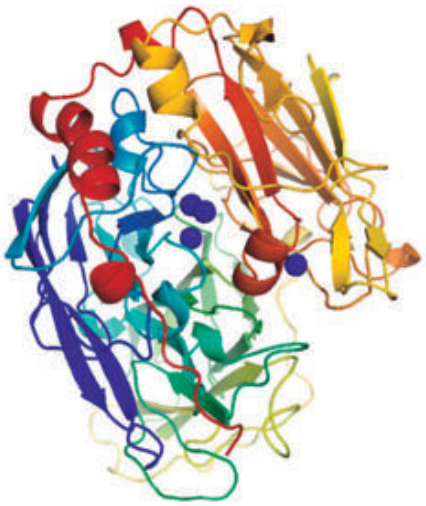
Martin Rühl

Laccases and other ligninolytic enzymes of the basidiomycetes *Coprinopsis cinerea* and *Pleurotus ostreatus*



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Nicht weil es schwer ist, wagen wir es nicht,
sondern weil wir es nicht wagen, ist es schwer
(Seneca)

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Summary

Organisms produce ligninolytic enzymes in order to degrade lignocellulosic materials. Mainly, such enzymes are found in the fungal kingdom and are particularly widespread in the phylum Basidiomycota. The ligninolytic system of fungi consists of peroxidases (manganese peroxidases - MnP, lignin peroxidases - LiP and versatile peroxidases - VP) and laccases which for substrate degradation are secreted by the fungus into the environment. Ligninolytic enzymes can be produced by cultivating adequate fungi either in submerged fermentation (SmF) or solid state fermentation (SSF). An introduction to these fermentation techniques is presented with regard to enzymes which are relevant for the wood processing industry. Laccases as main representative of the ligninolytic system can be used for delignification and bleaching in the pulp and paper industry, in the production of wood composites and to alter wood properties. Production and biochemical characterisations are in the focus of this PhD thesis.

As an example for the production of ligninolytic enzymes, the ligninolytic system of the white-rot fungus *Pleurotus ostreatus* showing laccase and peroxidases activity in SSF was experimentally studied. It was shown that the enzymatic activities of all the enzymes detected during SSF on wheat straw depend on the life cycle of the fungus. During vegetative growth phases of *P. ostreatus*, enzyme activities were high; they dropped upon initiation of fruiting body formation and were almost undectable after harvest of the mature fruiting bodies. In this study, maximum activities of 1.1-1.3 U/ml for laccase, 15-16 U/ml for MnP and 1.1-1.4 U/ml for VP were reached. Generally, the crude culture broth of the SSF on wheat straw had a brownish colour, which probably resulted from phenolic compounds and other impurities, restricting the biotechnological usage of the unpurified enzymes.

Industrial production of fungal enzymes and other metabolites is normally done in SmF, due to easier handling, better reproducibility and easy to establish standard operation processes. In liquid cultures during SmF, filamentous fungi can grow in free filamentous or in aggregated forms, known as pellets. The morphological type of growth is influenced by various factors, such as agitation or steering speed, cultivation temperature, pH and many more, and can influence production efficiencies of a fungal strain. In a combined study, I observed the morphology and laccase yield of the basidiomycete *Coprinopsis cinerea* FA2222 transformed with a plasmid containing the homologue laccase gene *lcc1* under control of the *Agaricus bisporus gpdII*-promoter in shaken flask and stirred bioreactor cultures. The

C. cinerea transformant showed pelleted growth in both types of cultivation, whereas in shake flask cultures at 120 rpm a more uniform and smaller pellet size was observed compared to the cultures grown in a stirred bioreactor at a stirring speed 120 rpm. In stirred bioreactor cultures, where distinct pH values were kept constant, a higher fragmentation rate of the pellets occurred at pH 7 and pH 8 as compared to pH 6. In contrast at pH 6, pellets of a more similar size occurred and highest laccase activities in stirred bioreactor cultures were obtained. In the shake flask cultures incubated at 25 °C and 37 °C highest total laccase activities of up to 10 U/ml were reached at the lower temperature of 25 °C. Microtome cuttings of the embedded pellets derived from shake flask cultures at 25 °C had a smooth appearance with a dense outer zone and a less dense inner region. On the other hand, pellets formed at 37 °C showed a hairy look with a broader and less dense surface.

