 mM sodium citrate pH 3. Elution buffer: 20 mM sodium citrate + 20% $(NH_4)_2SO_4$ pH 3.
Purification 2: The sample was adjusted to pH 4 with citric acid and diluted to 3 mS/cm with water. Binding buffer: 20 mM sodium citrate pH 4. Elution buffer: 20 mM sodium citrate + 40% $(NH_4)_2SO_4$ pH 4.

6.3 Ion Exchange Chromatography (IEX)

Ion exchange chromatography is used for the separation of ionic substances, like amino acids, organic acids or salts. The separation of proteins or peptides by IEX is based on interactions between the charged stationary phase of the gel and the corresponding charges of the amino acids on the surface of the protein. The stationary phase consists of small ionic groups covalently bound to a porous matrix consisting of highly polymeric molecules. The interactions are depending on the surface charges of the protein, the

Method	Strength	Functional group	Structure
Anion Exchange	soft	Diethylaminoethyl (DEAE)	-O-CH_2-CH_2-$N^+H(CH_2CH_3)_2$
Anion Exchange	strong	Quaternary ammonium (Q)	-O-CH_2-CHOH-CH_2-$N^+(CH_3)_3$
Cation Exchange	soft	Carboxymethyl (CM)	-O-CH_2-COO^-
Cation Exchange	strong	Methylsulphonate (S)	-O-CH_2-CHOH-$CH_2SO_3^-$

Table 6.1: Different methods for ion exchange chromatography.

pore size of the chromatography gel, as well as on the pH-value, the temperature and the kind and concentration of the salt used in the buffers. For applying the sample on the column, the salt concentration in the buffer has to be as low as possible so that no interfering reaction between the buffer and the gel matrix can occur. This favours ionic interaction between the protein and the gel. For eluting a buffer with increasing salt concentration has to be used, as the ionic groups on the gel are replaced by salt ions and the proteins are eluted. Both the pH and the type of salt can have a tremendous influence on the stability and activity of enzymes. This was considered for buffer preparation according to the Hofmeister series. The different methods for IEX and the corresponding gel matrices are given in table 6.1.

Protein separation was done at room temperature with an ÄKTA purifier from Amersham Pharmacia Biotech. During purification the pH value and conductivity were recorded. For protein detection the absorbance at 280 nm was recorded and the eluted samples were collected in fractions. The enzyme activity was determined with the standard ABTS activity assay. The fractions containing enzyme were pooled and stored at either 4°C overnight before performing the next purification step. For longer storage more than 12 hours, the samples were stored at -20°C, as laccase activity was rapidly decreasing during storage at 4°C.

Column cleaning: 2 CV (column volumes) 0.5 M NaOH, 2 CV H_2O, 2 CV 1 M acetic acid and 2 CV 20% ethanol for storage at room temperature. Prior to use the column was equilibrated with binding buffer.

6.4 Hydrophobic Interaction Chromatography (HIC)

The separation of proteins or peptides by HIC is based on interaction between the hydrophobic stationary phase of the gel and hydrophobic amino acids on the surface of the protein. The stationary phase consists of small non-polar groups (like phenyl-, butyl- or ethyl-groups) attached to a hydrophilic polymer backbone. The strength of the hydrophobic interaction between the protein and the gel matrix depends on the hydrophobic potency of the protein, the ligand of the chromatography gel, as well as on the pH-value, the temperature and the kind and concentration of the salt used in the buffers. The more hydrophobic the ligands are, the stronger the bond of the hydrophobic regions will be. For applying the sample on the column, the salt concentration in the buffer has to be high so that proteins bind to the matrix by a "salting-out" effect. For elution, a buffer with low salt concentration has to be used. Both, the pH and

the type of salt can have an influence on the stability and activity of enzymes. This was considered for buffer preparation, according to the Hofmeister series.

Protein separation was done at room temperature with an ÄKTA purifier from Amersham Pharmacia Biotech. During purification the pH value and conductivity were recorded. For protein detection the absorbance at 280 nm was recorded and the eluted samples were collected in fractions. The enzyme activity was determined with the standard ABTS activity assay. The fractions containing enzyme were pooled and stored at either 4°C overnight before performing the next purification step. For longer storage more than 12 hours, the samples were stored at -20°C, as laccase activity was rapidly decreasing during storage at 4°C.

Column cleaning: 2 CV (column volumes) 0.5 M NaOH, 2 CV H_2O, 2 CV 1 M acetic acid and 2 CV 20% ethanol for storage at room temperature. Prior to use the column was equilibrated with binding buffer.

6.5 Ultrafiltration with Amicon Ultra

The purified and pooled sample was desalted and further concentrated with an Amicon Ultra filtration tube equipped with a 10 kDa molecular weight cut-off ultra filtration membrane (Millipore). The sample was centrifuged (Megafuge 1.0R, Heraeus) at 4000 rpm and 15°C for 15 min. After each centrifugation step the tube was refilled with unconcentrated sample until the whole sample was concentrated step-by-step to less than 10 ml. The purified sample was rediluted in 50 mM NaH_2PO_4 pH 6.

6.6 Purification Scheme for *L. wrightii* Laccase

Purification 1 (table 9.6):
Anion exchange column DEAE Sepharose FF XK50 130 ml. Binding buffer: 50 mM Na-citrate pH 6.5. Elution buffer: 50 mM Na-citrate + 1 M NaCl pH 6.5. The sample was adjusted to pH 6.5 with K_2CO_3 and diluted to 13 mS/cm with binding buffer. Flowrate 25 ml/min. Gradient: 100% B in 2 CV, flowrate 20 ml/min. Fractions of 10 ml were collected.

Purification 2 (table 9.7):
Anion exchange column DEAE Sepharose FF XK16 24 ml. Binding buffer: 20 mM NaH_2PO_4 pH 5.5. Elution buffer: 500 mM NaH_2PO_4 pH 5.5. The sample was adjusted to pH 5 with H_3PO_4 and diluted to 3 mS/cm with water. Flowrate 5 ml/min. Gradient: 100% B in 2 CV. Fractions of 10 ml were collected.

Purification 3 (table 9.8):
Anion exchange column DEAE Sepharose FF XK16 24 ml. Binding buffer: 20 mM NaH_2PO_4 pH 5.5.

Elution buffer: 500 mM NaH_2PO_4 pH 5.5. The sample was adjusted to pH 5.5 with H_3PO_4 and diluted to 3 mS/cm with water. Flowrate 5 ml/min. Gradient: 100% B in 10 CV. Fractions of 10 ml were collected.

Purification 4 (table 9.9):
Anion exchange column DEAE Sepharose FF XK50 130 ml. Binding buffer: 20 mM NaH_2PO_4 pH 5.5. Elution buffer: 500 mM NaH_2PO_4 pH 5.5. The sample was adjusted to pH 5.5 with H_3PO_4 and diluted to 3 mS/cm with water. Flowrate 15 ml/min. Gradient: 100% B in 10 CV. Fractions of 10 ml were collected.

Purification 5 (table 9.10):
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM Na-citrate + 20% $(NH_4)_2SO_4$ pH 6. Elution buffer: 10 mM Na-citrate pH 6. The sample was adjusted to pH 6 with K_2CO_3 and to 112 mS/cm with $(NH_4)_2SO_4$ (about 30%). Flowrate 5 ml/min. Gradient: 100% B in 2 CV. Fractions of 5 ml were collected.

Purification 6 (table 9.11):
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. Elution buffer: 50 mM NaH_2PO_4 pH 6. The sample was adjusted to pH 5.8 with K_2CO_3 and to 170 mS/cm with $(NH_4)_2SO_4$ (about 40%). Flowrate 3 ml/min. Gradient: 100% B in 5 CV, flowrate 4 ml/min. Fractions of 5 ml were collected.

Purification 7 (table 9.12):
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. Elution buffer: 50 mM NaH_2PO_4 pH 6. The sample was adjusted to pH 6 with 2 M NaH_2PO_4 pH 6 and NaOH and adjusted to 180 mS/cm with $(NH_4)_2SO_4$ (about 45%). Flowrate 4 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.

Final purification (table 9.13):
Anion exchange column DEAE Sepharose FF XK50 130 ml. Binding buffer: 20 mM NaH_2PO_4 pH 5.5. Elution buffer: 500 mM NaH_2PO_4 pH 5.5. The sample was adjusted to pH 6 with K_2CO_3 and diluted to 4 mS/cm with binding buffer and water. Flowrate 20 ml/min. Gradient: 100% B in 8 CV. Fractions of 10 ml were collected.
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. Elution buffer: 50 mM NaH_2PO_4 pH 6. The sample was adjusted to pH 6 with 1 M NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6 and adjusted to 160 mS/cm with $(NH_4)_2SO_4$. Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.

Amicon Ultra. Buffer change to 50 mM NaH_2PO_4 pH 6.

6.7 Purification Scheme for *B. aclada* Laccase

Purification 1 (table 9.17):
Anion exchange column Q Sepharose FF 35 ml. Binding buffer: 20 mM NaH_2PO_4 pH 6. Elution buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. The sample was adjusted to pH 7 with K_2CO_3 and diluted to 6 mS/cm with water. Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 10 ml were collected.
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. Elution buffer: 50 mM NaH_2PO_4 pH 6. The sample was adjusted to pH 5.8 and 170 mS/cm with $(NH_4)_2SO_4$ (about 40%). Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.

Final purification (table 9.18):
Anion exchange column Q Sepharose FF XK50 50 ml. Binding buffer: 20 mM NaH_2PO_4 pH 6. Elution buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. The sample was turraxed at 24000 rpm for 1 min and centrifuged. Then the sample was adjusted to pH 7 with K_2CO_3 and diluted to 4 mS/cm with water. Flowrate 18 ml/min. Gradient: 100% B in 5 CV. Fractions of 10 ml were collected.
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6. Elution buffer: 50 mM NaH_2PO_4 pH 6. The sample was adjusted to pH 5.8 and 165 mS/cm with $(NH_4)_2SO_4$ (about 40%). Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.
Amicon Ultra. Buffer change to 50 mM NaH_2PO_4 pH 6.

6.8 Purification Scheme for Recombinant *B. aclada* Laccase

Purification of first fermentation (table 9.19):
Anion exchange column Q Sepharose FF 35 ml. Binding buffer: 20 mM NaH_2PO_4 pH 6.5. Elution buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6.5. The sample was adjusted to pH 6.8 with K_2CO_3, diluted to 9 mS/cm with water and filtrated. Flowrate 10 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.
Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6.5. Elution buffer: 50 mM NaH_2PO_4 pH 6.5. The sample was adjusted to pH 6.3 and 160 mS/cm with $(NH_4)_2SO_4$ (about 30%). Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.

Purification of second fermentation (table 9.20):

Hydrophobic interaction chromatography Phenyl-Source 22 ml. Binding buffer: 50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$ pH 6.5. Elution buffer: 50 mM NaH_2PO_4 pH 6.5. The sample was adjusted to pH 6 with K_2CO_3 and to 160 mS/cm with $(NH_4)_2SO_4$ (about 30%). Then the sample was centrifuged. Flowrate 5 ml/min. Gradient: 100% B in 5 CV. Fractions of 5 ml were collected.

Amicon Ultra. Buffer change to 50 mM NaH_2PO_4 pH 5.

Chapter 7

Gelelectrophoresis

7.0.1 Molecular Mass Determination by SDS-PAGE

Gelelectrophoresis separates proteins by the influence of an applied electric field. Molecules migrate at a speed determined by their mass to charge ratio. Electrophoretic separation is most commonly performed in polyacrylamide gels. These gels are casted between a pair of glass plates by polymerising a solution of acrylamide monomers and bisacrylamide into polyacrylamide chains and cross-linking. Due to the concentration of bisacrylamide, the pore size of the gel can be adjusted. The anionic detergent SDS (sodium dodecyl sulphate) has a denaturising effect on proteins, attaches to them and gives them a negative surface charge. Depending on the size of the protein a different amount of SDS can bind, influencing the overall negative charge. Hence, the size or mass determines the negative charge and therefore the migration rate of the protein. β-Mercaptoethanol has a reducing effect and breaks up disulfide bridges. The molecular weight of a protein can be estimated by comparison of the migration rate through the gel with the distances of known protein standards. To get a linear dependency it is necessary to plot the logarithm of the molecular weight against the migration distance (R_f). SDS-gels were also used to control the purity of enzyme preparations.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to table 7.1 using a Hoefer Mighty Small SE 250 dual vertical gel apparatus (Amersham Biosciences). All solutions for the separating gel were mixed, then filled to the tight cast gadget and covered with 2-butanol to smooth the surface. After 30 to 45 min of polymerisation the 2-butanol was removed completely, washed with water and excess drops carefully removed with a paper towel. Afterwards all solutions for the stacking gel were mixed and poured on top of the separating gel. A slot-comb was placed in the gel immediately. After 30 to 45 min of polymerisation the slot-comb was removed and the gel covered with running buffer.

The samples were diluted 1:2 with sample buffer containing SDS for denaturation according to Laemmli

Chemicals	Separating Gel	Stacking Gel
H_2O	4.8 ml	3.4 ml
Acrylamid 30%	3.96 ml	0.83 ml
1.5 M Tris pH 8.8	3 ml	-
1 M Tris pH 6.8	-	0.63 ml
SDS 10%	0.12 ml	50 μl
APS* 10%	0.12 ml	50 μl
TEMED*	5 μl	5 μl

Table 7.1: Preparation of separating and stacking gel for SDS-PAGE. *Ammoniumpersulphate. *N,N,N′,N′-Tetramethylethylendiamine.

(1970) and incubated at 90°C for 5 min. The protein content should exhibit a minimum of 1 μg protein per band for commassie staining. 5 μl of the molecular weight standard mixture (BioRad Precision Plus Protein^{TM}Standards or Fermentas Page Ruler Prestained Protein Ladder SM0671) as well as 20 μl of each sample were applied to the gel and run at 200 V and 40-50 mA. The electrophoresis was stopped when the Brompenol blue front reached the end of the separating gel. Afterwards, the protein bands were visualised by either coomassie staining or silver staining. The molecular masses of the bands were determined by comparison with the standard markers.

SDS-PAGE was also performed with the fully automated Phast System (Pharmacia Biosciences). Solution preparation and operation was done according to the manufacturer's manual.

7.0.2 Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) is a type of electrophoresis, where native proteins are separated by charge but not by mass. To perform IEF a polyacrylamide gel is rehydrated with a solution of ampholytes, a mixture of poly-anionic and poly-cationic molecules with a mass of 300 to 600 Da. The ampholytes are separating when placed in an electric field, forming a continuous pH gradient based on their net charge. Charged proteins will be driven by the electric field through the pH gradient until they reach their isoelectric point (pI), which characterises the pH value at which the protein charges are equally balanced.

Isoelectric focusing in the pH range of 2.5 to 10 was performed with the Multiphor II system using precast gels (CleanGel IEF, Amersham Biosciences) with gradient focusing at 300 V for 30 min. 20 μl of sample or standard were applied to sample application pieces and loaded for 1 h. Afterwards the sample application pieces were removed and IEF was carried out for 3 more hours at 500 V. In table 7.2 the pI markers used to determine the pI values of the proteins are shown.

Low pI (pH 2.5-6.5)		**Broad pI (pH 3-10)**	
Protein	pI	Protein	pI
Pepsinogen	2.80		
Amyloglucosidase	3.50	Amyloglucosidase	3.50
Methyl red*	3.75	Methyl red*	3.75
Glucose oxidase	4.15		
Trypsin inhibitor	4.55	Trypsin inhibitor	4.55
β-Lactoglobulin A	5.20	β-Lactoglobulin A	5.20
Carbonic anhydrase B (bovine)	5.85	Carbonic anhydrase B (bovine)	5.85
Carbonic anhydrase B (human)	6.55	Carbonic anhydrase B (human)	6.55
		Myoglobin, acidic band	6.85
		Myoglobin, basic band	7.35
		Lentil lectin, acidic band	8.15
		Lentil lectin, middle band	8.45
		Lentil lectin, basic band	8.65
		Trypsinogen	9.30

Table 7.2: Components of the pI Calibration Kit for IEF. *Methyl red does not appear in final stained gel.

7.0.3 Active Staining

After IEF, laccase bands could be visualised by active staining with ABTS, as this is a native electrophoresis and the enzymes are still active. The assay is a modification of the standard ABTS assay, as the gel was incubated in an aqueous 10 mM ABTS-solution until green bands appeared. For further staining the gel was fixed and destained with 10% trichloroacetic acid over night, in which the proteins precipitate. Before performing a silver staining the gel was rinsed with water.

7.0.4 Coomassie Blue Staining

The gel was covered with coomassie brilliant blue staining solution and incubated for 2 hours while softly agitated. Afterwards, the stain was decanted and the gel was rinsed with water. This was followed by a destaining step, until only blue bands were visible on the gel. Then the gel was soaked in conservation solution. All solutions were prepared according to the manufacturer's manual (Pharmacia Biosciences).

7.0.5 Silver Staining

According to Ansorge (1985), the solutions for silver staining are shown in table 7.3. Incubation was done on an orbital shaker with soft agitation.

Preparation		**Incubation**	
Solution	Composition	Solution	Time
Fixing solution	30% methanol	Fixing solution	15 min
	10% acetic acid	$CuCl_2$ solution	15 min
$CuCl_2$ solution	50% methanol	Washing I	15 min
	15% acetic acid	$KMnO_4$ solution	15 min
	2% copper(II)chloride	Washing I	15 min
Washing I	10% ethanol	Washing II	15 min
	5% acetic acid	H_2O	15 min
$KMnO_4$ solution	0.002% $KMnO_4$	Silver dyeing solution	15 min
Washing II	10% ethanol	H_2O	2 x 20 sec
Silver dyeing solution	0.2% $AgNO_3$	Developer	bands visible*
Developer	2% K_2CO_3 + 20 μl	H_2O	15 min
	37% formaldehyde/100 ml H_2O		

Table 7.3: Solutions and incubation conditions for silver staining. *decant if the solution gets hazy and continue with fresh developer.

Part III

Results

Chapter 8

Cultivation

Four different strains of fungi (*Lamprospora wrightii*, *Botrytis aclada*, *Phoma destructiva* and *Glomerella cingulata*) were subcultured in different media and under different conditions to examine growth and laccase production. All experiments were carried out in duplicates and the the results were calculated as mean values.

During the cultivation experiments it turned out that the subculturing conditions on the agar plates have a big influence on the formation of laccase. As proved with spot tests, only fungi subcultured on PDA were capable of laccase production, which shows that some ingredients of this media are necessary for enzyme production. Furthermore, some petridish cultures with already enzyme producing strains lost the ability of producing laccase during subculturing, even though the right media were used.

8.1 Cultivation of *Lamprospora wrightii*

8.1.1 Determination of optimum Shaking Flask Conditions

First of all the effects of different shaking conditions were examined. Therefore *L. wrightii* was cultivated in shaking flasks at 0 rpm and 90 rpm at 25°C. Results can be seen in figures 14.1 to 14.3 shown in the Appendix. A lot of oxygen is required for appropriate growth, even though no laccase production occured. An indirect correlation between increase of protein and pH progress can be denoted, which indicates acid formation during growth. Highest biomass production was found in baffled flasks at 90 rpm.

8.1.2 Cultivation in Basal Media

Results can be seen in table 8.1 and in figures 14.4 to 14.12 shown in the Appendix. The pH always stays between pH 5 and 8, whereas it sometimes increases and sometimes decreases during growth. There is always an increase in protein that levels off after 10 to 15 days of incubaton. As expected, the height of the protein content is directly correlated to the amount of soy peptone used in the media. It seems,

that a low peptone content in the media is correlated with a decrease in pH, whereas a higher petone concentration leads to an increase in pH during growth. Cell growth and protein formation seems to be enhanced by addition of copper. An addition of trace elements does not have any effect. As there is no laccase production in simple basal media another approach was chosen.

Media	Protein Maximum				Activity Maximum			
	Day	pH	Protein [mg/ml]	Activity [U/ml]	Day	pH	Protein [mg/ml]	Activity [U/ml]
F10P1	15	6.4	0.17	0	-	-	-	-
F10P5	15	6.9	0.72	0	-	-	-	-
F5P1	15	6.7	0.17	0	-	-	-	-
F5P5	15	8.7	0.42	0	-	-	-	-
F10P5-T	15	7.3	0.73	0	-	-	-	-
G10P5	15	8.1	0.59	0	-	-	-	-
Gly10P5	15	8.2	0.28	0	-	-	-	-
F5G5P5	27	8.1	0.58	0	-	-	-	-
F10G5P1-Cu	22	5.4	0.09	0	-	-	-	-

Table 8.1: Maxima of protein content and laccase activity during cultivation of *L. wrightii* in basal media. - no protein/activity was found.

8.1.3 Cultivation in Vegetable Media

As *L. wrightii* is known to be a plant pathogen the production of laccase may be associated to plant stimuli, like pectines or lignin. Therefore different substrates based on herbal origin were used. Copper was added as it is often mentioned to act as an inducer for laccase production [16, 20].

Results for cultivation on potato media can be seen in table 8.2 and in figures 14.13 to 14.20 shown in the Appendix. The pH always stays between 5 and 7. An increase in protein content can be followed with its maximum after 10 to 12 days of incubation, always correlated to a decrease in pH. In comparision to the basal media the protein content is not levelling off, but slowly degraded after reaching its maximum. Also much more protein could be detected, indicating that different extracellular proteins or enzymes were built. A different pH and protein progress occured when *L. wrightii* was cultivated in a petridish or with glucose, indicating different growth properties. Protein content was lowest in the petridish, indicating that without movement the oxygen transfer rate is not sufficient for proper growth and enzyme secretion. Glucose also seems not to be appropriate for cultivation, which can be seen on the low protein production, indicating reduced growth. Both copper and trace elements slightly increased protein production. However, this had no significant effect on laccase activity.

Results for cultivation on tomato and vegetable juice media can be seen in table 8.2 and in figures 14.21 to 14.25 shown in the Appendix. Laccase production starts at 2 days with its maximum after 5 days and shows a fast decrease afterwards, correlated with an increase in pH. Laccase production is faster the

higher the vegetable juice is diluted, but also is depleted earlier. Protein content and pH increase with laccase production and level off like laccase activity. Laccase is produced and active only over a short period at the beginning of cultivation and is rapidly inactivated afterwards. Tomato juice and vegetable juice gave compareable and satisfactory results. The cultivation experiments with *B. aclada* showed, that the enzyme maximum appeared much faster in the potato media than in the tomato juice. As the first enzyme assay in the potato media was performed at the third day of cultivation, it is possible that the maximum already passed. Visually can be ascertained, that in diluted vegetable juice the media gets darkbrown to black, whereas in pure vegetable juice the media stays red over the whole experiment.

Results for cultivation on apple and grape juice media can be seen in table 8.2 and in figures 14.26 and 14.27 shown in the Appendix. The pH starts at much lower values compared to the other media. No appreciable laccase production occured.