To analyse native production of laccases in *C. cinerea*, ten different monokaryotic strains of this fungus were tested at 25 °C and 37 °C for their ability to produce laccase in liquid shaken cultures in two different media, respectively. Overall, laccase yields were higher at 25 °C than at 37 °C for nine strains whilst one strain (LN118) produce no or only negligible amounts of laccase at both temperatures. In seven of the strains, laccase yields of around 1 U/ml or more were detected in glucose based media cultures at 25 °C. The laccase activity in these seven strains resulted from two or more laccase isoenzymes and/or isoforms as determined by LC-MS/MS. Five different isoenzymes were detected via LC-MS/MS in varying combinations in the different strains which were identified to be Lcc1, Lcc2, Lcc5, Lcc9 and Lcc10. In all strains, Lcc1 and Lcc5 were produced at highest amounts. Additionally to the native laccase production, improvement of recombinant laccase production of a single isoenzyme in *C. cinerea* was achieved. Culturing optimisation with different known *C. cinerea* media revealed that a glucose-based medium (modified Kjalke) gave highest laccase yields. In total, recombinant laccase activity from culture supernatants were improved from values of around 3 U/ml for a *C. cinerea* FA2222 laccase *lcc1* transformant in to more than 10 U/ml for a *C. cinerea* LN118 laccase *lcc1* transformant both cultivated at 37 °C.

Other than Lcc1, laccases Lcc5, Lcc6 and Lcc7 were recombinantly produced in *C. cinerea* FA2222 at 37 °C. Laccases were purified from the supernatant of shake flask cultures. The optimal reaction temperature, optimal pH value and kinetic parameters for four different laccase substrates were analysed. Stability tests regarding temperature, pH and organic solvents were conducted, revealing Lcc5 to be a good candidate for reactions in high concentrations of organic solvents. Additionally, the 3-dimensional structure of Lcc5 was determined via X-ray.

Zusammenfassung

Ligninolytische Enzyme werden von Mikroorganismen produziert mit dem Zweck, Lignocellulose abzubauen. Verschiedene ligninolytische Enzyme, wie Peroxidasen (Mangan-Peroxidasen - MnP, Lignin-Peroxidasen - LiP und versatile-Peroxidasen - VP) und Laccasen werden überwiegend von Pilzen, insbesondere Basidiomyceten zum Substratabbau in ihre Umgebung sekretiert.

Ligninolytische Enzyme können industriell durch Kultivierung von entsprechenden Pilzen sowohl in submersen Fermentationsverfahren (SmF) als auch in Feststoffermentationen (SSF) hergestellt werden. Zu Beginn dieser Arbeit werden beide Fermentationssysteme dargestellt und ihr Einsatz bei der Produktion von Enzymen, die für die Holzwerkstoffindustrie von Interesse sind, diskutiert. Der Schwerpunkt der Betrachtung liegt dabei auf den Laccasen, die als eine der wichtigsten Enzymklassen im ligninolytischen System von Pilzen zum Delignifizieren und Bleichen von Papier und Pülpe, zur Herstellung von Holzwerkstoffen und zur Veredelung von Hölzern eingesetzt werden. Die Produktion von Laccasen und deren biochemische Charakterisierung stehen daher im Fokus dieser Doktorarbeit. Für diese Arbeit wurde die Produktion von ligninolytischen Enzymen beispielhaft am ligninolytischen System des Weißfäulepilzes *Pleurotus ostreatus* untersucht. Während der SSF auf Weizenstroh wurden Peroxidase- und Laccaseaktivitäten nachgewiesen, deren Intensität sich nach dem Lebenszyklus des Pilzes richtete. Die Enzymaktivitäten in besiedeltem Substrat waren in den vegetativen Wachstumsphasen von *P. ostreatus* hoch, fielen danach mit aufkommender Fruktifikation ab und waren nach der Ernte der Pilzfruchtkörper kaum mehr messbar. Im Verlauf der Studie wurden maximale Aktivitäten von 1.1-1.3 U/ml für Laccase, 15-16 U/ml für MnP and 1.1-1.4 U/ml für VP erreicht. Extrahierte Laccase-haltige Kulturbrühe der SSF hatte eine braune Farbe, die womöglich aus phenolischen Komponenten und anderen Verunreinigungen resultierte. Diese Verfärbung schränkt eine biotechnologische Nutzung der unaufgereinigten Enzymlösung ein.

Normalerweise findet eine industrielle Produktion von pilzlichen Enzymen und anderen Metaboliten in SmF statt, weil hier eine technisch einfachere Handhabung und bessere Reproduzierbarkeit in standardisierten Verfahren möglich sind. Filamentöse Pilze können in solchen Flüssigkulturen entweder als freie Filamente oder als Hyphenaggregate, sog. Pellets, wachsen. Die Art der Morphologie des Pilzes in der Flüssigkultur hängt von verschiedenen Faktoren ab, wie z.B. von Schüttel- oder Rührgeschwindigkeit, Kultivierungstemperatur oder pH-Wert, und sie kann die Produktionseffizienz eines Stammes entscheidend beeinflussen.