	Protein Maximum				**Activity Maximum**			
Media	Day	pH	Protein [mg/ml]	Activity [U/ml]	Day	pH	Protein [mg/ml]	Activity [U/ml]
Potato Infusion								
PotF10	12	5.1	0.67	0.01	10	5.2	0.61	0.01
PotF10Cu0.01	10	5.6	0.62	0.01	10	5.6	0.62	0.01
PotF10-Cu	12	5.7	0.59	0.01	12	5.7	0.59	0.01
PotF10Cu5	10	5.4	0.61	0.01	10	5.4	0.61	0.01
PotF10T	12	5.3	0.74	<0.01	10	5.4	0.61	0.01
PotF10Petri	15	7.0	0.23	<0.01	3	7.4	<0.01	0.01
PotG10	15	6.0	0.47	<0.01	3	6.8	0.08	0.01
PotF5G5	12	5.3	0.65	<0.01	-	-	-	-
Vegetable Juice								
TomF10	31	8.0	0.28	0.01	5	4.6	<0.01	0.39
Tom1:2F10P5	14	8.1	0.43	0.05	5	6.5	0.17	0.60
Tom1:5F10P5	10	7.9	0.43	<0.01	3	5.2	<0.01	0.14
Tom1:10F10	27	5.3	0.34	<0.01	-	-	-	-
VeggyF10	31	8.4	0.28	0.03	5	4.6	<0.01	0.30
Fruit Juice								
AppleF10	14	6.9	0.41	<0.01	6	3.5	<0.01	0.08
GrapeF10	14	4.0	0.20	0.01	10	3.7	<0.01	0.02

Table 8.2: Maxima of protein content and laccase activity during cultivation of *L. wrightii* in vegetable juice media. - no protein/activity was found.

8.2 Cultivation of *Botrytis aclada*

Results for cultivation experiments can be seen in table 8.3 and in figures 15.1 to 15.6 shown in the Appendix. There is mentionable laccase production in potato, tomato and maltose media. The amount is compareable to *L. wrightii*. Interestingly, the maximum of laccase production in tomato juice was reached after 12 days, whereas in potato and maltose media the maximum was reached in less than 6

days. Laccase production is not directly related to the protein content. Contrary, in most cases the occurence of laccase is correlated to low protein contents, which led to the assumption, that this laccase has a very high specific activity. In tomato juice the degradation of laccase is correlated to an increase of protein. During growth there is always an increase in pH up to 7. Only in apple and grape juice the pH is not rising above pH 4. In grape juice there is a measureable protein content at the beginning, which has to be degraded before laccase production occurs. Anyway, there is no appreciable laccase production in grape or apple juice.

Furthermore *B. aclada* was cultivated in a petridish, but no mentionable protein and laccase production occured. During growth the fungi builded up a thick gel under the surface of the mycelium, due to an excessive formation of polysaccharides. This increase of viscosity led to problems during purification.

Media	**Protein Maximum**				**Activity Maximum**			
	Day	pH	Protein [mg/ml]	Activity [U/ml]	Day	pH	Protein [mg/ml]	Activity [U/ml]
PotF10	-	-	-	-	6	5.1	<0.1	0.22
TomF10	27	7.8	0.18	0.04	12	5.6	<0.1	0.25
Maltose	-	-	-	-	6	4.0	<0.1	0.21
Petridish*	-	-	-	-	9	5.0	<0.1	0.02
AppleF10	-	-	-	-	10	3.6	<0.1	0.04
GrapeF10	3	3.4	0.12	0	22	3.9	<0.1	0.05

Table 8.3: Maxima of protein content and laccase activity during cultivation of *B. aclada* under different conditions. *equals PotF10, but was cultivated in a petridish without shaking. - no protein/activity was found.

8.3 Cultivation of *Glomerella cingulata*

Results for cultivation experiments can be seen in table 8.4 and in figures 16.1 to 16.6 shown in the Appendix. No reasonable laccase production could be achieved under selected conditions. Highest laccase production occured in tomato juice with about 0.1 U/ml correlated to a high protein content and high pH. In comparison to the other strains, laccase production was very slow, but was stable and not degraded over time. The pH always increased during growth, reaching pH 7 in potato and tomato media. In apple and grape juice the pH stayed at very low levels of about 4. Furthermore *G. cingulata* was cultivated in maltose media (recommended by CBS) and in a petridish, but no protein and laccase production occured.

8.4 Cultivation of *Phoma destructiva*

Results for cultivation experiments can be seen in table 8.5 and in figures 17.1 to 17.6 shown in the Appendix. No laccase production could be achieved under selected conditions. In potato, tomato and maltose media the protein content was increasing with an increase in pH up to 9. In the petridish, apple

Media	Protein Maximum				Activity Maximum			
	Day	pH	Protein [mg/ml]	Activity [U/ml]	Day	pH	Protein [mg/ml]	Activity [U/ml]
PotF10	36	7.0	<0.1	0.01	36	7.0	<0.1	0.01
TomF10	36	7.5	0.34	0.07	36	7.5	0.34	0.07
Maltose	36	6.5	0.11	0	-	-	-	-
Petridish*	-	-	-	-	-	-	-	-
AppleF10	-	-	-	-	14	3.8	0	0.01
GrapeF10	6	3.4	0.14	0	14	3.6	0.12	0.03

Table 8.4: Maxima of protein content and laccase activity during cultivation of *G. cingulata* under different conditions. *equals PotF10, but was cultivated in a petridish without shaking. - no protein/activity was found.

and grape juice the pH stayed at very low levels of about 4. During performing the enzyme assays it could be observed that the activity always decreased and levelled off after a short period.

The cultivation experiments showed, that only for *L. wrightii* and *B. aclada* reasonable laccase production could be achieved. Therefore further experiments were only done with these two strains.

Media	Protein Maximum				Activity Maximum			
	Day	pH	Protein [mg/ml]	Activity [U/ml]	Day	pH	Protein [mg/ml]	Activity [U/ml]
PotF10	36	8.4	0.34	0	-	-	-	-
TomF10	36	8.8	0.55	0	-	-	-	-
Maltose	36	8.3	0.20	0	-	-	-	-
Petridish*	-	-	-	-	-	-	-	-
AppleF10	-	-	-	-	-	-	-	-
GrapeF10	3	3.3	0.12	0	-	-	-	-

Table 8.5: Maxima of protein content and laccase activity during cultivation of *P. destructiva* under different conditions. *equals PotF10, but was cultivated in a petridish without shaking. - no protein/activity was found.

8.5 Fermentation Experiments

8.5.1 Fermentation of *L. wrightii*

As already indicated by the cultivation experiments, soy peptone is not necessary for growth. However, in diluted tomato juice the laccase is degraded very quickly, most likely due to proteases. Therefore, soy peptone was added to stabilise the enzyme activity. Results were summarised in table 8.6 and in figure 8.1. In the first fermentation experiment it seems that laccase production occurs even more quickly than in shaking flasks. As the first enzyme assay was done after 3 days, it is possible that the laccase maximum already passed unnoticed. In the second fermentation experiment less soy peptone was used, as no significant effect could be seen on the stabilisation of the enzyme. $CuSO_4$ was used an inducer of laccase production. Nevertheless, the yield of enzyme was better at the first fermentation. In general,

Hrs.	pH	Temp. [°C]	O_2 %	Laccase [U/ml]	Protein [mg/ml]	Harvest* [l]	Batch [U]	Spec. Activity [U/mg]
0	4.4	25	100					
3	4.4	24.8	84					
69	5.7	24.1	36	0.22	0.09	7.25	1618	2.4
78	6.0	25.2	51	0.18	0.10	7.25	1270	1.8

Hrs.	pH	Temp. [°C]	O_2 %	Laccase [U/ml]	Protein [mg/ml]	Harvest* [l]	Batch [U]	Spec. Activity [U/mg]
0	4.8	25	110					
2	4.8	24.9	92					
18	4.6	25	3	0.11	<0.1			
26	4.6	24.9	1	0.12	<0.1			
49	5.0	25	1	0.16	<0.1			
67	5.2	25	2	0.16	<0.1			
71	5.2	25	2	0.15	<0.1	15.4	2360	>1.5

Table 8.6: **Top** First fermentation of *L. wrightii* in tomato juice with 10 g/l fructose and 5 g/l soy peptone. **Bottom** Second fermentation of *L. wrightii* in tomato juice with 10 g/l fructose and 1 g/l soy peptone. *remaining fermentation broth after centrifugation and filtration.

most enzyme productivity for *L. wrightii* could be reached under shaking flask conditions.

At both approaches the fermentation broth turned from red to brown, which has a negative effect on column material staining during purification.

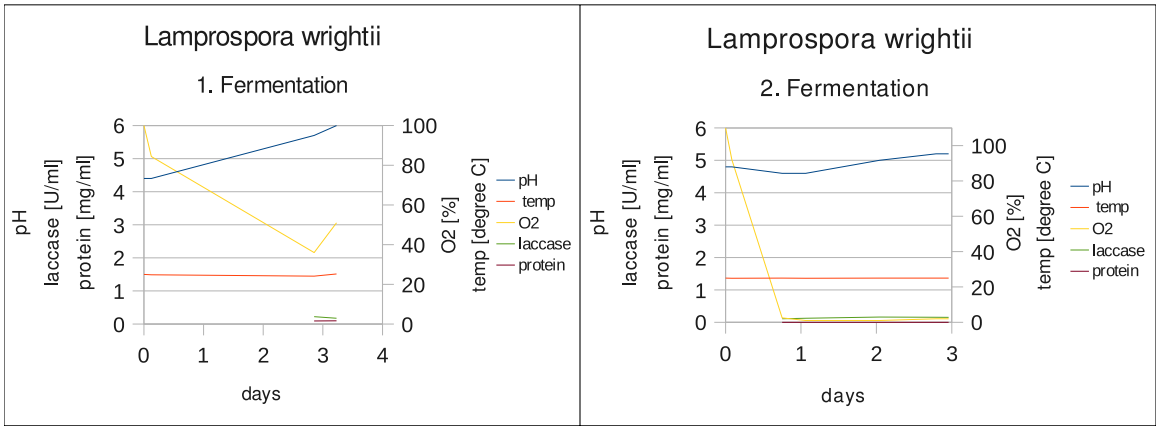

Figure 8.1: **Left figure** First fermentation of *L. wrightii* in tomato juice with 10 g/l fructose and 5 g/l soy peptone. **Right figure** Second fermentation of *L. wrightii* in tomato juice with 10 g/l fructose and 1 g/l soy peptone.

8.5.2 Fermentation of *B. aclada*

Results for fermentation can be seen in table 8.7 and in figure 8.2. Laccase production occured on the first day and increased steadily during the whole fermentation process. Due to time shortage the fermentation was stopped after 8 days, even though the laccase production could have been enhanced. A better overall

Hrs.	pH	Temp. [°C]	O_2 %	Laccase [U/ml]	Protein [mg/ml]	Harvest* [l]	Batch [U]	Spec. Activity [U/mg]
0	4.2	25	80					
1	4.2	25	73					
17	4.1	25.1	2	0.04	<0.1			
25	4.1	25	1	0.05	<0.1			
48	4.1	25	0	0.07	<0.1			
66	4.1	25	0	0.12	<0.1			
73	4.1	25	0	0.13	<0.1			
89	4	25	0	0.17	<0.1			
115	4.1	25.8	0	0.21	<0.1			
141	4.1	28.8	0	0.22	<0.1			
157	4.2	24	0	0.25	<0.1			
164	4.2	25.5	0	0.27	<0.1			
185	4.2	24.9	0	0.28	<0.1			
206	4.3	25.5	0	0.36	<0.1	13.2	4741	(4.3)

Table 8.7: Fermentation of *B. aclada* in tomato juice with 10 g/l fructose and 1 g/l soy peptone. *remaining fermentation broth after centrifugation and filtration. Number in brackets are estimated values, as the protein content was below the validated detection limit.

activity could be achieved compared to *L. wrightii*. The pH remained at lower values than in the shaking flask cultivation experiments, possibly stabilising enzyme activity. The fermentation broth turned again from red to brown. A higher impeller speed could be used next time, as the mycelium grew mainly at the bottom of the fermenter.

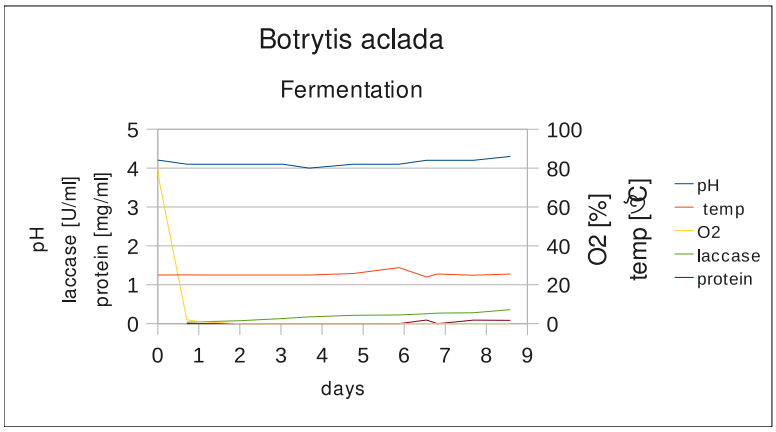

Figure 8.2: Fermentation of *B. aclada* in tomato juice with 10 g/l fructose and 1 g/l soy peptone

Chapter 9

Purification

9.1 Fractionated Precipitation

For *L. wrightii* the proteins in the culture supernatant start to precipitate at about 40 to 50% ammonium sulfate saturation, whereas laccase acitivity is decreasing continuously. At about 60 to 70% of ammonium sulfate the laccase starts to precipitate, which can be seen in table 9.1 and figure 9.1. It seems, that the laccase is inhibited by the salt, but is not precipitating until very high salt concentrations. This is confirmed by the fact that minimum specific activity in the supernatant is at about 40% saturation, whereas minimum specific activity in the precipitate is reached at about 60% saturation. This gap can be explained by protein precipitation at 40% salt concentration, which is not laccase. Even though it was shown that the laccase starts to precipitate at around 60% saturation, still a reasonable amount of laccase can be found in the supernatant at even higher concentrations. A maximum of 35% recovery could be achieved by precipitation with no increment of specific activity.

For *B. aclada* no laccase activity data in the pellet could be obtained, as the exopolysaccaride produced during growth made it impossible to resolve the pellet after precipitation. Results are shown in table 9.2 and figure 9.2. Only at very low salt concentrations up to 20% a slight increase in specific activity can be annotated. With higher ammonium sulfate concentrations laccase activity is decreasing constantly, whereas the protein content is more or less stable. This leads to a drastic decrease in specific activity, which can be explained by inhibition of the enzyme without any precipitation. In general, ammonium sulfate precipitation is not recommendable as a purification step due to inhibition of laccase by higher salt concentrations and its undefined precipitation characteristics.

Even though higher ammounium sulfate concentrations inhibit the enzyme, long term experiments showed that during storage the decrease in laccase activity was less distinct at higher salt concentrations. Anyway, the effect of inhibition is much more crucial than the stabilisation during storage. It also could be shown, that the inhibition of laccase by ammonium sulfate is reversible at small levels up to about 15%.

	Supernatant			**Pellet**		
$(NH_4)_2SO_4$ %	Activity [U/ml]	Protein [mg/ml]	Spec. Activity [U/mg]	Activity [U/ml]	Protein [mg/ml]	Spec. Activity [U/mg]
0	0.47	0.14	3.37	-	-	-
20	0.34	0.14	2.35	0.01	<0.1	0.37
30	0.30	0.15	1.94	0.01	<0.1	0.73
40	0.26	0.15	1.71	0.01	<0.1	0.62
50	0.23	0.13	1.74	0.01	<0.1	0.45
60	0.22	0.11	2.03	0.03	<0.1	0.49
70	0.19	0.08	2.45	0.10	0.08	1.30
80	0.16	0.05	3.34	0.17	0.10	1.72

	Laccase			**Protein**		
$(NH_4)_2SO_4$ %	Recovery Supernatant	Recovery Pellet	Recovery %	Recovery Supernatant	Recovery Pellet	Recovery %
20	73	2	74	104	16	120
30	64	2	66	111	8	119
40	55	2	56	108	9	116
50	50	3	53	97	19	116
60	46	6	52	77	38	114
70	40	22	61	54	56	111
80	33	35	69	34	69	103

Table 9.1: Fractionated precipitation of *L. wrightii* laccase with $(NH_4)_2SO_4$.

	Supernatant			**Recovery**	
$(NH_4)_2SO_4$ %	Activity [U/ml]	Protein [mg/ml]	Spec. Activity [U/mg]	Laccase %	Protein %
0	0.59	0.16	3.77	-	-
20	0.52	0.09	5.61	89	60
30	0.50	0.10	4.93	85	65
40	0.48	0.10	5.00	81	61
50	0.42	0.11	3.97	71	68
60	0.26	0.10	2.62	43	62
70	0.14	0.09	1.55	24	58
80	0.04	0.08	0.55	7	49

Table 9.2: Fractionated precipitation of *B. aclada* laccase with $(NH_4)_2SO_4$.

	L. wrightii		*B. aclada*
	Stability	**Reversibility**	**Stability**
$(NH_4)_2SO_4$ %	Recovery %	Recovery %	Recovery %
0	-19	-	-2
20	-12	+1	-4
30	-9	+3	-5
40	+2	+8	-6
50	+2	+10	0
60	+9	+14	+3
70	+20	+5	-42
80	+20	+16	-74

Table 9.3: Stability of laccase at the presence of different $(NH_4)_2SO_4$ concentrations for *L. wrightii* and *B. aclada*.

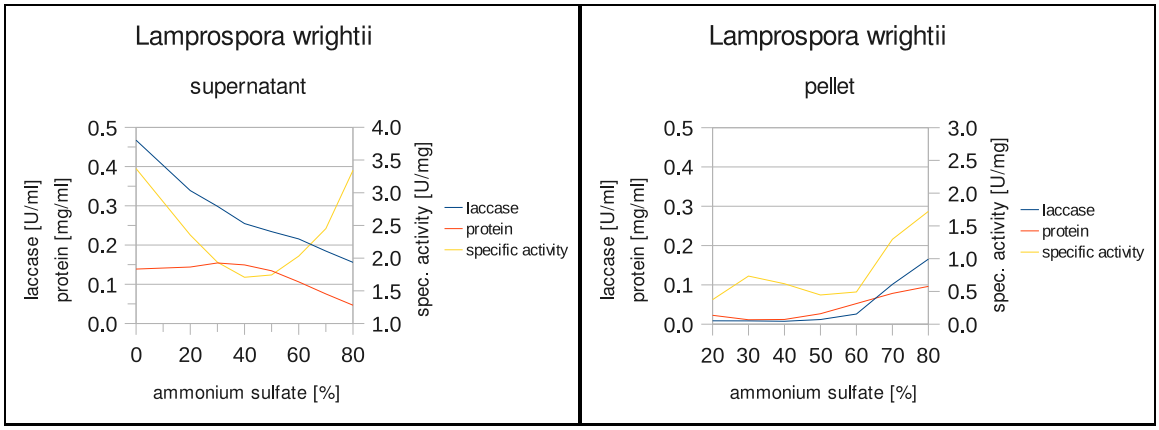

Figure 9.1: Fractionated precipitation of *L. wrightii* laccase with $(NH_4)_2SO_4$.

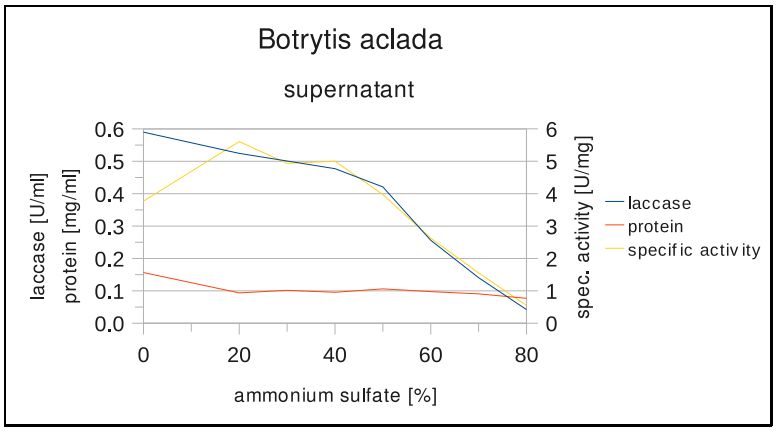

Figure 9.2: Fractionated precipitation of *B.aclada* laccase with $(NH_4)_2SO_4$.

9.2 *Lamprospora wrightii*

9.2.1 Preliminary Experiments

Stability Experiments

It was shown that laccase is very stable in all buffers used between a pH range of 5 to 6 (see table 9.4). Concering the salt stability laccase from *L. wrightii* is most active and stable in NaH_2PO_4 and K_2SO_4. Unfortunately, K_2SO_4 has a very low solubility in water and is therefore inappropriate for purification purposes. The higher the salt concentration the lower the laccase activity. Any additional salt addition should be avoided if possible, as it decreases laccase stability significantly. It has to be noticed that Na_2HPO_4 is less soluble than NaH_2PO_4 which has the consequence, that a 2 M NaH_2PO_4 buffer starts to precipitate at pH>6.

Buffer/Salt	pH	Applied Activity [U/ml]	Remaining Activity [U/ml]	Recovery %	Recovery after 6h %
20 mM Pyridine-acetate	3	0.16	0.15	92	-25
20 mM Pyridine-acetate	4	0.16	0.16	97	-16
20 mM Pyridine-acetate	5	0.16	0.19	118	-4
20 mM Pyridine-acetate	6	0.16	0.21	130	-8
20 mM Piperazine-acetate	5	0.16	0.19	115	-1
20 mM Piperazine-acetate	6	0.16	0.20	126	-7
20 mM Tris-acetate	7.5	0.16	0.18	109	-31
20 mM Sodium-acetate	4	0.16	0.16	98	-4
20 mM Sodium-acetate	5	0.16	0.20	124	+7
1 M $(NH_4)_2SO_4$	5.5	0.26	0.21	81	+6
1 M $(NH_4)_2HPO_4$	5.5	0.26	0.20	78	+2
1 M Na_2SO_4	5.2	0.26	0.20	78	+4
0.5 M NaH_2PO_4 + 1 M $(NH_4)_2SO_4$	6.2	0.065	0.045	68	
1 M NaH_2PO_4	5.3	0.069	0.073	105	
1 M NaH_2PO_4	5.5	0.26	0.25	96	-10
1 M NaH_2PO_4	6.4	0.069	0.067	96	
1 M NaH_2PO_4	7.1	0.069	0.063	91	
1 M NaH_2PO_4 + 0.5 M $(NH_4)_2SO_4$	6.2	0.065	0.055	84	
1 M NaH_2PO_4 + 1 M $(NH_4)_2SO_4$	5.4	0.069	0.049	70	
1 M NaH_2PO_4 + 2 M $(NH_4)_2SO_4$	5.4	0.069	0.036	51	
1 M NaH_2PO_4 + 0.6 M K_2SO_4	6.2	0.065	0.052	79	
1.5 M NaH_2PO_4	6.2	0.065	0.071	108	
2 M NaH_2PO_4	5.4	0.069	0.067	97	
2 M NaH_2PO_4	6.2	0.069	0.057	82	
2 M NaH_2PO_4	7.1	0.069	0.056	81	
3 M NaH_2PO_4	5.4	0.069	0.058	84	
3 M NaH_2PO_4	6.1	0.069	0.049	70	
1 M Na-acetate	5.1	0.26	0.16	61	-20
0.5 M Na-citrate	5.3	0.26	0.18	70	-10
0.33 M K_2SO_4	5.7	0.26	0.25	98	-10
0.65 M KH_2PO_4	5.4	0.26	0.25	96	-19

Table 9.4: Preliminary experiments: stability of *L. wrightii* laccase in different buffers and with different salts. Applied initial laccase activities varied and are indicated in the table.

Binding and Elution Experiments

The experiments were carried out at small scale in Eppendorf tubes measuring laccase activity in the supernatant. Results are summarised in table 9.5. If the remaining enzyme activity was below 50% these conditions were listed as "binding conditions". If the remaining enzyme activity was above 50% these conditions were listed as "elution conditions". With all buffers and salt combinations used adequate IEX binding and elution conditions were achieved. The most effective and easiest way to do it is to simply vary the molarity of a NaH_2PO_4 buffer. It can be seen that the binding of laccase on a HIC column is forced by either higher buffer strength or higher pH. Very effective is also the addition of at least 1 M $(NH_4)_2SO_4$. No sufficient binding occured on a butyl-sepharose FF material, as it has less hydrophobic capacity than a phenyl residue.

9.2.2 Purification Experiments

Different abbreviations are used to describe the different stages during purification. *Start* means the sample after storage and adjusting to the particular purification conditions. *Pool* means the collected, pooled fractions, where laccase was found in an adequate amount and purity. *Waste* means the solution collected from sample application to the column and rinsing with binding buffer afterwards.

Purification experiment 1 (table 9.6). The total loss of enzyme was tremendously high with about 90%. The major part was lost during storage of the sample over night at 4 °C due to proteolytic activity in the clarified concentrated supernatant. Concering the first purification step, about 50% of the enzyme was lost. Na-citrate buffer is protonated 3 times at pH 6.5 and thereby strongly negatively charged. Maybe this leads to interactions with the anion exchange column. But due to the fact that the laccase could not be found in the breakthrough it is possible that the addition of buffer or NaCl leads to a loss of function. Anyway, a higher pH in the sample will increase its negative charge and lead to a better binding to the column. On the other hand, a pH above 6 negatively affects the stability of the enzyme.

Purification Experiment 2 (table 9.7). Ultrafiltration was done to concentrate the sample and to get rid of small molecules and contaminants such as salts and dyes. It can be seen that ultrafiltration is a effective method to concentrate the sample and also leads to a small increment in purity of the enzyme. However, the AIEX leads to a lot of loss of enzyme but in turn does not lead to any increase in purity. During elution two peaks could be detected, which could not be clearly separated. Next time the elution gradient should be extended to try to separate the peaks completely. Interestingly, the protein content, indicated by 280 nm, was not directly correlated with the detection of laccase. This leads to the assumption, that the sample is very impure, which also can be seen by the dark brown colour of the crude extract.

			Binding Conditions		Elution Conditions	
Buffer/Salt	pH	Column	Recovery %	Activity [U/ml]	Recovery %	Activity [U/ml]
20 mM Pyridine-acetate	3.6	DEAE FF			73	0.118
20 mM Pyridine-acetate	4.4	DEAE FF	14	0.023		
20 mM Pyridine-acetate	5.4	DEAE FF	8	0.013		
20 mM Pyridine-acetate	5.7	DEAE FF	5	0.009		
20 mM Pyridine-acetate	6.6	DEAE FF	5	0.009		
20 mM Pyridine-acetate + 0.6 M K_2SO_4	5.3	DEAE FF			73	0.127
20 mM Piperazine-acetate	5.3	DEAE FF	14	0.022		
20 mM Piperazine-acetate	5.5	DEAE FF	5	<0.01		
20 mM Piperazine-acetate	6.2	DEAE FF	12	0.019		
20 mM Piperazine-acetate + 0.6 M K_2SO_4	5.5	DEAE FF			73	0.127
20 mM Tris-acetate	7.5	DEAE FF	4	<0.01		
20 mM Tris-acetate	7.7	DEAE FF	1	<0.01		
20 mM Tris-acetate + 0.6 M K_2SO_4	7.6	DEAE FF			64	0.110
20 mM Sodium-acetate	4.8	DEAE FF	15	0.024		
20 mM Sodium-acetate	5.8	DEAE FF	14	0.023		
20 mM NaH_2PO_4	6.4	DEAE FF	7	0.013		
100 mM NaH_2PO_4	6.1	DEAE FF	21	0.036		
500 mM NaH_2PO_4	5.9	DEAE FF			85	0.146
1 M NaH_2PO_4	5.7	DEAE FF			82	0.142
1.5 M NaH_2PO_4	5.5	DEAE FF			78	0.134
2 M NaH_2PO_4	5.4	DEAE FF			75	0.130
0.5 M NaH_2PO_4	4.1	Phenyl FF			68	0.027
0.5 M NaH_2PO_4 + 1 M $(NH_4)_2SO_4$	6.2	Phenyl FF	36	0.024		
1 M NaH_2PO_4	4.3	Phenyl FF			63	0.026
1 M NaH_2PO_4	5.1	Phenyl FF			72	0.050
1 M NaH_2PO_4	6.3	Phenyl FF			87	0.060
1 M NaH_2PO_4	7.1	Phenyl FF			69	0.048
1 M NaH_2PO_4 + 0.5 M $(NH_4)_2SO_4$	6.2	Phenyl FF	44	0.029		
1 M NaH_2PO_4 + 1 M $(NH_4)_2SO_4$	5.4	Phenyl FF	6	<0.01		
1 M NaH_2PO_4 + 2 M $(NH_4)_2SO_4$	5.4	Phenyl FF	0	<0.01		
1 M NaH_2PO_4 + 0.6 M K_2SO_4	6.2	Phenyl FF	24	0.016		
1 M NaH_2PO_4	5.3	Butyl FF			81	0.056
1.5 M NaH_2PO_4	6.2	Phenyl FF			58	0.038
2 M NaH_2PO_4	4.3	Phenyl FF			65	0.027
2 M NaH_2PO_4	5.3	Phenyl FF	24	0.017		
2 M NaH_2PO_4	6.2	Phenyl FF	10	<0.01		
2 M NaH_2PO_4	7.1	Phenyl FF	2	<0.01		
2 M NaH_2PO_4	5.4	Butyl FF			69	0.048
3 M NaH_2PO_4	4.3	Phenyl FF	30	0.012		
3 M NaH_2PO_4	5.3	Phenyl FF	1	<0.01		
20 mM Pyridine + 0.6 M K_2SO_4	4.3	Phenyl FF			58	0.024

Table 9.5: Preliminary experiments: conditions for binding and elution of *L. wrightii* laccase at DEAE Sepharose FF (0.1616 and 0.1731 U/ml, respectively). Conditions for binding and elution of *L. wrightii* laccase at Phenyl- or Butyl-Sepharose FF (0.0406, 0.0694 and 0.0654 U/ml, respectively).

Purification experiment 3 (table 9.8). It can be seen that it is necessary to apply a very long gradient to resolve the laccase in an appropriate purity. However, the relation between gain of purity and loss of enzyme is not sustainable. There is a loss of about 75% laccase during purification. However, most laccase (about 50%) got lost during ultrafiltration. This happened to due several interruptions and long storage during the ultrafiltration process.

Purification experiment 4 (table 9.9). Major problems were pH shifts during sample application and the dark brown colour of the crude extract. It can be speculated, that the dye is interacting with the Bradford assay and the UV detector signal at 280 nm. However, most laccase (about 50%) was lost during ultrafiltration. This happened to due several interruptions and long storage during the ultrafiltration process. Overall, DEAE only leads to a 2 fold increase in purity with a total recovery of about 60%.

Purification experiment 5 (table 9.10). The sample was not binding to the column due to the low conductivity of the sample. The sample has to be mixed with $(NH_4)_2SO_4$ to achieve a conductivity of more than 120 mS/cm. It is also adviseable to change the buffer to NaH_2PO_4, as this stabilises the enzyme.

Purification experiment 6 (table 9.11). With a conductivity of 170 mS/cm the enzyme was now binding to the column, even though a big amount still went through. To account for that only an increase in pH or conductivity is possible. Laccase is eluted from the column as a peak, but can be found in small amounts in all fractions afterwards. This fact contributes to the two pools collected.

Purification experiment 7 (table 9.12). The conductivity was increased to about 45% $(NH_4)_2SO_4$, which still has no mentionable effect on enzyme stability, but leads to a good binding to the column. Overall, HIC leads to a 6 fold increase in purity with a total recovery of about 85%.

Final purification experiment (table 9.13). It is not usefull to dilute the sample for DEAE with binding buffer to decrease conductivity. On one hand the ionic strength is too low to achieve any buffering effect, on the other hand the ionic strength is high enough that it's not possible to decrease the conductivity to a proper extend. The purification was satisfactory with a 26 fold increase in purity, 35% recovery and a specific activity of 150 U/mg. This enzyme preparation was used for further characterisation studies.

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	200	0.49	98	<0.1	-	-	100
Start AIEX	400	0.06	23	<0.1	-	-	24
Pool AIEX	50	0.06	3	<0.1	-	-	3
Waste AIEX	800	0.01	9	<0.1	-	-	9

Table 9.6: Purification (1) of laccase from *L. wrightii* using an anion exchange column DEAE Sepharose FF XK50 130 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	1050	0.10	110	0.10	1.1	-	100
Ultrafiltration	240	0.52	125	0.13	3.9	3.5	114
Start AIEX	700	0.15	106	<0.1			96
Pool AIEX	70	0.83	58	0.22	3.8	3.5	53
Waste AIEX	170	0.02	4	<0.1			4

Table 9.7: Purification (2) of laccase from *L. wrightii* using an anion exchange column DEAE Sepharose FF XK16 24 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	1290	0.20	260	0.09	2.2		100
Ultrafiltration	300	0.40	120	0.17	2.4	1.1	46
Start AIEX	900	0.12	110	<0.1	(2.4)		42
Pool 1 AIEX	40	0.30	12	0.10	2.9	1.3	5
Pool 2 AIEX	60	0.13	7	<0.1	(1.5)		3
Pool 3 AIEX	130	0.32	42	<0.1	(5.2)	(2.4)	16
Waste AIEX	180	0.02	3	<0.1			1

Table 9.8: Purification (3) of laccase from *L. wrightii* using an anion exchange column DEAE Sepharose FF XK16 24 ml. Number in brackets are estimated values, as the protein content was below the validated detection limit.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	5390	0.20	1095	0.09	2.2		100
Ultrafiltration	1250	0.40	500	0.17	2.4	1.1	46
Start AIEX	3380	0.14	480	<0.1			44
Pool 1 AIEX	160	0.34	54	0.13	2.6	1.2	5
Pool 2 AIEX	440	0.15	68	0.12	1.3	0.6	6
Pool 3 AIEX	350	0.49	170	0.15	3.2	1.5	16
Waste AIEX	3700	0.01	44				4

Table 9.9: Purification (4) of laccase from *L. wrightii* using an anion exchange column DEAE Sepharose FF XK50 130 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	100	0.18	18	0.10	1.8	-	100
Start HIC	130	0.09	11	0.15	0.55	-	61
Pool HIC	-	<0.01	-	-	-	-	
Waste HIC	130	0.08	11	0.10	0.87	1.6	61

Table 9.10: Purification (5) of laccase from *L. wrightii* using a hydrophobic interaction chromatography column Phenyl-Source 22 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Pool DEAE	400	0.38	150	0.12	3.2		100
Start HIC	1000	0.12	120	<0.1			80
Pool 1 HIC	35	1.64	57	0.27	6.1	1.9	38
Pool 2 HIC	80	0.21	16	0.25	0.8	0.3	11
Waste HIC	1000	0.06	58				39

Table 9.11: Purification (6) of laccase from *L. wrightii* using a hydrophobic interaction chromatography column Phenyl-Source 22 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Pool DEAE	340	0.41	140	0.09	4.4		100
Start HIC	850	0.14	120	<0.1			86
Pool 1 HIC	25	4.24	105	0.16	27	6.1	75
Pool 2 HIC	50	0.27	14	0.32	0.9	0.2	10
Waste HIC	950	<0.01					

Table 9.12: Purification (7) of laccase from *L. wrightii* using a hydrophobic interaction chromatography column Phenyl-Source 22 ml.

Sample	Volume [ml]	Activity [U/ml]	Total Activity [U]	Protein [mg/ml]	Spec. Activity [U/mg]	Purification [fold]	Yield %
Crude Extract	7060	0.15	1080	<0.1			100
Ultrafiltration	1100	0.85	935	0.15	5.8		86
Start AIEX	2200	0.31	680	<0.1	(3.8)		63
Pool 1 AIEX	250	0.32	80	0.10	3.2	0.6	7
Pool 2 AIEX*	350	1.11	390	<0.1	(15)	(2.6)	36
Waste AIEX	2400	<0.01		<0.1			
Start HIC	950	0.31	300	<0.1	(12)	(2.1)	28
Pool 1 HIC*	40	8.34	330	<0.1	(96)	(17)	31
Pool 2 HIC	60	0.25	15	0.20	1.2	0.2	1
Waste AIEX	970	<0.01					
Amicon	15	24	355	0.16	150	26	33

Table 9.13: Final purification of laccase from *L. wrightii* using an anion exchange column DEAE Sepharose FF XK50 130 ml, a hydrophobic interaction chromatography column Phenyl-Source 22 ml and Amicon Ultra. *these fractions were used for further purification. Number in brackets are estimated values, as the protein content was below the detection limit.

Buffer/Salt	pH	Applied Activity [U/ml]	Remaining Activity [U/ml]	Recovery %
20 mM NaH_2PO_4	4.0	0.470	0.622	132
20 mM NaH_2PO_4	5.0	0.470	0.584	124
20 mM NaH_2PO_4	6.0	0.470	0.428	91
20 mM NaH_2PO_4	7.0	0.470	0.481	103
20 mM NaH_2PO_4	8.0	0.470	0.362	77
20 mM NaH_2PO_4	9.0	0.470	0.250	53
20 mM NaH_2PO_4	10	0.470	0.155	33
20 mM Tris-acetate	7.3	0.146	0.144	98
20 mM Tris-acetate	8.2	0.146	0.104	71
20 mM Tris-acetate	9.2	0.146	0.061	42
20 mM Pyridine-acetate	6.1	0.146	0.156	107
50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$	5.0	0.194	0.164	85
50 mM NaH_2PO_4 + 40% $(NH_4)_2SO_4$	6.0	0.194	0.164	85

Table 9.14: Preliminary experiments: stability of *B. aclada* laccase in different buffers. Applied initial laccase activities varied and are indicated in the table.

9.3 *Botrytis aclada*

9.3.1 Preliminary Experiments

Stability Experiments

Results can be seen in table 9.14. It can be stated that enzyme activity is decreasing with increasing pH. All denoted buffers can be used and show similar effects on laccase activity. $(NH_4)_2SO_4$ seems to have a positive effect on enzyme stability, as activity stays stable at all tested pH values.

Binding and Elution Experiments

The experiments were carried out at small scale in Eppendorf tubes measuring laccase activity in the supernatant. Results are summarised in table 9.15. If the remaining enzyme activity was below 50% these conditions were listed as “binding conditions”. If the remaining enzyme activity was above 50% these conditions were listed as “elution conditions”. With a weak anion exchange column (DEAE) a very high pH is needed to achieve satisfactory binding. Remaining activity in the supernatant is decreasing with increasing pH, which correlates to enzyme stability in the corresponding buffers. However, there seems to be a dinstinct pH at which the enzyme starts to bind to the column independent from its activity behaviour due to pH. This is indicated by the rapid decrease of activity in the supernatant between a pH of 7.0 and 8.5, whereas the stability of the enzyme in this pH range is much higher. With a strong anion exchange column (Q) a strong binding already occurs at pH 6. A weak cation exchange column (CM) seems to be inadequate for enzyme purification. For HIC pH 6 is sufficient for proper binding under stated conditions.

Further experiments were performed using a strong cation exchange column (S) for enzyme purification.

			Binding Conditions		Elution Conditions	
Buffer/Salt	pH	Column	Recovery %	Activity [U/ml]	Recovery %	Activity [U/ml]
20 mM NaH_2PO_4	3.4	DEAE			78	0.159
20 mM NaH_2PO_4	4.6	DEAE			96	0.194
20 mM NaH_2PO_4	5.6	DEAE			88	0.179
20 mM NaH_2PO_4	6.1	DEAE			59	0.119
20 mM NaH_2PO_4	7.0	DEAE			68	0.137
20 mM NaH_2PO_4	7.8	DEAE	29	0.058		
20 mM NaH_2PO_4	8.5	DEAE	2	0.003		
20 mM NaH_2PO_4	9.2	DEAE	2	0.003		
20 mM NaH_2PO_4	6.1	Q	4	0.007		
20 mM NaH_2PO_4	7.0	Q	3	0.006		
20 mM NaH_2PO_4	7.8	Q	1	0.001		
20 mM NaH_2PO_4	4.7	CM			96	0.196
20 mM NaH_2PO_4	5.3	CM			95	0.192
20 mM NaH_2PO_4	5.9	CM			58	0.119
20 mM NaH_2PO_4	6.9	CM			68	0.138
50 mM NaH_2PO_4 + 40 percent $(NH_4)_2SO_4$	5.2	phenyl	4	0.008		
50 mM NaH_2PO_4 + 40 percent $(NH_4)_2SO_4$	6.0	phenyl	3	0.005		
50 mM NaH_2PO_4 + 40 percent $(NH_4)_2SO_4$	6.9	phenyl	3	0.006		
20 mM Tris-acetate	7.4	DEAE			56	0.082
20 mM Tris-acetate	8.3	DEAE	14	0.020		
20 mM Tris-acetate	9.2	DEAE	4	0.006		
20 mM Pyridine-acetate	6.2	DEAE			72	0.105

Table 9.15: Preliminary experiments: buffer and pH for binding of *B. aclada* laccase at DEAE Sepharose FF, Q Sepharose FF, CM and HIC (0.2029, 0.1565, 0.1463 and 0.1935 U/ml, respectively).

A ready-to-use 1 ml CIEX column from PALL was used. Results can be seen in table 9.16. Fifty percent of laccase activity can be retrieved at pH 3, but the rest is still bound to the column. Therefore, a higher salt concentration for elution was used. At pH 4 the laccase did not bind to the column properly. This means that for using a cation exchange column a pH of 3 has to be adjusted, which in turn limits enzyme stability.

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Yield %	Volume [ml]	Activity [U/ml]	Total Act. [U]	Yield %
Start CIEX	17	0.47	8		22	0.45	10	
Pool CIEX	5	0.84	4	50	5	0.01	0	0
Waste CIEX	18	0.07	1	13	22	0.27	6	60

Table 9.16: Preliminary experiments: purification of laccase from *B. aclada* with a cation exchange column PALL S HyperD F 1 ml. **Left** Purification 1 at pH 3. **Right** Purification 2 at pH 4.

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Protein [mg/ml]	Spec. Act. [U/mg]	Purification [fold]	Yield %
Crude Extract	1450	0.36	520	<0.1	(4.3)		100
Crossflow	330	1.37	450	0.23	6.0	1.4	87
Start Q	700	0.52	360	0.13	4.1		69
Pool Q	30	2.33	70	0.26	9	2.1	14
Waste Q	800	0.29	230				44
Start HIC	50	0.97	48	0.10	9.5	2.2	9
Pool HIC	25	1.47	37	<0.1	(120)	(28)	7
Waste HIC	100	<0.01					

Table 9.17: Purification (1) of laccase from *B. aclada* using an anion exchange column Q Sepharose FF 35 ml and a hydrophobic interaction chromatography column Phenyl-Source 22 ml. Number in brackets are estimated values, as the protein content was below the validated detection limit.

9.3.2 Purification Experiments

Purification experiment 1 (table 9.17). The 2-fold purification with Q Sepharose is satisfactory, but the yield of laccase activity is quite poor. For binding on the Q Sepharose column a pH of 6 is at the lower limit. During washing with binding buffer the pH was sometimes declining under the critical pH, which could have led to an early elution of already bound laccase. The recovery for HIC exhibits about 75% with a 13 fold increase in purity. A lot of losses during storage can be annotated. Overall a 28 fold increase in purity could be achieved, with a recovery of 7%.

Final purification experiment (table 9.18). A bigger column was packed to lower the backpressure and increase the flow. The sample showed a very high viscosity which leaded to problems due to loading the sample onto the column. Therefore the sample was mixed wih an Ultraturrax to break polysaccharides and reduce viscosity. There was no increase in purity with Q sepharose, but the recovery was much better (30%) this time. Still the binding was very poor. A specific activity of 27 U/mg could be achieved with HIC. Even though in previous experiments using the same conditions a specific activity of 120 U/mg could be reached, this enzyme preparation was used for further characterisation studies due to its higher total activity. Also a lot of activity losses during storage can be annotated. Overall a 6-fold increase in purity could be achieved, with a recovery of about 10%.

Purification of Recombinant *B. aclada* Laccase

Results from the first fermentation can be seen in table 9.19. The crude extract was first centrifuged to separate the biomass. The fermentation broth showed both a high volumetric and specific activity, indicating a good expression of laccase by *Pichia pastoris*. While adjusting the pH for anion exchange protein precipitation occured, which was removed by filtrating the sample through a paper filter. It can be seen that adjusting the pH led to an increase in specific activity. Unfortunately nearly the whole

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Protein [mg/ml]	Spec. Act. [U/mg]	Purification [fold]	Yield %
Crude Extract	4490	0.36	1610	<0.1	(4.3)		100
Crossflow	1020	1.37	1395	0.23	6.0	1.4	87
Start Q	2150	0.39	845	<0.1	(5.1)	(1.2)	53
Pool Q	80	3.33	265	0.67	5.0	1.2	17
Waste Q	1800	0.23	410				26
Start HIC	125	1.88	235	0.45	4.1		15
Pool HIC	25	7.62	190	0.28	27	6.3	12
Waste HIC	160	<0.01					
Amicon	12	12	145	0.44	27	6.3	9

Table 9.18: Final purification of laccase from *B. aclada* using an anion exchange column Q Sepharose FF XK50 50 ml, a hydrophobic interaction chromatography column Phenyl-Source 22 ml and Amicon Ultra. Number in brackets are estimated values, as the protein content was below the validated detection limit.

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Protein [mg/ml]	Spec. Act. [U/mg]	Purification [fold]	Yield %
Crude Extract	90	19.6	1767	0.94	20.9		100
Start Q	1600	1.07	1706	<0.1	(29.8)	(1.4)	97
Pool Q	25	1.34	33	1.15	1.2	0.06	2
Waste Q	1610	<0.01					
Start HIC	37	0.73	27	0.66	1.1		2
Pool HIC	10	2.30	23	0.75	3.1	0.15	1
Waste HIC		<0.01					

Table 9.19: Purification of laccase from *B. aclada* recombinant first fermentation using an anion exchange column Q Sepharose FF 35 ml and a hydrophobic interaction chromatography column Phenyl-Source 22 ml. Number in brackets are estimated values, as the protein content was below the validated detection limit.

enzyme got lost during anion exchange chromatography, leading to a drastic decrease in specific activity. HIC showed a overall good performance, leading to a 3-fold purification with good recovery. Using a Q Sepharose under the described conditions is not useful for purification purposes.

In a second approach only the hydrophobic interaction chromatography step was used, followed by ultrafiltration. Results can be seen in table 9.20. The volumetric and specific activity from the second fermentation can be compared to the first one. During sample application onto the HIC column the backpressure constantly increased and new precipitate occured. A 3-fold increase in purity could be achieved with HIC, but recovery was quiet poor. The ultrafiltration step also led to an increase in purity, but also to losses in yield. Overall a recovery of 20% and a specific activity of 60 U/mg could be achieved.

Sample	Volume [ml]	Activity [U/ml]	Total Act. [U]	Protein [mg/ml]	Spec. Act. [U/mg]	Purification [fold]	Yield %
Crude Extract	18	15.6	281	0.91	17.2		100
Start HIC	25	9.48	237	0.61	15.5		84
Pool HIC	15	6.93	104	0.15	46.9	2.7	37
Waste HIC		<0.01					
Amicon	2.5	22.7	57	0.37	61.2	3.6	20

Table 9.20: Purification of laccase from *B. aclada* recombinant second fermentation using a hydrophobic interaction chromatography column Phenyl-Source 22 ml and Amicon Ultra.

Chapter 10

Isoelectric Focusing and SDS-PAGE

Isoelectric focusing of *L. wrightii* and *B. aclada* laccase can be seen in picture 10.1. The active staining shows, that the *L. wrightii* laccase has a more acidic pI than the *B. aclada* laccase. The protein content was not enough to get any bands visible on silver staining. Unfortunately the bands, including the standards, run very uneven which could be attributed to a high salt content in the sample. Therefore the pI could only be roughly estimated to be 4.0 for *L. wrightii* laccase and 5.2 for *B. aclada* laccase. Isoforms could not be detected for any of these strains.

The progress of laccase purification from fermentation to ultrafiltration for *L. wrightii* and *B. aclada* was observed by SDS-PAGE using the vertical gel apparatus. In picture 10.2 hardly any bands can be seen on coomassie as well as on silver staining for *L. wrightii*. This can be attributed to a very low protein content in the sample. However, the overall activity is satisfactory, as the specific activity is very high. For *B. aclada* the protein content was much higher, resulting in a lower specific activity. Anyway, for both strains a band with the size of about 75 kDa was concentrated, even though the *B. aclada* laccase doesn't seem to be as pure as desired. It has to be annotated, that some proteins with a higher molecular weight were also concentrated to a remarkable extend. Bands at about 100, 120 and 170 kDA can be detected.

The *B. aclada* laccase produced from the wildtype and expressed in *Pichia pastoris* were compared by SDS-PAGE using the Phast system. Picture 10.3 shows, that for the recombinant enzyme a proper purification could be achieved, enriching two proteins with a molecular weight of about 100 and 75 kDa. A third protein with a molecular weight of about 37 kDa is still present, which could not be removed by purification. Comparing the wildtype and the recombinant samples both show a distinct band at about 100 kDa. For the wildtype laccase there is also a remarkable band visible at about 37 kDa. Interestingly, on previous gels using the vertical gel apparatus a distinct band at 75 kDa was visible, but non at lower mass units. This shows, that the two different SDS-PAGE methods used are not compareable in terms

of molecular mass determination. Most likely the 75 kDa band on the vertical gel apparatus corresponds to the 100 kDa band on the Phast system. Furthermore, the 37 kDa band seems to be a degradation product of the laccase formed during storage. Anyway, deglycosilation of both *B. aclada* wildtyp and recombinant enzymes gave a distinct band of about 65 kDa, even though the pattern on SDS-Page in the crude samples are quite different and hard to interpret.

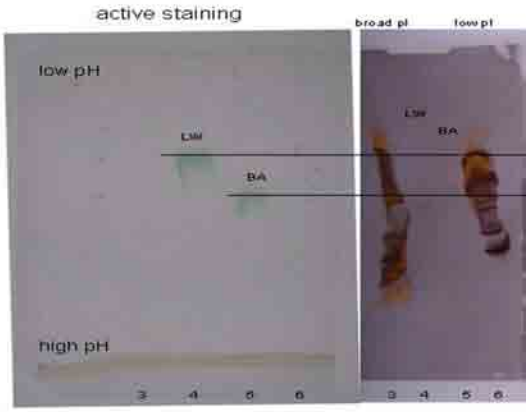

Figure 10.1: Isoelectric focussing from *L. wrightii* and *B. aclada* laccase. **Lane 3** - Broad pI Standard. **Lane 4** - *L. wrightii* Fermentation broth. **Lane 5** - *B. aclada* Fermentation broth. **Lane 6** - Low pI Standard.

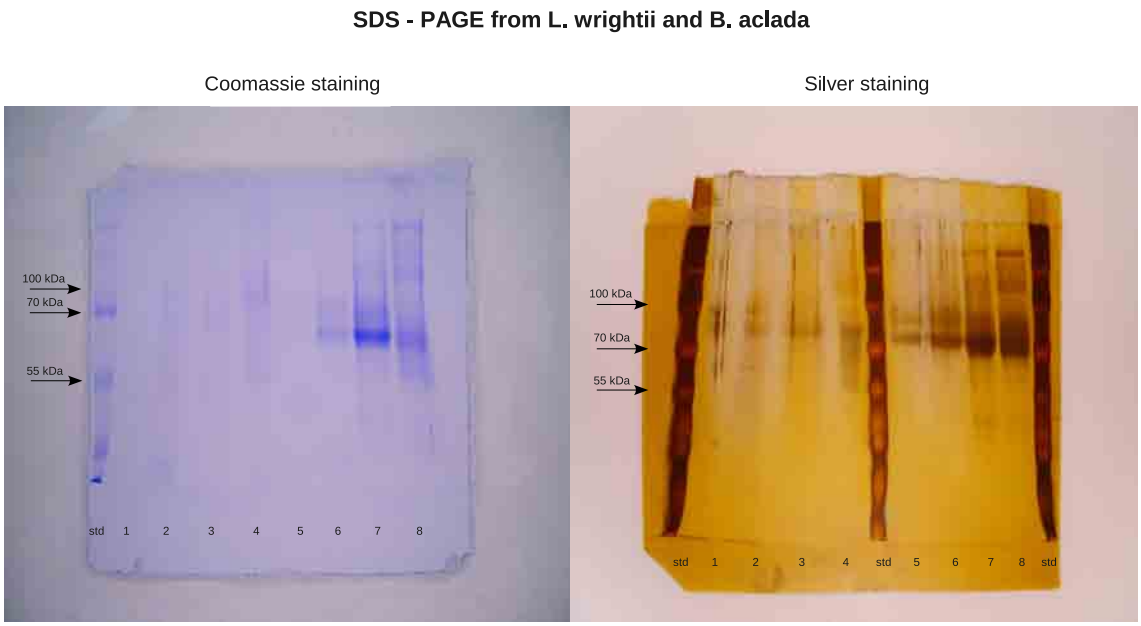

Figure 10.2: SDS-PAGE on vertical gel from *L. wrightii* and *B. aclada* with coomassie (left) and silver staining (right). **std** - Standard Marker Fermentas SM0671. **Lane 1** - *L. wrightii* Fermentation broth. **Lane 2** - *L. wrightii* Crossflow. **Lane 3** - *L. wrightii* DEAE. **Lane 4** - *L. wrightii* Phenyl HIC and Amicon Ultra. **Lane 5** - *B. aclada* Fermentation broth. **Lane 6** - *B. aclada* Crossflow. **Lane 7** - *B. aclada* Q Sepharose. **Lane 8** - *B. aclada* Phenyl HIC and Amicon Ultra.

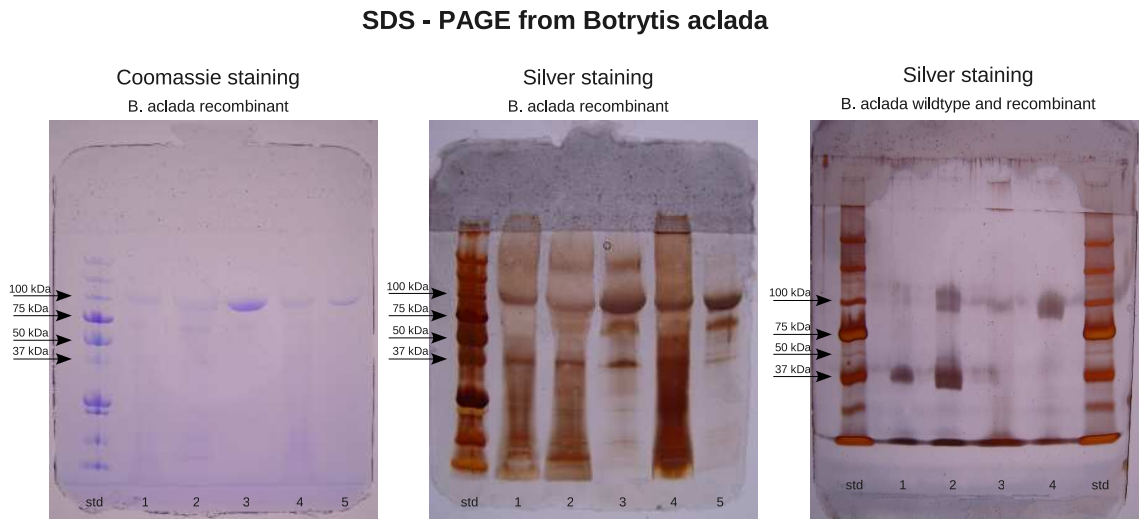

Figure 10.3: SDS-PAGE with Phast system from *B. aclada*. Figure 1 and 2 **std** - Standard Marker BioRad Dual Color. **Lane 1** - Fermentation broth first trial. **Lane 2** - Q Sepharose. **Lane 3** - Phenyl HIC. **Lane 4** - Fermentation broth second trial. **Lane 5** - Phenyl HIC. Figure 3 **std** - Standard Marker BioRad Dual Color. **Lane 1** - Wildtype after Crossflow. **Lane 2** - Wildtype after Amicon Ultra. **Lane 3** - Recombinant Fermentation broth. **Lane 4** - Recombinant after Amicon Ultra.

Chapter 11

Kinetic Properties

11.1 pH Optima

The pH optima for all strains and all substrates measured are summarised in table 11.1 and figure 11.1. For ABTS the pH optima and profiles are similar for all strains. At higher pH values the *B. aclada* laccase shows better stability. For syringaldazine the pH optima are the same for all strains, only the profiles differ a bit. The recombinant *B. aclada* laccase shows very good stability at lower pH, whereas the *B. aclada* wildtype laccase shows a good stability over a very broad pH range. The enzyme activities with catechol differ for all strains concerning pH profile and optima. The pH optimum for TEMPO could not be measured, as the reaction rate with this substrate was too low to get any reasonable results.

Sample	Subtrate	pH Optimum [pH]	Spec. Activity [U/mg]
L. wrightii	ABTS	3.0	153
B. aclada wildtype	ABTS	3.0	21
B. aclada recombinant	ABTS	3.0	109
L. wrightii	syringaldazine	6.0	4.6
B. aclada wildtype	syringaldazine	6.0	3.5
B. aclada recombinant	syringaldazine	6.0	48
L. wrightii	catechol	5.0	3.7
B. aclada wildtype	catechol	6.5	0.84
B. aclada recombinant	catechol	3.5	11
L. wrightii	TEMPO	-	-
B. aclada wildtype	TEMPO	-	-
B. aclada recombinant	TEMPO	-	-

Table 11.1: pH profile determined for all enzymes with different substrates.

11.2 Temperature Optima

The data obtained from the temperature assays are summarised in table 11.2 and figure 11.2. It can be seen, that laccase from *B. aclada* species have a higher temperature maximum than from *L. wrightii*. For

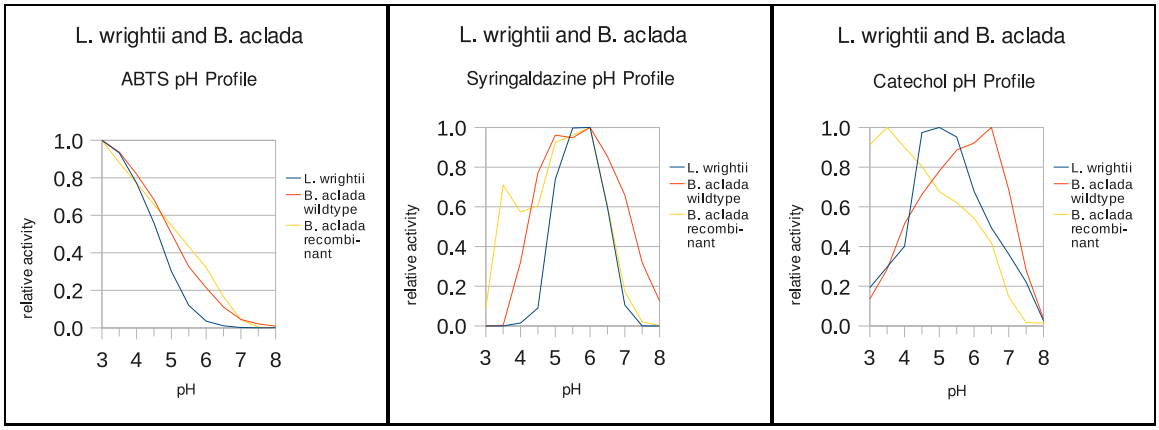

Figure 11.1: pH profiles of *L. wrightii* and *B. aclada* laccases. **Figure 1** pH profile with ABTS as substrate. **Figure 2** pH profile with syringaldazine as substrate. **Figure 3** pH profile with catechol as substrate.

all enzymes the activity is slightly increasing with temperature, but as soon as the temperature optimum is reached, the activity is rapidly decreasing. The activation energy is highest for *L. wrightii* laccase and shows similar values for both *B. aclada* laccases. The sudden drop of the reaction rate constant in the arrhenius plot indicates enzyme inactivation by temperature for all strains. The breakpoint of the arrhenius curve marks the temperature optimum $1/T_{opt}$ of the corresponding enzyme.

Sample	Temp. Optimum [°C]	Spec. Activity [U/mg]	Activation Energy [kJ/K· mol]
L. wrightii	35	5.97	35.7
B. aclada wildtype	45	4.48	20.3
B. aclada recombinant	45	59.0	16.6

Table 11.2: Temperature optimum and activation energy E_a determined for all enzymes with the ABTS assay.

11.3 Inhibition

Table 11.3 with I_{50} values for all inhibitors examined shows the effects of different salts on laccase activity. *B. aclada* laccase seems to be less susceptible to NaCl than *L. wrightii* laccase. However, the inhibtion with NaCl seems also to be pH dependent. The inhibitory effect was less distinct at pH 6 for all enzymes. For *B. aclada* the recombinant enzyme seems to be less affected by NaCl than the wildtype. The inhibitory effect of NaN_3 is not as pronounced for the *B. aclada* wildtype laccase than for the *L. wrightii* enzyme. Interestingly, the recombinant enzyme is affected most by NaN_3 and NaF, indicating different structure or glycosilation characteristics. Generally the overall inhibitory effect is highest for NaF.

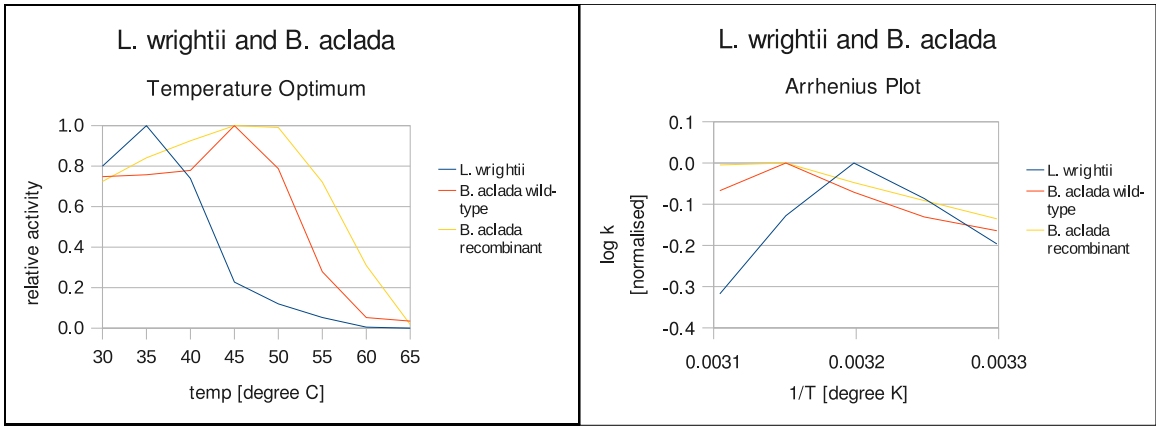

Figure 11.2: **Left figure** Temperature optimum determined for all enzymes with the ABTS assay. **Right figure** Arrhenius plot to determine the activation energy E_a.

Sample	NaCl pH3 [mM]	NaCl pH6 [mM]	NaN_3 [mM]	NaF [mM]
L. wrightii	36	162	1.1	0.2
B. aclada wildtype	171	500	1.7	0.5
B. aclada recombinant	300	570*	0.05	0.05

Table 11.3: I_{50} values for NaCl, NaF and NaN_3. *this is an extrapolated value, as data were only recorded until 500 mM NaCl concentration.

11.4 Exchange of Metal Ions

There is no significant difference in enzyme activity due to exchanging the catalytic metal ions. This shows, that there is no recovery of enzymatic activity of laccase molecules with an incomplete set of copper atoms. Furthermore, there is no exchange of copper to other metal ions, indicating a strong bond inside the active center. A slight decrease in enzyme activity can be observed at the presence of all metal ions during the incubation experiment, indicating an inhibitory or denaturing effect at higher concentrations and longer exposure. In general can be said, that no significant activation or reconstitution occured due to the presence of different metal ions.

11.5 Michaelis-Menten Kinetics

The kinetic constants are summarised in table 11.4. Comparing the kintetic constants from *L. wrightii* and *B. aclada* wildtype it can be seen that *L. wrightii* shows higher v_{max} values, whereas *B. aclada* has the lower K_m values. This means, that *L. wrightii* laccase has a higher turnover rate, whereas the laccase from *B. aclada* works more specific and has a higher substrate affinity. Comparing the two *B. aclada* species it can be seen, that the recombinant enzyme shows much better values for both v_{max} and K_m. In general can be said, that the recombinant *B. aclada* laccase shows much better kinetic properties than

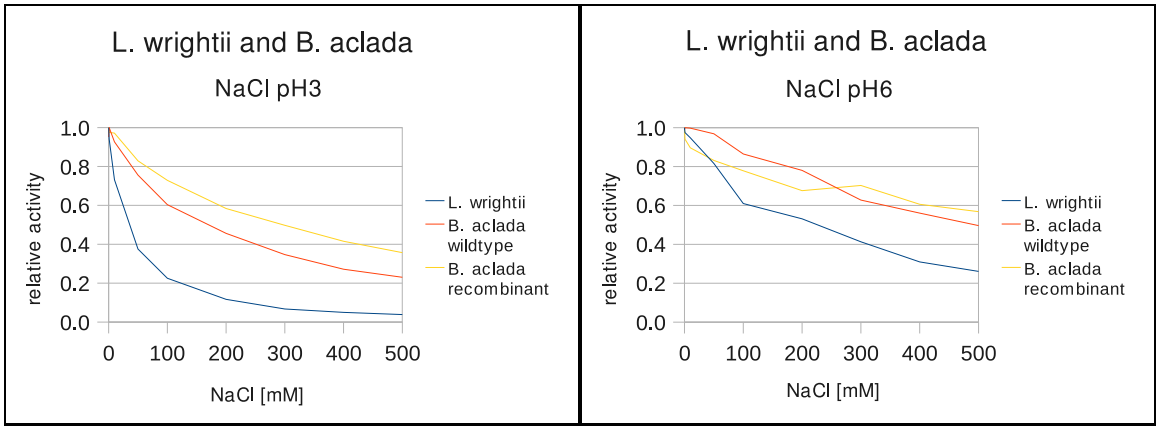

Figure 11.3: **Left figure** Inhibition of laccase with NaCl at pH3. **Right figure** Inhibition of laccase with NaCl at pH6.

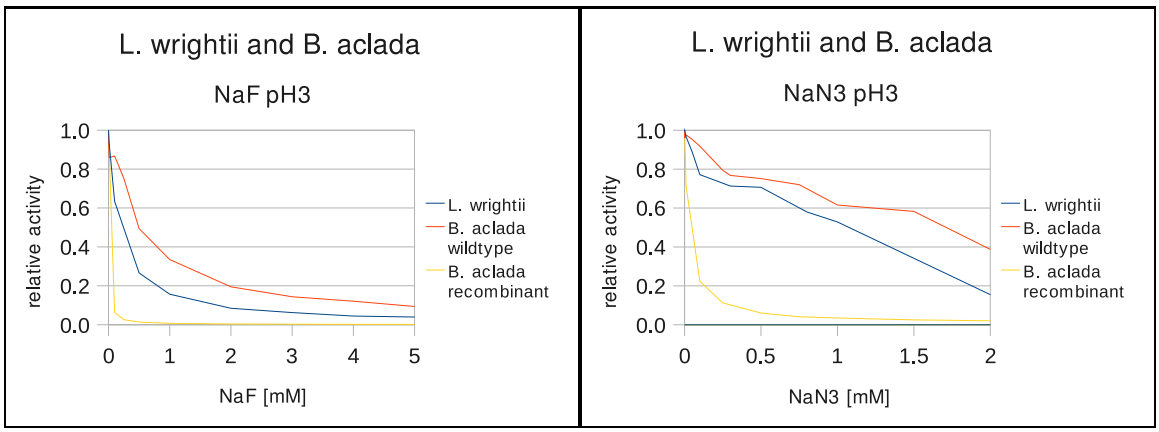

Figure 11.4: **Left figure** Inhibition of laccase with NaF. **Right figure** Inhibition of laccase with NaN_3.

the other enzymes. Comparing the v_{max} values for all enzyme preparations reveal, that the *B. aclada* wildtype laccase is less pure, which is also indicated by its low specific activity.

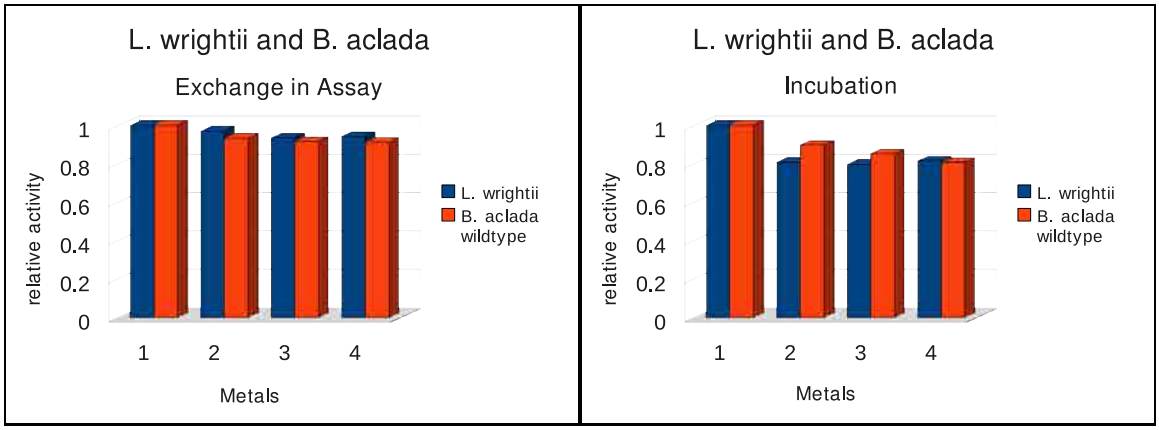

Figure 11.5: **Left figure** Effect of metal ions on enzymatic activity. **Right figure** Effect of metal ions on enzymatic activity after 15 min of incubation. **Lane 1** - natural. **Lane 2** - cobalt. **Lane 3** - zinc. **Lane 4** - copper.

Sample	Substrate	v_{max} [U/mg]	K_m [μM]
L. wrightii	ABTS pH3	174	60.4
B. aclada wildtype	ABTS pH3	26	14.2
B. aclada recombinant	ABTS pH3	153	5.0
L. wrightii	ABTS pH5	77	1410
B. aclada wildtype	ABTS pH5	11	108
B. aclada recombinant	ABTS pH5	84	19.6
L. wrightii	Catechol pH5	8.93	4000
B. aclada wildtype	Catechol pH5	1.65	2070
B. aclada recombinant	Catechol pH5	12.9	435
B. aclada wildtype	Syringaldazine pH6	6.61	16.8
B. aclada recombinant	Syringaldazine pH6	49	2.0

Table 11.4: Kinetic constants determined for all enzymes for ABTS, catechol and syringaldazine.

Part IV

Discussion

Chapter 12

Cultivation and Purification

As expected, a high dissolved oxygen concentration and therefore a high oxygen transfer rate is necessary for appropriate growth. For standard cultivation conditions an Erlenmeyer flask with an agitation frequency of 90 rpm was used. This was taken into account to use an amount of medium that yields a big surface area to promote oxygen diffusion. However, growth and laccase production could be optimised by using baffled flasks or higher agitation rates.

12.1 Cultivation in Basal Media

It could be seen, that high peptone concentrations in the media resulted in a pH increase during growth. As high pH values are not suitable for laccase stability, higher soy peptone concentrations should be avoided. Lowering the pH could also be achieved by increasing the sugar concentration accompanied by a decrease in peptone. Fructose concentrations of about 10 g/l were able to stabilise peptone concentrations up to 5 g/l in terms of pH. Neither glucose nor glycerin showed such good acidification properties in the fermentation process than fructose.

In most experiments 0.1 mM Cu_2SO_4 was added during inoculation. While protein formation was slightly enhanced, this had no significant effect on laccase activity. As shown by Galhaup *et al.* the optimal copper concentration for laccase formation of *Trametes pubescens* was found to be 1.5-2-0 mM [16]. Furthermore, the time point of addition influenced laccase synthesis, while supplementation of copper at the time of inoculation did not lead to any growth inhibition for low concentrations of 0.1-1 mM. Addition of copper after more than 4 days of cultivation was not sufficient to enhance laccase activity during growth. According to these results a higher copper concentration is recommended for distinct laccase induction, but it has to be considered, that ascomycetes tolerate less copper sulphate.

12.2 Cultivation in Vegetable Media

In the tomato, vegetable and fruit juice media the pH started at low levels and went up during fungal growth. Laccase production was always observed very early and at a low pH. The activity maximum usually appeared at around pH 5. Interestingly, in the potato media the pH profile and laccase production behaved very different for the different strains. For *L. wrightii* the initial pH was around 7 and went down during cultivation, whereas no laccase production occured. For the other strains, the pH profile was the same as for the juice media and *B. aclada* even produced a considerable amount of laccase. It is not clear why the initial pH in the potato infusion and the pH progress differ that much between the experiments. However, these results indicate that pH is an important factor in laccase expression and stability. It seems that laccase is always induced at low pH correlated to an increase in pH afterwards. Appropriate media, especially for Botrytis strains, were described for optimised enzyme production by [42] and [12]. For *G. cingulata* and *P. destructiva* no conditions could be found for satisfactory laccase production. Botrytis and Glomerella species are well known to be grape and apple pathogens, so it could be expected that they would produce laccase in the corresponding juices, however this was not the case.

Laccase is always produced at the beginning of cultivation and active only over a short period. This indicates, that the enzyme is built to sustain the depletion of the plant cell matrix and is not used anymore during later stages. The laccase maximum always appears at low protein concentrations, whereas most of the protein is produced during the subsequent accumulation of fungal mycelium. During performing the enzyme assays for *P. destructiva* it was observed, that at the beginning the activity always decreased and levelled off after a short period.

12.3 Fermentation Experiments

The browning during fermentation can be attributed to the action of laccase on phenolic compounds in the medium. According to Baldrian, laccases were described to be associated to spore morphogenesis, which could also explain the appearance of brown colour [3]. However, spore formation could not be confirmed under the microscope. For *L. wrightii* a comparison of enzyme production demonstrates, that the fermentation process never produced as much enzyme as shaking flasks. The laccase emerged much faster in the fermentation experiment, but the enzyme activity also vanished earlier. The increase of pH during fermentation was not that distinct for the second fermentation. This is in accordance to the cultivation experiments, where higher peptone concentrations led to a faster increase in pH. Even though a higher peptone concentration results in a higher pH during growth, which is inappropriate for laccase stability, soy peptone was added to counteract laccase degradation by proteinases. However, the addition of peptone had no significant influence on laccase stability. Interestingly, the oxygen consumption rate in the first fermentation is less than in the second one, indicating less growth, but laccase production is

enhanced.

B. aclada produced slightly higher activities in the fermenter compared to the shaking flask cultivation experiments, which can be attributed to the addition of peptone. Anyway, the fermentation time should be elongated to at least 12 days. Also other media as potato or maltose based media could be used to accelerate enzyme production. As shown by Slomczynski *et al.* a faster impeller speed of 500 rpm could be used, which probably would lead to a higher oxygen transfer rate and a better homogenisation of the fermentation broth [42]. As seen in our experiments an impeller speed of 200 rpm led to an accumulation of mycelium on the bottom of the fermenter, indicating insufficient agitation. However, the shear sensitivity of the mycelium has also be considered.

When comparing the fermentation experiments of the two different strains, the pH during *L. wrightii* fermentation increased much faster than the pH during *B. aclada* fermentation, which remained constant, in accordance to the shaking flask cultivation experiments. These differences in the pH profile could reflect differences in growth properties and can also affect laccase stability.

12.4 Purification Experiments

In general, it can be said, that most losses during purification (about 50-80%) occured during anion exchange chromatography (AIEX) and about 20% during hydrophobic interaction chromatography (HIC). Losses of about 20% of enzymatic activity are due to freezing and thawing processes necessary for sample storage.

For *L. wrightii* laccase a 26-fold purification with a yield of 33% and a specific activity of 150 U/mg could be achieved. With AIEX purity could be increased by a factor of 4, whereas HIC led to an 8-fold increase. Also ultrafiltration had a small but positive effect on purification.

For *B. aclada* laccase a 28-fold purification with a yield of 7% and a specific activity of 120 U/mg could be achieved. AIEX played a negligible role in purification, whereas HIC led to an 13-fold increase in purity. Also ultrafiltration showed a positive effect on purification. Unfortunately, in a second trial *B. aclada* laccase could only be purified 6-fold with a specific activity of 27 U/mg. It is not absolutely clear why HIC showed such poor performance in the second experiment, but maybe the use of the ultraturrax produced a lot of polysaccharide fragments that interacted with the chromatography column, lowering its selectivity. As reported by Slomczynski *et al.* an extraction step with 10% acetone was used to remove the 1,3-β-glucan exopolysaccharides from *Botrytis cinerea* [42]. It is recommended to carry out such extraction instead of using the ultraturrax to remove the highly viscose matrix. It is likely that this step

would improve the purification performance, but in turn can also lead to enzyme denaturation.

For the recombinant *B. aclada* laccase a 4-fold purification with a yield of 20% and a specific activity of 61 U/mg could be achieved. During the first purification approach nearly the whole enzymatic activity got lost during AIEX. There is evidence, that the enzyme was still bound to the column and could not be eluted under the given conditions. In the second purification step the backpressure was constantly increasing during sample loading onto the HIC column and new precipitate occured. A fractionated precipitation with a subsequent ultracentrifugation or filtration step with a mixed-cellulose filter could be advantageous in this case. The laccases from *B. aclada* (wildtype and recombinant) had different binding and elution properties, indicating different protein surfaces or glycosylation. This can result from a different gene or different posttranslational modifications due to the expression in a different host.

Overall the purification yields are quiet similar for *L. wrightii* and *B. aclada* laccases. However, the different binding conditions for AIEX announce differences in protein structure for both laccases. It seems, that *B. aclada* laccase exhibit less ionic residues on the surface than *L. wrightii*. For HIC the same conditions can be used, but *B. aclada* laccase is binding much better than *L. wrightii* laccase. That suggests more hydrophobic residues on *B. aclada* laccase.

B. aclada as well as *L. wrightii* laccase were purified 25-30-fold, which seems to be satisfactory compared to other works [42, 43], even though SDS-Page does not indicate a homogeneous enzyme preparation. Additionally, a purified laccase extract should be pale-blue due to the T1 copper atom. All enzyme preparations appeared light brown, which is an indication for low molecular weight impurities. This brown colour appeared during cultivation only in diluted tomato juice media. Maybe an additional filter or dialisation step could further remove non-specific lower polypeptides and contaminants. Anyway, there seems to be a potential in optimising the procedures of the purification process. For *B. aclada* it seems adaquate to use an extraction step with 10% acetone after removing the fungal mycelia. Crossflow filtration is appropriate to concentrate the sample and to get rid of low molecular weight contaminants. According to Slomczynski *et al.*, for *B. aclada* a ultrafiltration unit equipped with a 30 kDa molecular-weight-cutoff filter should be used to remove remaining sticky material [42]. Due to the high salt stability of the laccases an ammonium sulfate saturation of about 30% could be tested to precipitate other proteins. After filtration with a mixed-cellulose filter, to remove particulate matter, a direct HIC step can be performed. In each case it has to be considered if AIEX is still necessary, as HIC appears to be the chromatographic method of choice.

Chapter 13

Characterisation

13.1 Isoelectric Focusing and SDS-PAGE

The differences in the pI values of 4.0 for *L. wrightii* and 5.2 for *B. aclada* laccases reflect the fact, that different conditions for purification had to be used, and indicate different protein surfaces. No isoenzymes were detected in cultures of both strains. As the isoelectric focusing did not run very nicely, a longer prefocusing time for the ampholytes is recommended (about 4 h), which will enhance separation.

Problems with the assignment of bands and their reproducibility occured during performing SDS-PAGE. For SDS-PAGE the samples were diluted 1:2 with sample buffer and incubated at 90°C for 5 min. It is recommended to restrict heating to 1 minute to avoid degradation of proteins into smaller fragments. Furthermore, it is hard to compare gels from different systems, as the gels for the vertical SDS-PAGE were prepared by hand, whereas the Phast system was used with precasted gels. It is likely, that the 75 kDa band on the vertical gel preparation corresponds to the 100 kDa band on the Phast system. Differences in band distribution at different gels can also be attributed to degradation products built over time. The *B. aclada* wildtype preparation for example showed a distinct band at about 37 kDa after several weeks of storage.

According to Thurston, all fungal laccases, like most fungal extracellular enzymes, are glycoproteins and carbohydrate content may vary from about 15 to 80% [46]. As reported by Slomczynski *et al.* SDS-PAGE shows anomalous migration of glycopeptides [42]. SDS-PAGE from a *Botrytis cinerea* laccase resulted in a 100 kDa band, whereas a denaturing size exclusion chromatography determined a molecular mass of about 74 kDa. This indicates, that the 100 kDa band found for *L. wrightii* and *B. aclada* laccases is likely to correspond to the 74 kDa band. Mayer *et al.* attributes differences in enzyme molecular masses to be a function of the inducers used [33].

So far most laccases were purified from wood-rotting white-rot basidiomycetes with monomeric structures,

molecular masses of approximately 60-70 kDa and an isoelectric point around pH 4.0. It seems that there is considerable heterogeneity in the properties of laccases isolated from ascomycetes, especially with respect to molecular weight [3]. Thakker *et al.* showed in *Monocillium indicum*, that a single band of 100 kDa after gel filtration resolved into three proteins of 24, 56 and 72 kDa on SDS-PAGE [45]. Palmieri *et al.* showed, that laccases from *Pleurotus ostreatus* exhibit three different polypeptides of 67, 18 and 16 kDA, whereas the native proteins behave homogeneously, as demonstrated with a single band in IEF and native-PAGE [34].

Property	Median	*Lamprospora wrightii*	*Botrytis aclada* wildtype	*Botrytis aclada* recombinant
Molecular Weight (kDa)	66	75	75	100
pI	3.9	4.0	5.2	-
Temp Optimum (°C)	55	35	45	45
pH Optimum				
ABTS	3.0	3.0	3.0	3.0
Syringaldazine	6.0	6.0	6.0	6.0
$\mathbf{K}_m$ (μM)				
ABTS	39	60	14	5
Syringaldazine	36	-	17	2

Table 13.1: Comparision of properties of the purified laccases to mean values calculated for laccases from [3].

13.2 pH Optima

The determined pH optima of 3.0 for ABTS and 6.0 for syringaldazine correspond to the published values of laccases by Baldrian [3]. Only the enzyme activities for catechol differ between all strains concerning pH gradient and optima, respectively. For characterisation the *B. aclada* wildtype laccase with a specific activity of 27 U/mg was used. This enzyme preparation was not pure enough to give reliable signals in the catechol assay, as catechol is a very bad substrate anyway. On the other hand, the recombinant laccase expressed in *Pichia pastoris*, did show different behaviour.

A general model of pH influencing laccase activity was developed by Xu [48], explaining the bell shaped pH profile of phenolic compounds by two opposing effects. The oxidation process is enhanced the bigger the redox potential difference between the substrate and the T1 copper is. The potential of a phenol decreases when pH increases due to the proton release. However, during pH change the potential of the phenol decreases much more than the potential for the laccase. Therefore, the driving force is increasing and oxidation enhanced. On the other hand, the enzyme activity is decreasing with pH by the binding of a hydroxide to the copper ions, which interferes with the intramolecular electron transfer.

13.3 Temperature Optima

According to Baldrian the temperature profiles of laccases correspond to other extracellular ligninolytic enzymes with optima between 50°C and 70°C [3]. Both *L. wrightii* and *B. aclada* laccase show temperature optima far below the average with 35°C and 45°C. The activation energy of 35 kJ/K mol for the *L. wrightii* laccase is about double than that for the *B. aclada* species (20 kJ/K mol). All enzymes show a sudden drop of the reaction rate in the Arrhenius plot, indicating enzyme inactivation at 40°C and 45°C for the laccases from *L. wrightii* and *B. aclada* wildtype. The recombinant enzyme is more heatresistant and inactivated at a temperature of 60°C. There is evidence, that all laccases examined are temperature sensitive, which could lead to limitations concerning their usage in industrial applications.

13.4 Inhibition and Exchange of Metal Ions

For all inhibitors tested, *B. aclada* laccase shows less inhibition than *L. wrightii* laccase. Interestingly, the recombinant enzyme from *B. aclada* behaves quite different to the wildtype. Although the recombinant laccase is less affected by NaCl, it is very unstable in the presence of the other salts. There is evidence, that *B. aclada* has different genes coding for laccase and likely, the expressed and characterised laccase from the wildtype does not correspond to the cloned one in *Pichia pastoris*. It is also possible, that during fermentation the wildtype strain produced different types of laccase with different properties, resulting in an enzyme mixture. Anyway, it has to be mentioned, that no isoenzymes could be detected by isoelectric focusing. Compared to other works [25, 42, 43] all characterised laccases show a good resistance against sodium azide.

There is no significant difference in enzyme activity due to exchanging the catalytic metal ions. This shows, that no activation or reconstitution occured due to the presence of different metal ions. No recovery of enzymatic activity due to an incomplete set of copper atoms could be observed. Furthermore, no exchange of copper to other metal ions could be achieved, indicating a strong bond inside the active center.

13.5 Michaelis-Menten Kinetics

In general, it can be said that the recombinant *B. aclada* laccase has much better kinetic properties than the other enzymes. Comparing the results to Baldrian shows, that the K_m values for the used substrates are very low for all enzymes [3]. The K_m values of about 5 μM for the recombinant *B. aclada* laccase belongs to the lowest refered. Concerning v_{max} it can be seen, that the laccase from the *B. aclada* wildtype strain is of poor purity. The v_{max} values for *L. wrightii* and *B. aclada* recombinant laccases are quite similar, indicating sufficient purity. By comparing the purification folds of 26 for *L. wrightii* and

4 for *B. aclada* it is obvious, that the fermentation of *P. pastoris* for recombinant enzyme production is much more efficient than using the wildtype strain.

Part V

Summary

Extracellular laccases from the two fungal strains *Lamprospora wrightii* and *Botrytis aclada* were cultivated, purified and characterised. Additionally, a recombinant *B. aclada* laccase expressed in *Pichia pastoris* was examined. Medium composition is crucial for laccase induction, which seems to be associated to plant stimuli, like pectines or lignin. A tomato juice media containing fructose, soy peptone, copper sulphate and trace elements is appropriate for sufficient laccase production. Additionally, a high oxygen saturation is necessary for suitable growth. Laccase production was examined at small scale in shaking flasks and in 20 L fermentation experiments. *L. wrightii* showed highest laccase concentrations in shaking flasks with 90 rpm at 25°C (0.60 U/ml), whereas *B. aclada* showed best productivity during fermentation (0.36 U/ml). Purification had to be done under various conditions for all laccases examined, indicating different structural properties. *L. wrightii* laccase was purified with a weak anion exchange chromatography, followed by hydrophobic interaction chromatography (HIC) and an ultrafiltration step, achieving a 26-fold increase in purity with a specific activity of 150 U/mg. *B. aclada* laccase from the wildtype strain was purified with a strong anion exchange chromatography, followed by HIC and an ultrafiltration step, achieving a 28-fold increase in purity with a specific activity of 120 U/mg. Problems in the reproducibility of *B. aclada* purification emerged from the excessive production of an extracellular polysaccharide during cultivation, leading to a drastic increase in viscosity. The recombinant *B. aclada* laccase was purified with HIC followed by an ultrafiltration step, achieving a 4-fold increase in purity with a specific activity of 61 U/mg. Isoelectric focusing showed different pI values of 4.0 for *L. wrightii* and 5.2 for *B. aclada* laccase. No isoenzymes were detected in cultures of both strains. The molecular weight of all laccases examined by SDS-PAGE was found to be around 75 kDa. All laccases have typical pH optima of 3.0 and 6.0 for ABTS and syringaldazine, respectively, with low temperature optima of 35°C and 45°C for *L. wrightii* and *B. aclada* laccase. The activation energy of 35 kJ/K mol for the *L. wrightii* laccase is two-fold higher than that of the *B. aclada* laccases. All enzymes show a sudden drop of the reaction rate in the Arrhenius plot, indicating enzyme inactivation at 40°C and 45°C for the laccases from *L. wrightii* and *B. aclada* wildtype. The recombinant enzyme is more heatresistant and inactivated at a temperature of 60°C. For all inhibitors and substrates tested, both *B. aclada* laccases show preferable kinetic constants to *L. wrightii* laccase. In particular, the recombinant enzyme with the lowest K_m values of about 5 μM for ABTS and syringaldazine has to be mentioned.

Zusammenfassung

Die zwei pflanzenpathogenen Pilze *Lamprospora wrightii* und *Botrytis aclada* wurden hinsichtlich ihrer Laccaseproduktion untersucht und optimiert. Zusätzlich wurde aus *B. aclada* eine Laccase rekombinant in *Pichia pastoris* exprimiert und ebenfalls charakterisiert. Die Wahl des richtigen Kultivierungsmedium war ausschlaggebend für die Induktion der Laccaseproduktion. Ein Tomatensaftmedium mit Fructose, Pepton, Kupfersulfat und Spurenelementen in Verbindung mit einer hohen Sauerstoffsättigung führt zu einer zufriedenstellenden Laccaseproduktion. Wachstumsversuche wurden im Kleinmaßstab mit Schüttelkolben und in 20 L Fermentationsansätzen durchgeführt. Die beste Laccaseproduktion für *L. wrightii* wurde in Schüttelkolben bei 90 rpm und 25°C erzielt (0.60 U/ml). Für *B. aclada* konnte mit Hilfe der Fermentation die beste Enzymausbeute erreicht werden (0.36 U/ml). Bei der Proteinreinigung mussten unterschiedliche Bedingungen gewählt werden, was auf verschiedene strukturelle Eigenschaften der einzelnen Enzyme hindeutet. Die *L. wrightii* Laccase konnte mit Hilfe eines schwachen Anionenaustauschers, gefolgt von einer hydrophoben Interaktionschromatographie (HIC) und einem Ultrafiltrationsschritt um das 26-fache auf 150 U/mg aufgereinigt werden. Die Laccase vom *B. aclada* Wildtyp konnte mit Hilfe eines starken Anionenaustauschers, gefolgt von einer HIC und einem Ultrafiltrationsschritt um das 28-fache auf 120 U/mg aufgereinigt werden. Probleme bei der Reproduzierbarkeit ergaben sich durch die Produktion eines extrazellulären Polysaccharides während der *B. aclada* Kultivierung, wodurch sich die Viskosität der Kulturlösung stark erhöht hat. Die rekombinante Laccase konnte mit Hilfe einer HIC, gefolgt von einem Ultrafiltrationsschritt um das 4-fache auf 61 U/mg aufgereinigt werden. Die pI Werte beider Enzyme sind mit 4.0 für *L. wrightii* und 5.2 für *B. aclada* recht unterschiedlich. Bei beiden Pilzspezies konnten keine Isoenzyme gefunden werden. Alle Laccasen haben ein Molekulargewicht von circa 75 kDa und zeigen typische pH-Optima bei 3.0 für ABTS und 6.0 für Syringaldazin. Die Temperaturoptima mit 35°C und 45°C für *L. wrightii* und *B. aclada* Laccasen sind generell sehr niedrig. Wie sich im Arrhenius Plot zeigt, führen Temperaturen von 40°C beziehungsweise 45°C zu einer schnellen Enzyminaktivierung bei *L. wrightii* und *B. aclada* Wildtyp. Nur die rekombinante Laccase zeigt mit 60°C eine bessere Hitzeresistenz. Die *L. wrightii* Laccase hat mit 35 kJ/K mol eine doppelt so große Aktivierungsenergie wie beide *B. aclada* Enzyme. Bezogen auf die Inhibierungs- und Michaelis-Menten Kinetik weisen die *B. aclada* Laccasen geringere Beeinflussung durch verschiedene Anionen und niedrigere K_m-Werte für alle getesteten Substrate auf. Hervorzuheben sei das rekombinante Enzym mit einem besonders niedrigen K_m-Wert von 5 μM für ABTS und Syringaldazin.

Part VI

Bibliography

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Part VII

Appendix

Chapter 14

Cultivation of *Lamprospora wrightii*

14.1 Determination of optimum Shaking Flask Conditions

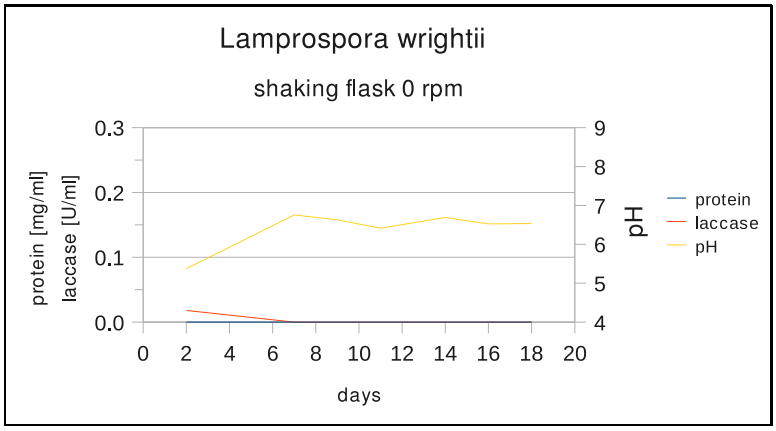

Figure 14.1: *Lamprospora wrightii* cultivated in a shaking flask at 0 rpm.

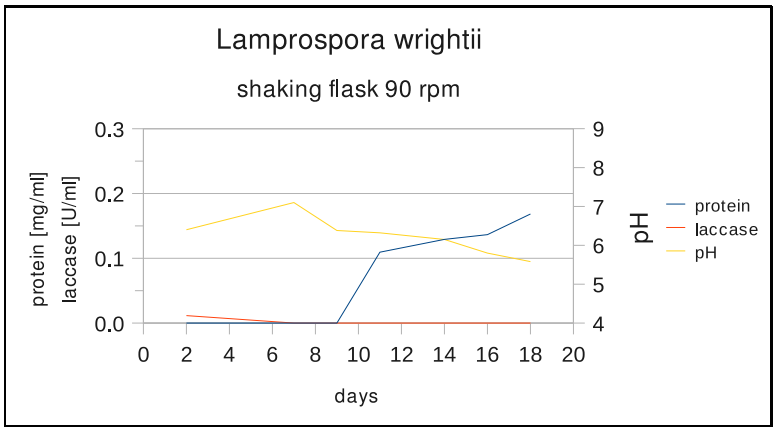

Figure 14.2: *Lamprospora wrightii* cultivated in a shaking flask at 90 rpm.

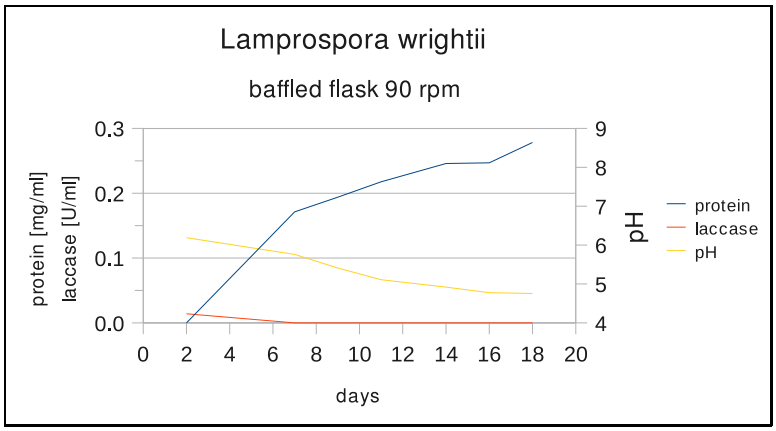

Figure 14.3: *Lamprospora wrightii* cultivated in a baffled shaking flask at 90 rpm.

14.2 Cultivation in Basal Media

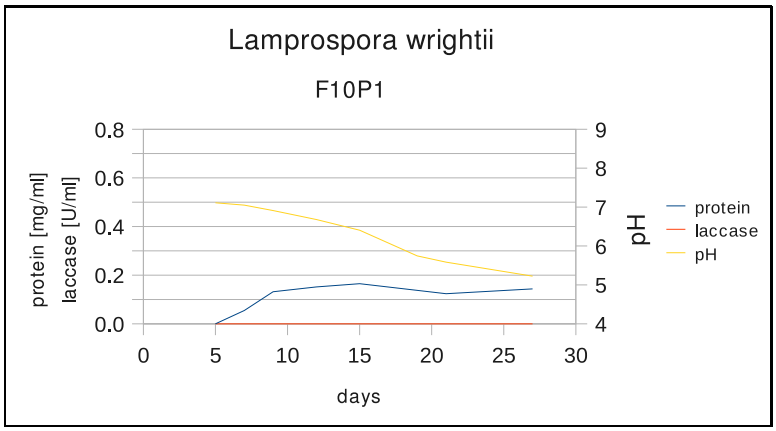

Figure 14.4: *L. wrightii* cultivated in 10 g/l fructose, 1 g/l soy peptone and 0.1 mM copper sulphate.

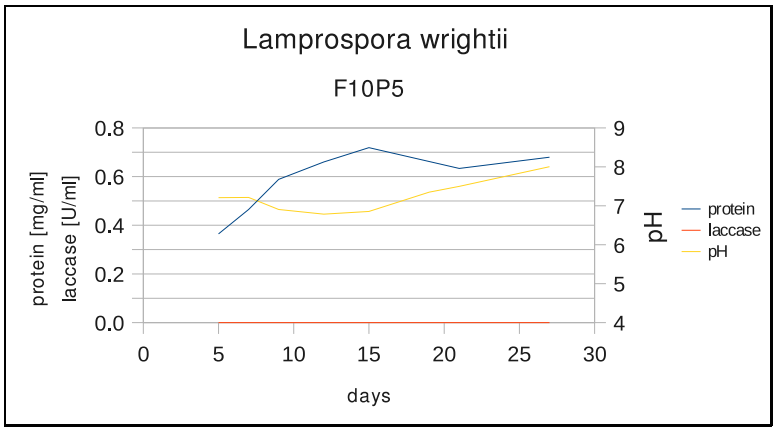

Figure 14.5: *L. wrightii* cultivated in 10 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

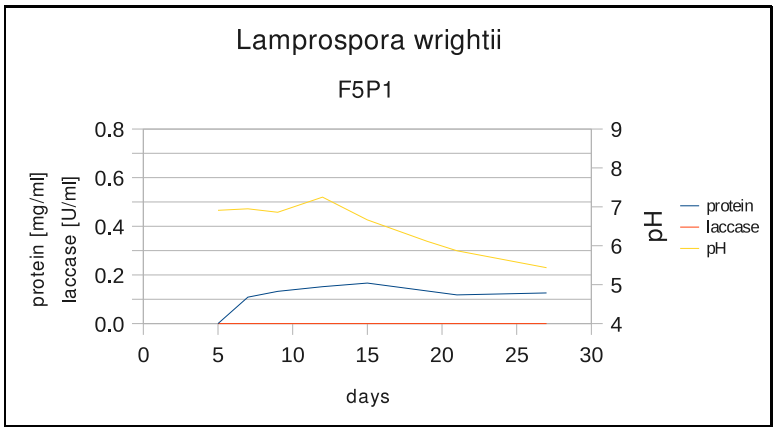

Figure 14.6: *L. wrightii* cultivated in 5 g/l fructose, 1 g/l soy peptone and 0.1 mM copper sulphate.

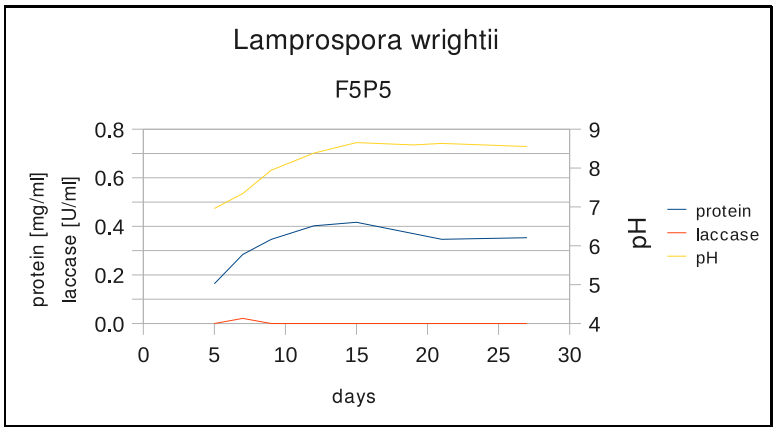

Figure 14.7: *L. wrightii* cultivated in 5 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

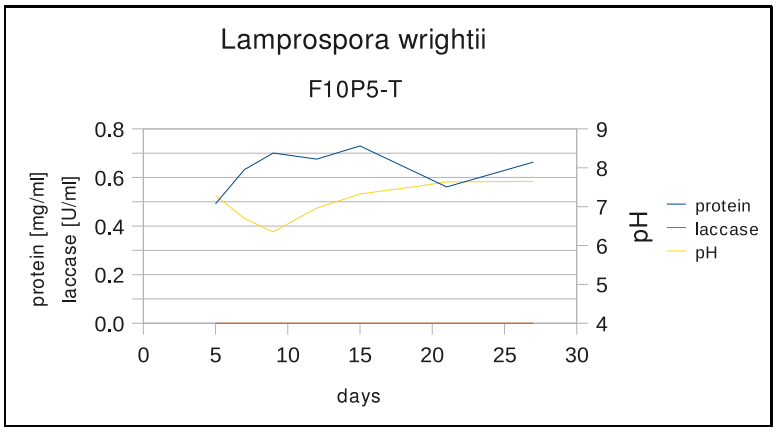

Figure 14.8: *L. wrightii* cultivated in 10 g/l fructose, 5 g/l soy peptone, 0.1 mM copper sulphate and without trace elements.

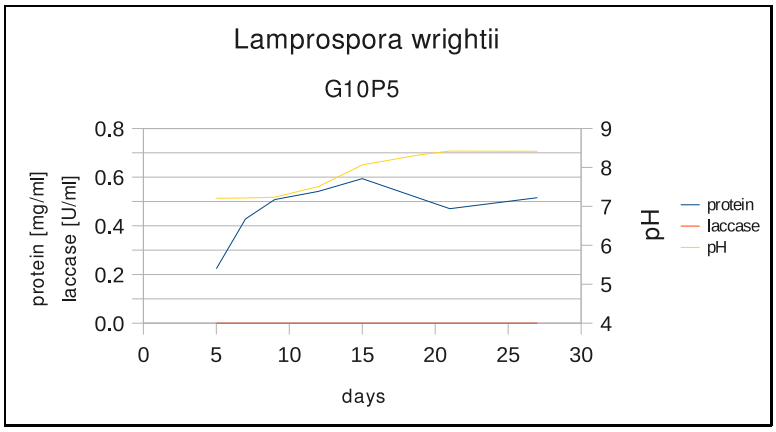

Figure 14.9: *L. wrightii* cultivated in 10 g/l glucose, 5 g/l soy peptone and 0.1 mM copper sulphate.

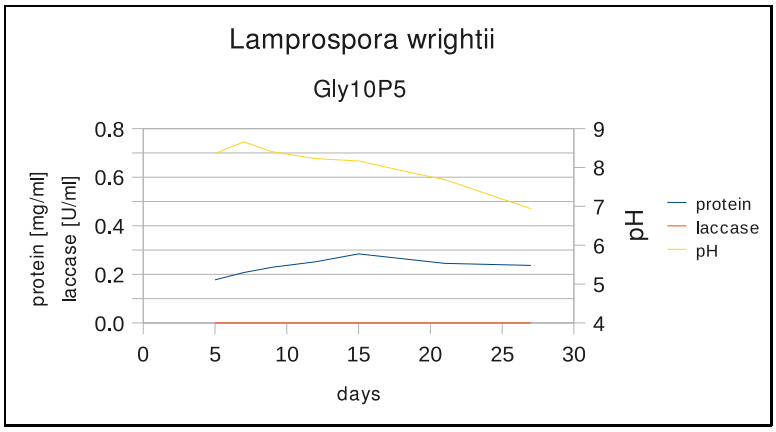

Figure 14.10: *L. wrightii* cultivated in 10 g/l glycerine, 5 g/l soy peptone and 0.1 mM copper sulphate.

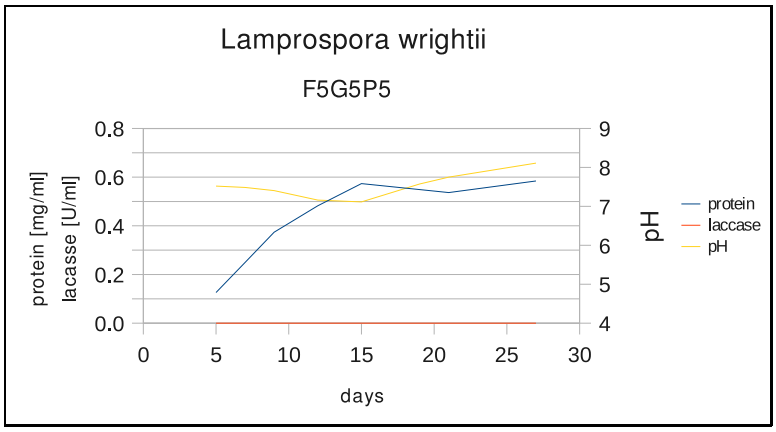

Figure 14.11: *L. wrightii* cultivated in 5 g/l fructose, 5 g/l glucose, 5 g/l soy peptone and 0.1 mM copper sulphate.

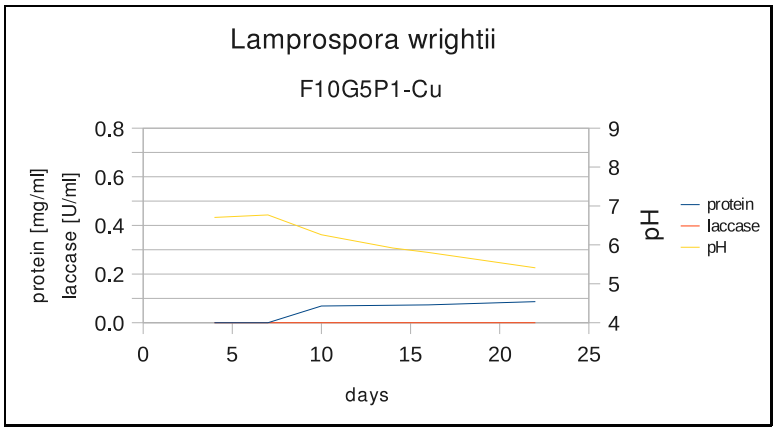

Figure 14.12: *L. wrightii* cultivated in 10 g/l fructose, 5 g/l glucose, 1 g/l soy peptone and without copper sulphate.

14.3 Cultivation in Vegetable Media

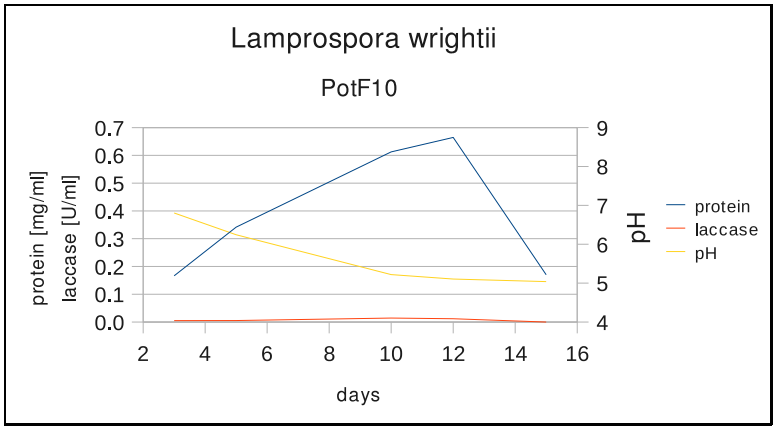

Figure 14.13: *Lamprospora wrightii* cultivated in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

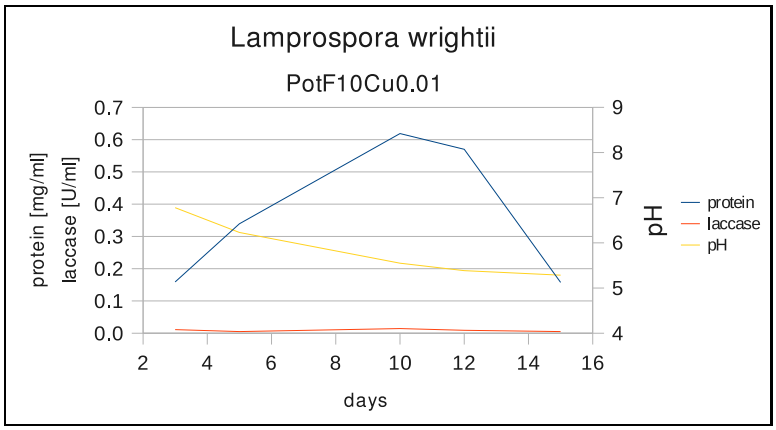

Figure 14.14: *L. wrightii* cultivated in potato media with 10 g/l fructose and 0.01 mM copper sulphate.

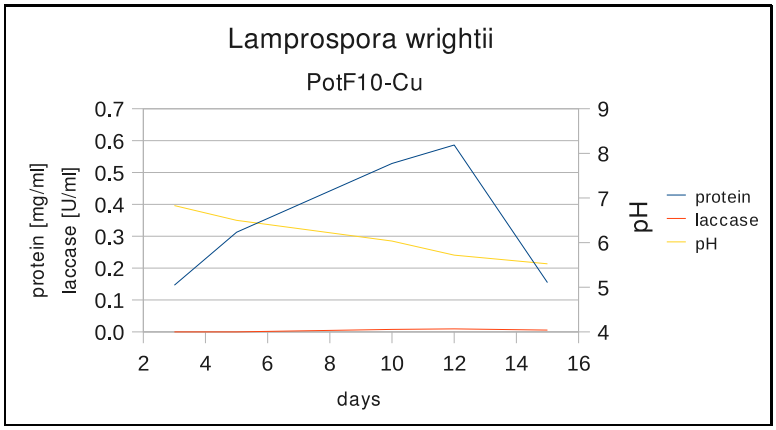

Figure 14.15: *L. wrightii* cultivated in potato media with 10 g/l fructose.

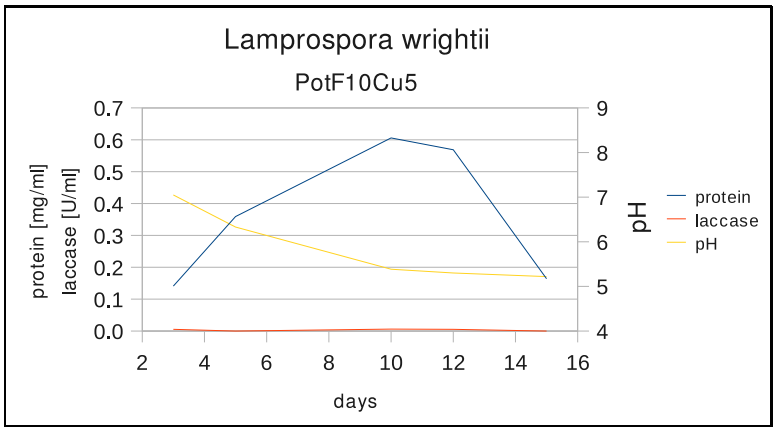

Figure 14.16: *L. wrightii* cultivated in potato media with 10 g/l fructose. Induction with 0.1 mM copper sulphate after 5 days.

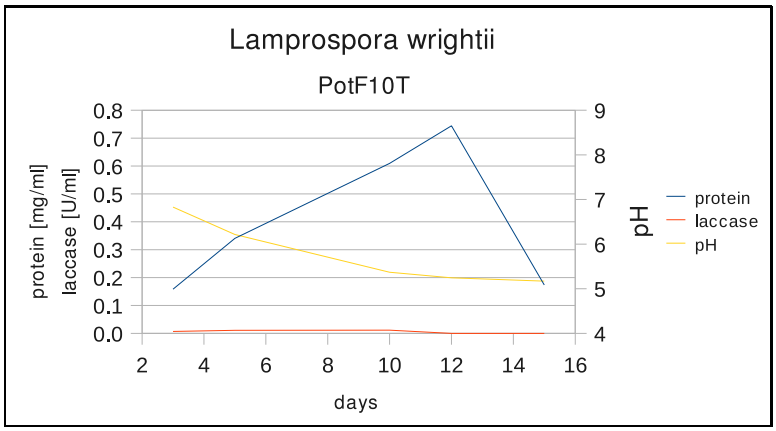

Figure 14.17: *L. wrightii* cultivated in potato media with 10 g/l fructose, 0.1 mM copper sulphate and trace elements.

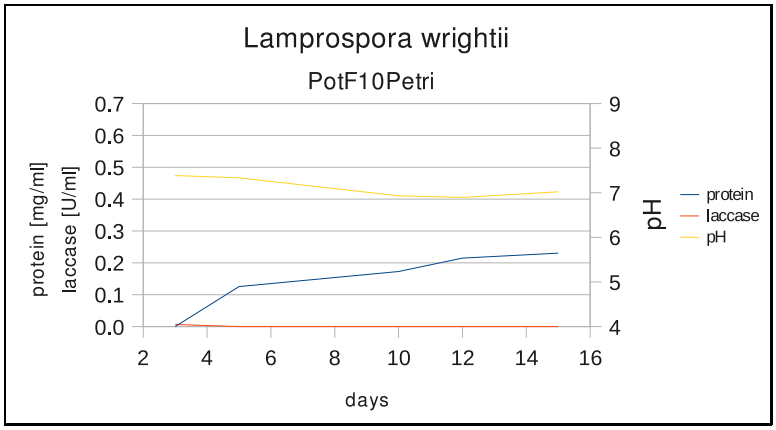

Figure 14.18: *L. wrightii* cultivated in an petridish in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

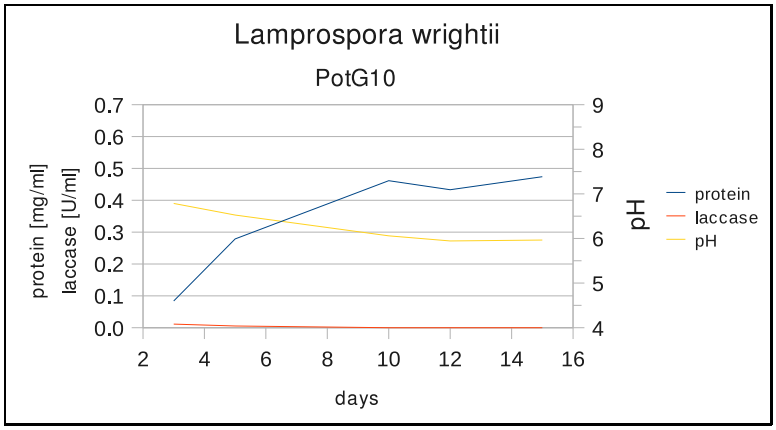

Figure 14.19: *L. wrightii* cultivated in potato media with 10 g/l glucose and 0.1 mM copper sulphate.

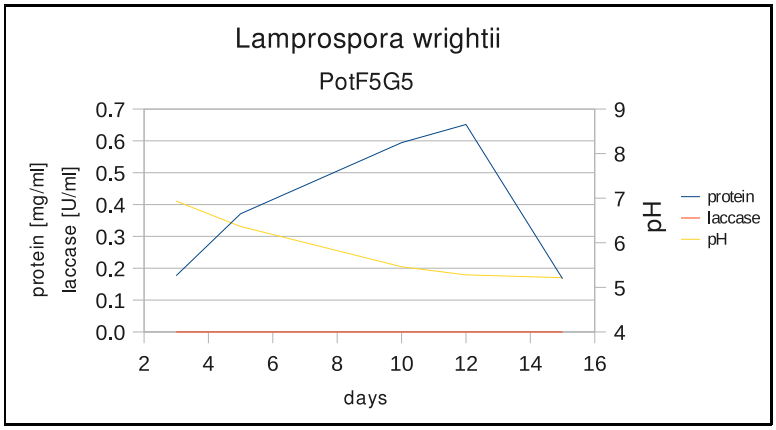

Figure 14.20: *L. wrightii* cultivated in potato media with 5 g/l fructose, 5 g/l glucose and 0.1 mM copper sulphate.

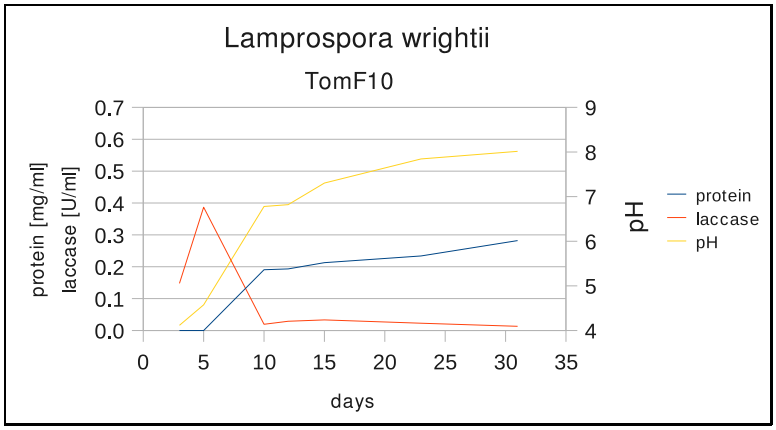

Figure 14.21: *L. wrightii* cultivated in tomato juice with 10 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

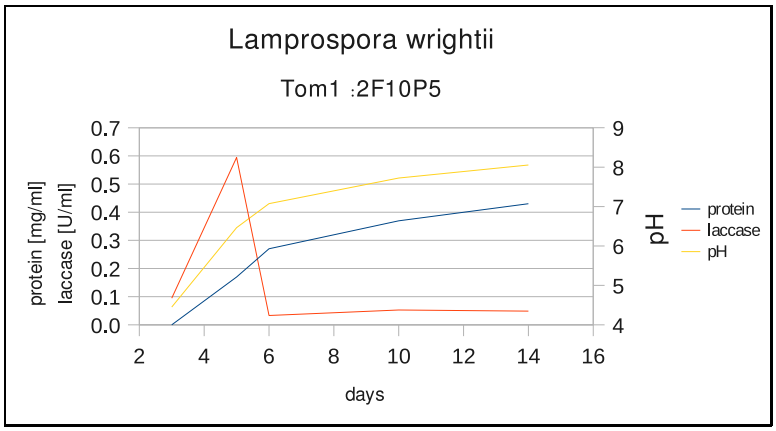

Figure 14.22: *Lamprospora wrightii* cultivated in tomato juice (1:2) with 10 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

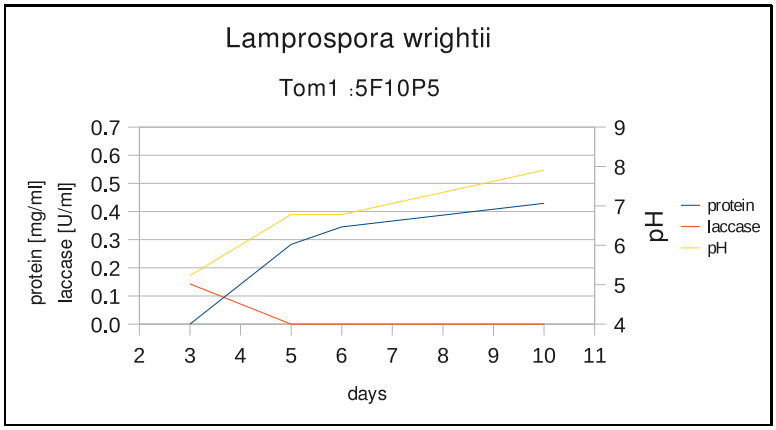

Figure 14.23: *L. wrightii* cultivated in tomato juice (1:5) with 10 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

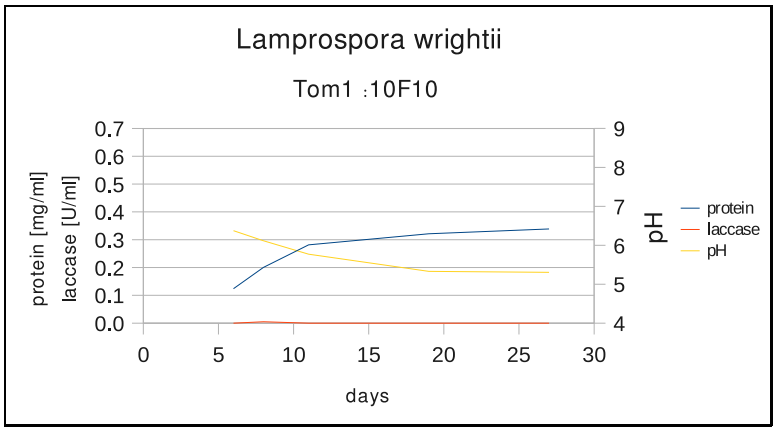

Figure 14.24: *L. wrightii* cultivated in tomato juice (1:10) with 10 g/l fructose, 5 g/l soy peptone and 0.1 mM copper sulphate.

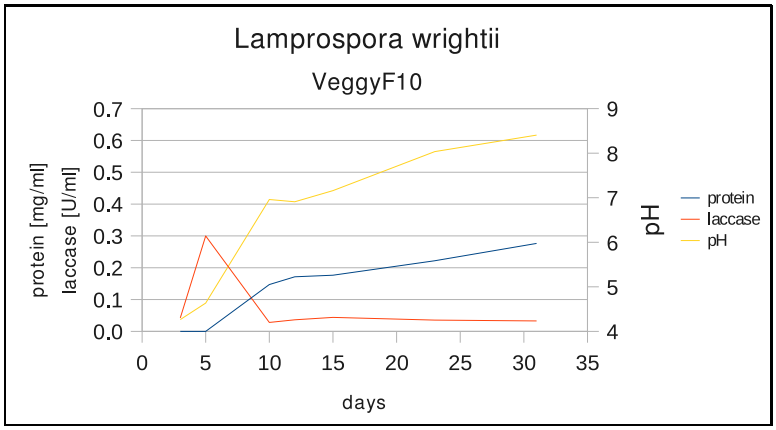

Figure 14.25: *L. wrightii* cultivated in vegetable juice with 10 g/l fructose and 0.1 mM copper sulphate.

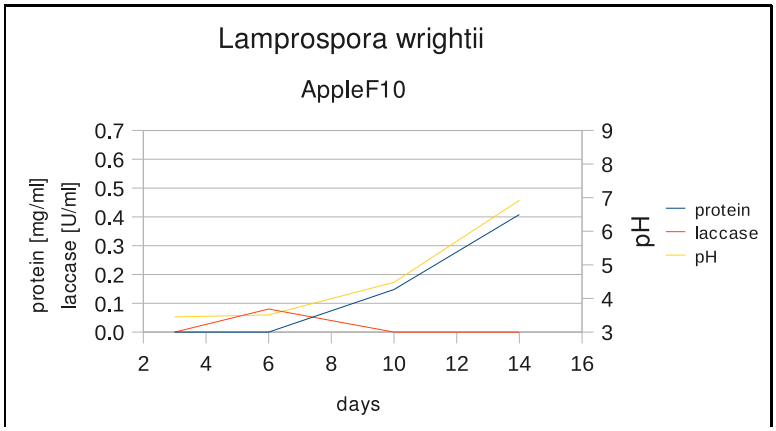

Figure 14.26: *L. wrightii* cultivated in apple juice with 10 g/l fructose and 0.1 mM copper sulphate.

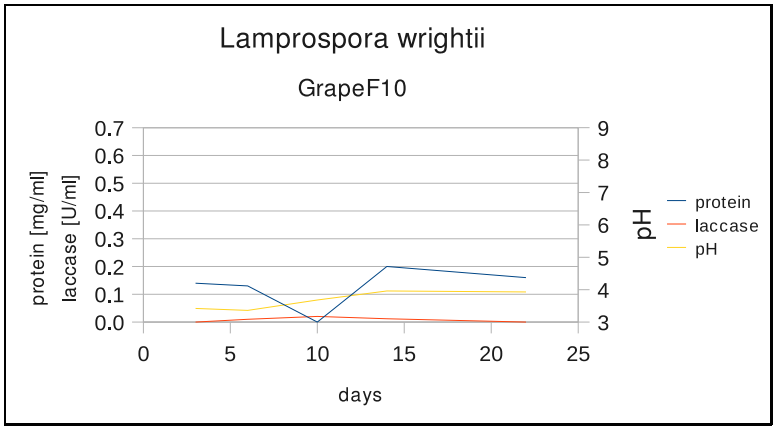

Figure 14.27: *L. wrightii* cultivated in grape juice with 10 g/l fructose and 0.1 mM copper sulphate.

Chapter 15

Cultivation of *Botrytis aclada*

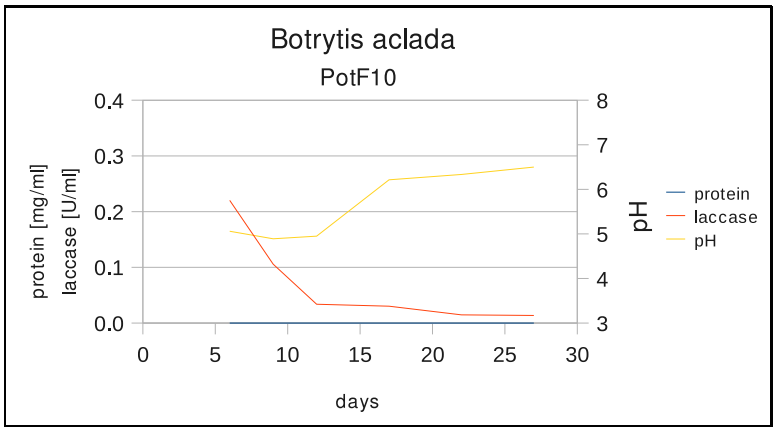

Figure 15.1: *B. aclada* cultivated in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

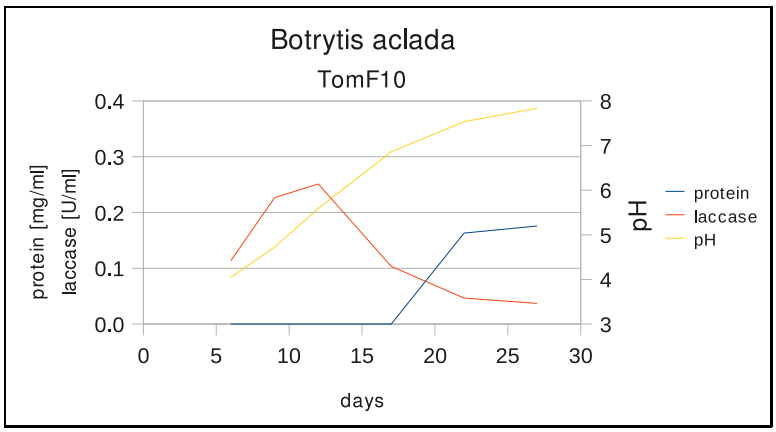

Figure 15.2: *B. aclada* cultivated in tomato juice with 10 g/l fructose and 0.1 mM copper sulphate.

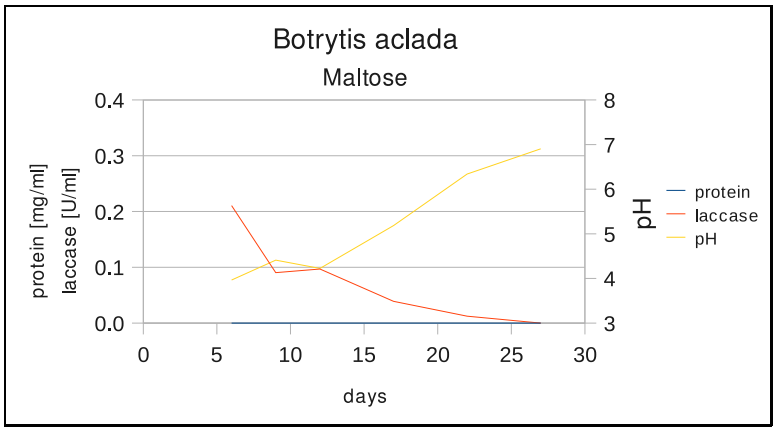

Figure 15.3: *B. aclada* cultivated with 30 g/l malt extract, 3 g/l soy peptone and 0.1 mM copper sulphate.

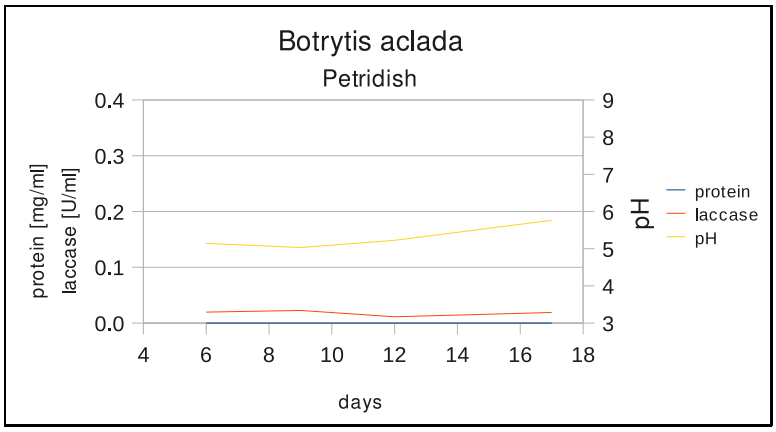

Figure 15.4: *B. aclada* cultivated in a petridish in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

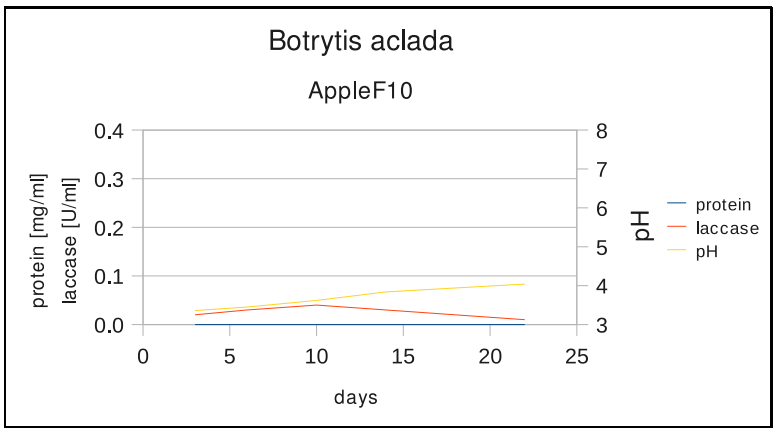

Figure 15.5: *B. aclada* cultivated in apple juice with 10 g/l fructose and 0.1 mM copper sulphate.

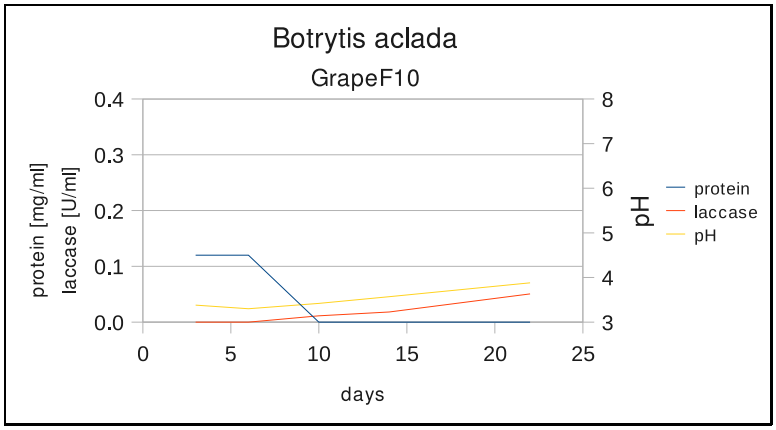

Figure 15.6: *B. aclada* cultivated in grape juice with 10 g/l fructose and 0.1 mM copper sulphate.

Chapter 16

Cultivation of *Glomerella cingulata*

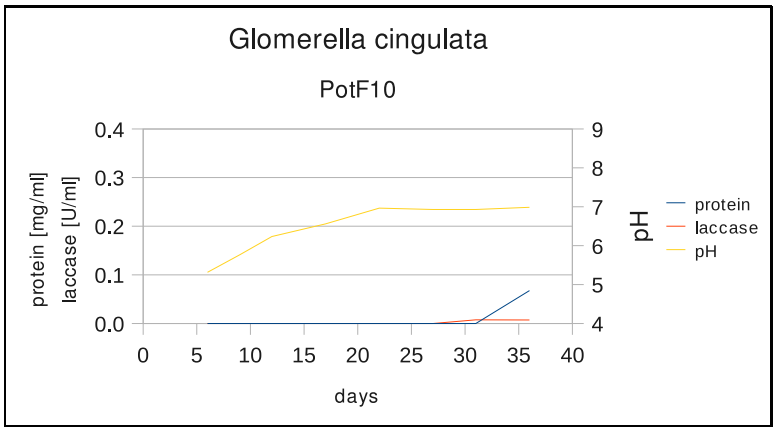

Figure 16.1: *G. cingulata* cultivated in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

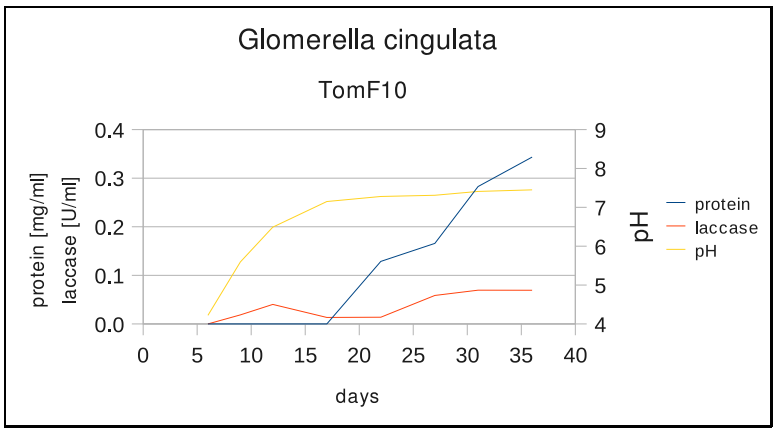

Figure 16.2: *G. cingulata* cultivated in tomato juice with 10 g/l fructose and 0.1 mM copper sulphate.

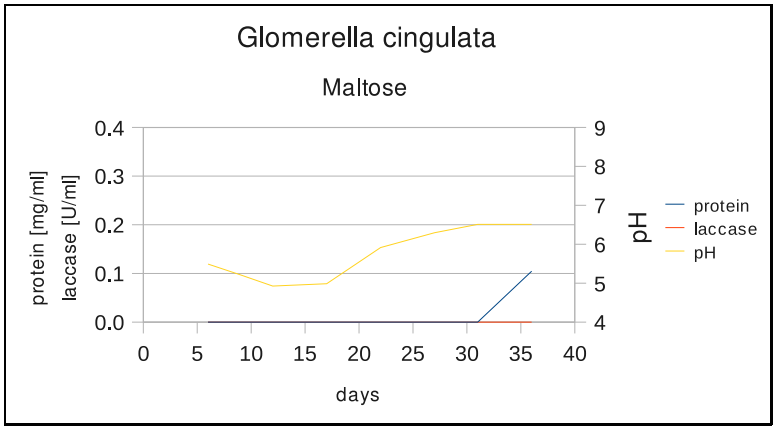

Figure 16.3: *G. cingulata* cultivated with 30 g/l malt extract, 3 g/l soy peptone and 0.1 mM copper sulphate.

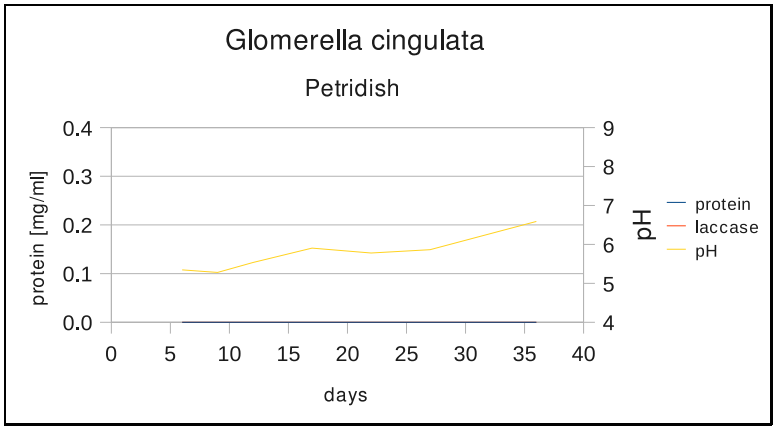

Figure 16.4: *G. cingulata* cultivated in a petridish in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

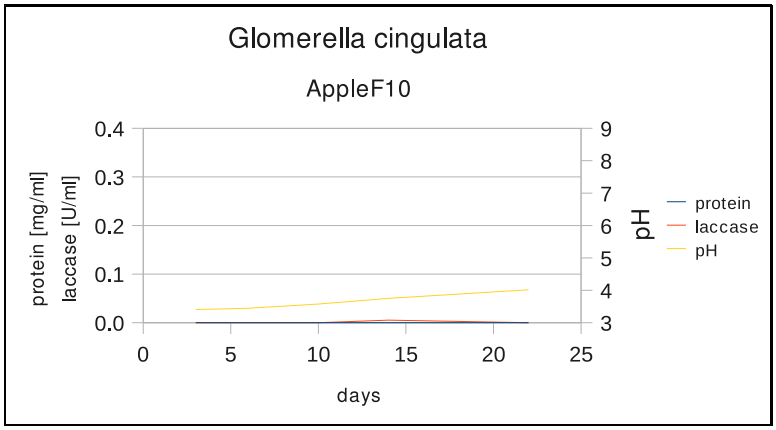

Figure 16.5: *G. cingulata* cultivated in apple juice with 10 g/l fructose and 0.1 mM copper sulphate.

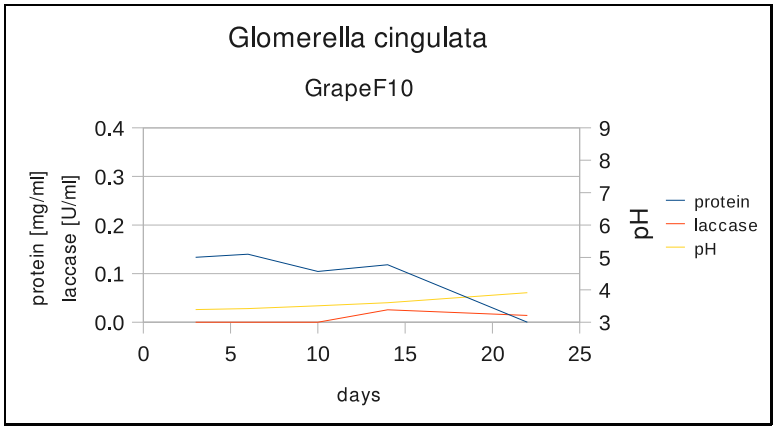

Figure 16.6: *G. cingulata* cultivated in grape juice with 10 g/l fructose and 0.1 mM copper sulphate.

Chapter 17

Cultivation of *Phoma destructiva*

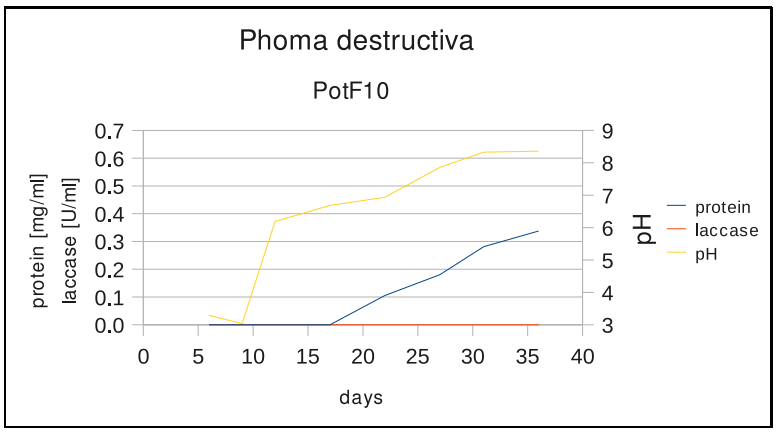

Figure 17.1: *P. destructiva* cultivated in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

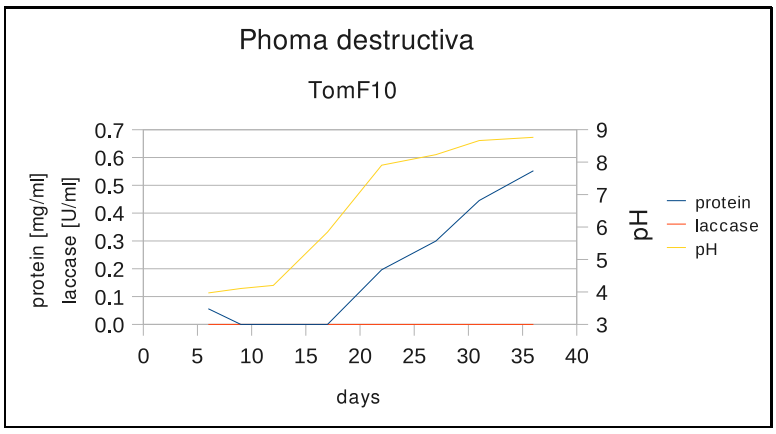

Figure 17.2: *P. destructiva* cultivated in tomato juice with 10 g/l fructose and 0.1 mM copper sulphate.

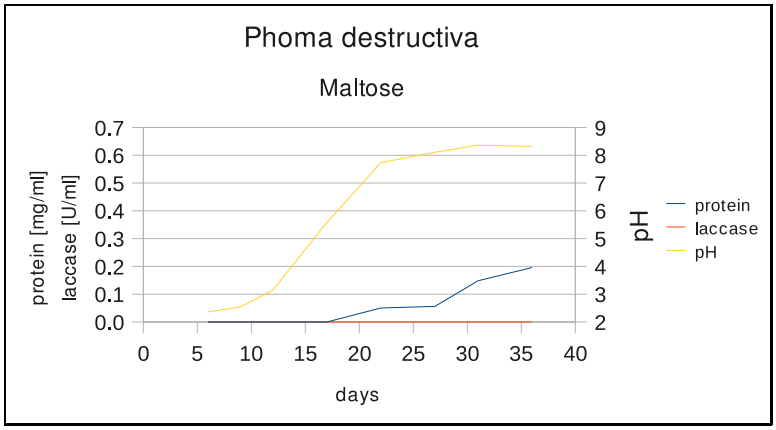

Figure 17.3: *P. destructiva* cultivated with 30 g/l malt extract, 3 g/l soy peptone and 0.1 mM copper sulphate.

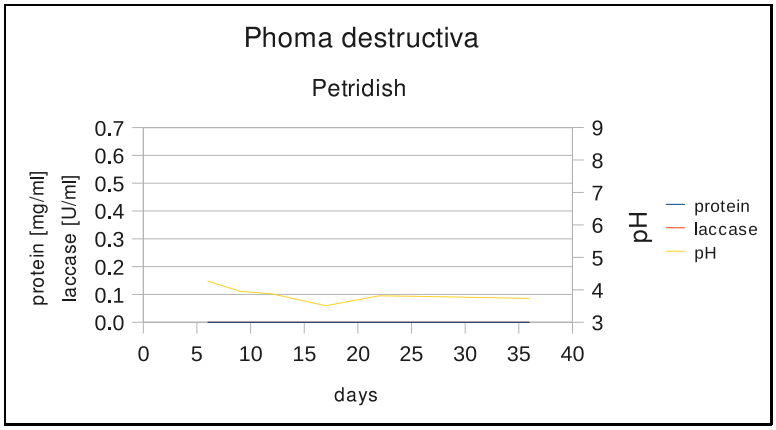

Figure 17.4: *P. destructiva* cultivated in a petridish in potato media with 10 g/l fructose and 0.1 mM copper sulphate.

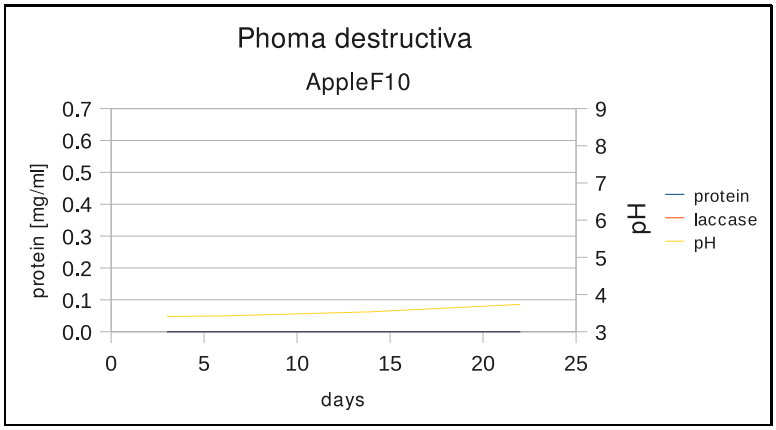

Figure 17.5: *P. destructiva* cultivated in apple juice with 10 g/l fructose and 0.1 mM copper sulphate.

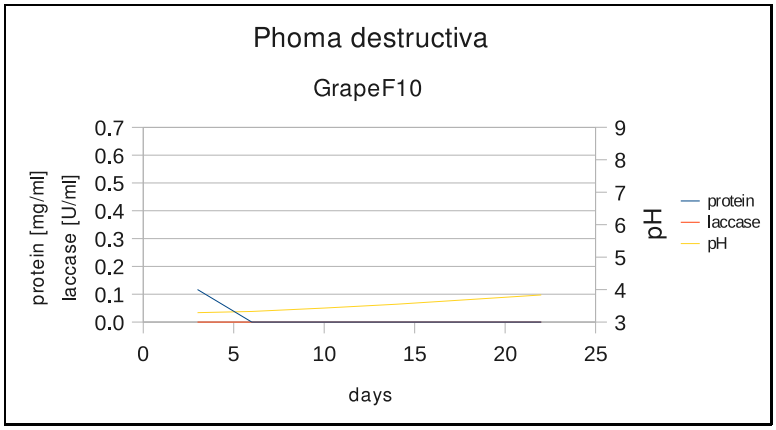

Figure 17.6: *P. destructiva* cultivated in grape juice with 10 g/l fructose and 0.1 mM copper sulphate.

Chapter 18

Michaelis-Menten Kinetics

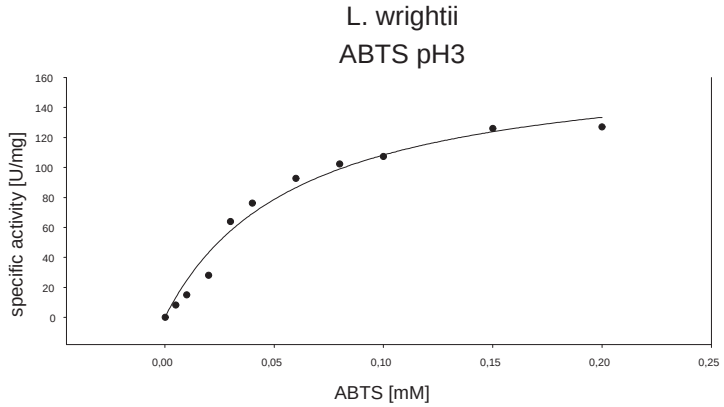

Figure 18.1: Michaelis Menten plot from *L. wrightii* laccase for ABTS at pH 3.0.

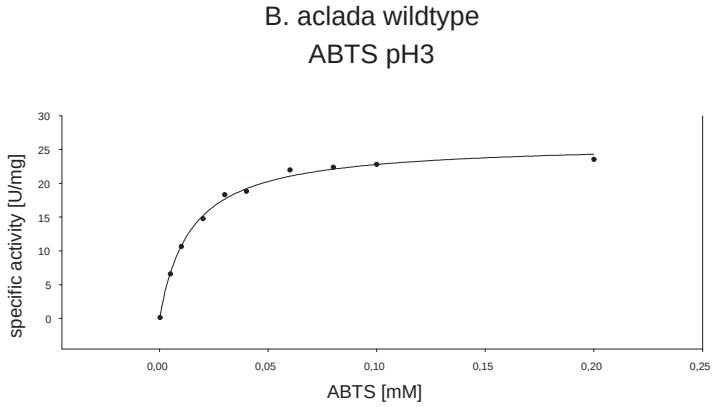

Figure 18.2: Michaelis Menten plot from *B. aclada* wildtype laccase for ABTS at pH 3.0.

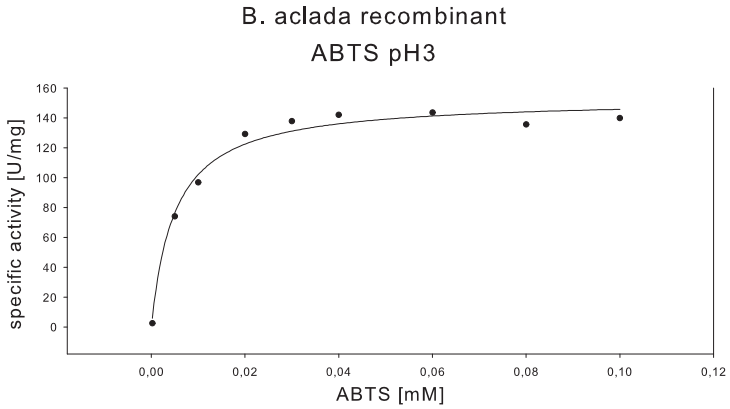

Figure 18.3: Michaelis Menten plot from *B. aclada* recombinant laccase for ABTS at pH 3.0.

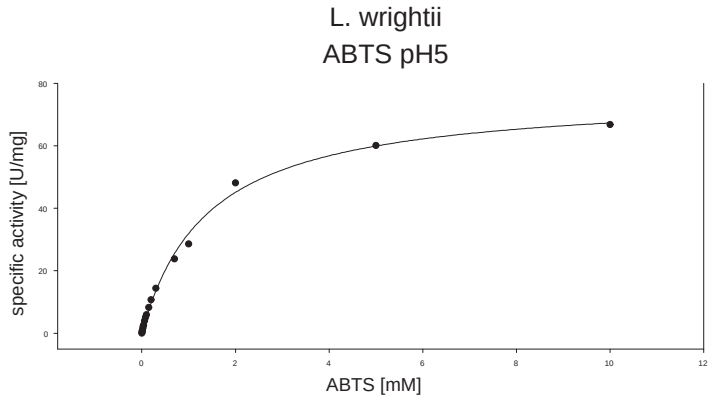

Figure 18.4: Michaelis Menten plot from *L. wrightii* laccase for ABTS at pH 5.0.

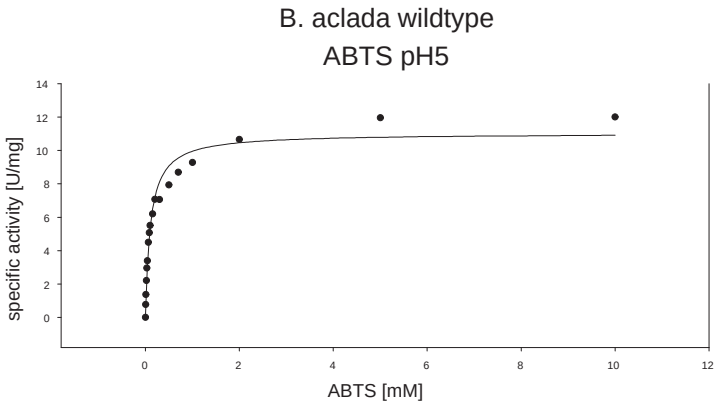

Figure 18.5: Michaelis Menten plot from *B. aclada* wildtype laccase for ABTS at pH 5.0.

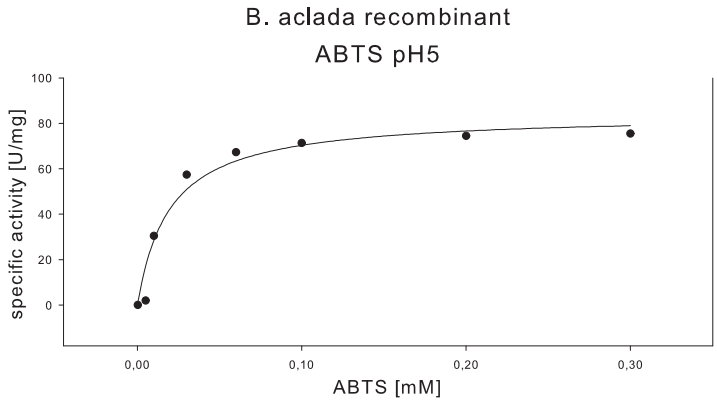

Figure 18.6: Michaelis Menten plot from *B. aclada* recombinant laccase for ABTS at pH 5.0.

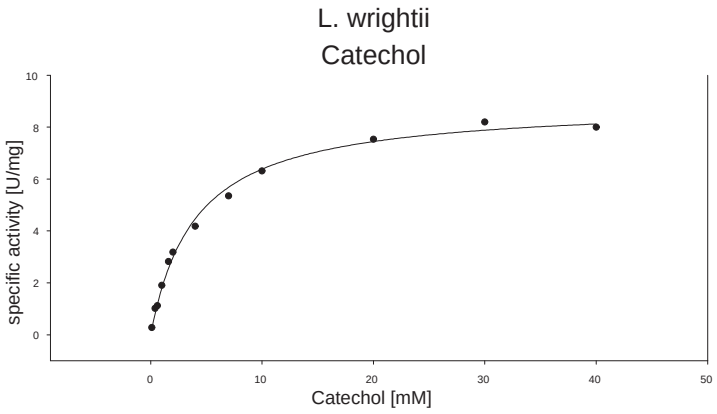

Figure 18.7: Michaelis Menten plot from *L. wrightii* laccase for catechol at pH 5.0.

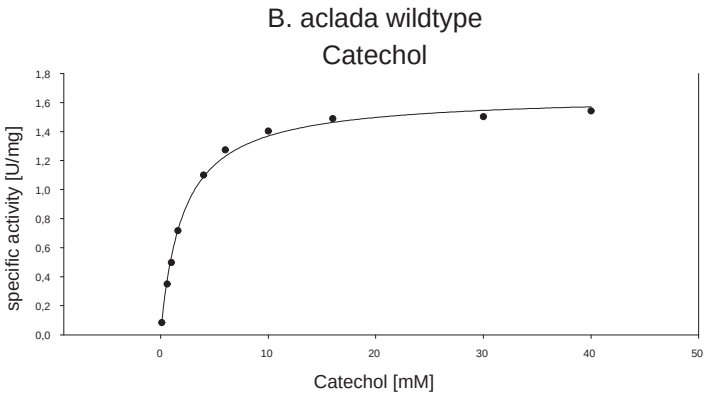

Figure 18.8: Michaelis Menten plot from *B. aclada* wildtype laccase for catechol at pH 5.0.

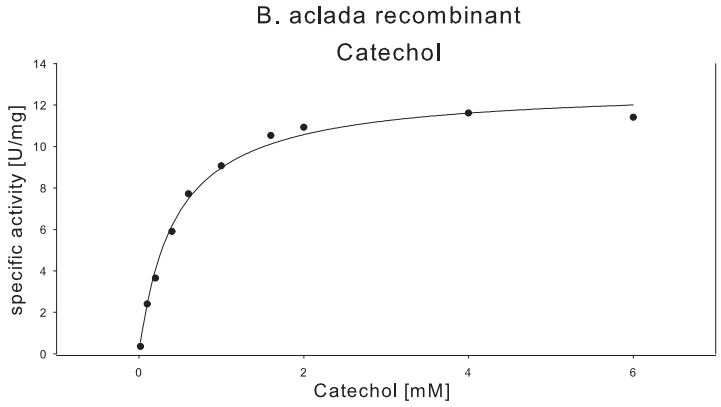

Figure 18.9: Michaelis Menten plot from *B. aclada* recombinant laccase for catechol at pH 5.0.

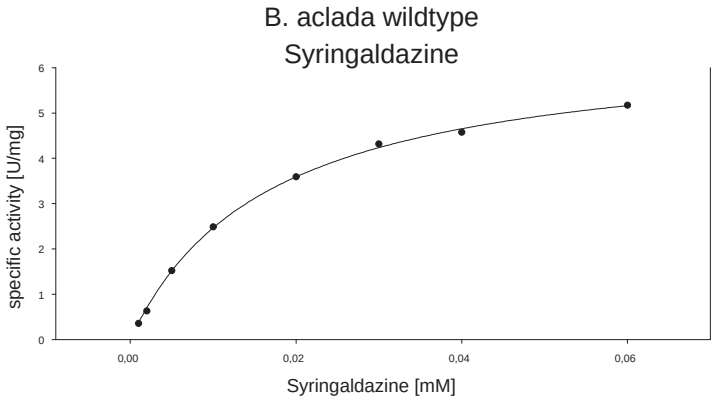

Figure 18.10: Michaelis Menten plot from *B. aclada* wildtype laccase for syringaldazine at pH 6.0.

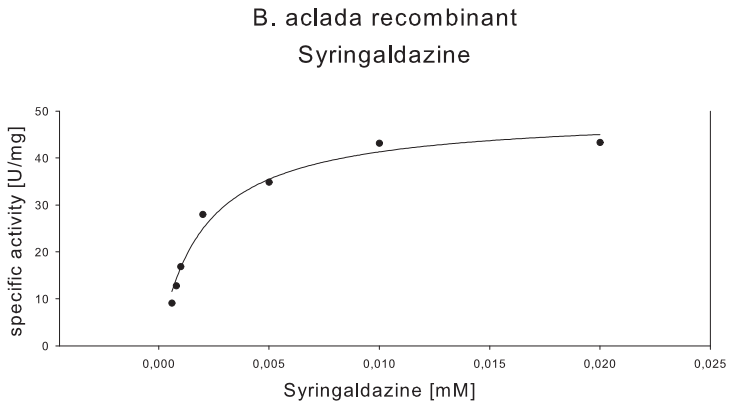

Figure 18.11: Michaelis Menten plot from *B. aclada* recombinant laccase for syringaldazine at pH 6.0.