In einer vergleichenden Studie zwischen Schüttelkolben und Bioreaktor wurden Morphologie und Laccaseausbeute eines mit dem Plasmid pYSK7 transformierten Stammes des Basidiomyceten *Coprinopsis cinerea* untersucht. Das verwendete Plasmid beinhaltet das *C. cinerea* Laccase Gen *lcc1*, das in seiner Expression durch den *Agaricus bisporus gpdII* Promotor kontrolliert wird. Der *C. cinerea* Transformant wuchs in beiden Kultivierungsformen in Form von Pellets, wobei die Pellets in den Schüttelkulturen bei 120 upm (Umdrehung pro Minute) gleichmäßiger und kleiner waren als jene, die sich im Bioreaktor bei einer Rührgeschwindigkeit von 120 upm ausbildeten. Im Rührkesselreaktor, in dem der pH Wert konstant bei pH 6, pH 7 oder pH 8 gehalten wurde, konnte man eine höhere Fragmentierung der Pellets bei den höheren pH Werten pH 7 und pH 8 feststellen. Bei pH 6 zeigten die Pellets allerdings eine gleichmäßigere Größe. Außerdem wurden die höchsten Laccaseaktivitäten in den Bioreaktorkulturen bei pH 6 gemessen. In den Schüttelkulturen, die bei 25 °C und 37 °C inkubiert wurden, konnten Laccaseaktivitäten von bis zu 10 U/ml bei der niedrigeren Kultivierungstemperatur von 25 °C erreicht werden. Mikrotomschnitte von in Wachs eingebetteten Pellets zeigten, dass Pellets aus den Schüttelkulturen bei 25 °C aussen vergleichsweise glatt waren. Ein dichter Ring von kompaktem Myzel umgibt einen weniger dichten inneren Bereich. Im Gegensatz dazu waren Pellets bei 37 °C vom Erscheinungsbild her eher aufgerauht und der äußere Myzelring war breiter und nicht so dicht gepackt wie bei Pellets bei 25 °C.

Um die natürliche Laccaseproduktion von *C. cinerea* zu testen, wurden zehn unterschiedliche Monokaryonten bei jeweils 25 °C und 37 °C in zwei unterschiedlichen Medien kultiviert. Insgesamt waren bei neun der Stämme die Laccaseausbeuten bei 25 °C höher als bei 37 °C, wobei ein Stamm (LN118) keine oder nur sehr geringe Mengen an Laccase produzierte. In sieben der Stämme wurden Laccaseaktivitäten von 1 U/ml und mehr erreicht, wenn diese in Glukose-haltigem Medium bei 25 °C kultiviert wurden. Anhand einer LC-MS/MS Analyse konnte gezeigt werden, dass die Laccaseaktivität dieser sieben Stämme aus zwei oder mehreren verschiedenen Isoenzymen und/oder Isoformen resultiert. Fünf verschiedene Isoenzyme (Lcc1, Lcc2, Lcc5, Lcc9 und Lcc10) die in verschiedenen Zusammensetzungen in den einzelnen Stämmen vorkommen, konnten nachgewiesen werden. Lcc1 und Lcc5 wurden von allen Stämmen in hohen Mengen produziert.

Weitere Experimente wurden durchgeführt, um die rekombinante Produktion eines einzelnen Isoenzyms in *C. cinerea* zu erhöhen. Versuche zur Optimierung der Kultivierungsbedingungen mit verschiedenen *C. cinerea* Kulturmedien ergaben, dass das Glukose-basierte Medium (modified Kjalke) die höchsten Laccaseausbeuten lieferte. Durch

verschiedene Optimierungsversuche konnte für einen Lcc1 Transformanten des *C. cinerea* Stammes FA2222 die Laccaseaktivität im Kulturüberstand insgesamt von ungefähr 3 U/ml auf über 10 U/ml erhöht werden. Laccase Erträge von bis zu 11 U/ml wurden auch für den Lcc1 Transformanten des *C. cinerea* Stammes LN118 erzielt.

Neben der Laccase Lcc1 wurden auch die Laccasen Lcc5, Lcc6 und Lcc7 rekombinant in *C. cinerea* FA2222 bei 37 °C produziert. Die Laccasen wurden aus dem Kulturüberstand der Schüttelkolben aufgereinigt. Ihre optimale Reaktionstemperatur, ihr optimaler pH Wert und ihre kinetischen Parameter wurden für vier verschiedene Laccasesubstrate analysiert. Stabilitätstests bezüglich Temperatur, pH und organischen Lösungsmitteln zeigten, dass Lcc5 insbesondere für Reaktionen bei hohen Konzentrationen von organischen Lösungsmitteln geeignet ist. Zusätzlich wurde die 3-dimensionale Struktur von Lcc5 mit Hilfe der Röntgenstrukturanalyse bestimmt.

Abbreviations

ABTS	2,2-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid)
ACN	acetonitrile
APS	ammonium peroxodisulfate
AU	arbitrary units
BE	biological efficiency
BPA	bisphenol A
BSM	basal medium
C/N	carbon nitrogen ratio
CCD	charge-coupled device
DEAE	diethylaminoethyl cellulose
DMAB	3-dimethylaminobenzoic acid
DMP	2.6-dimethoxyphenol
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DW	dry weight
E°	electron potential
EDTA	ethylenediaminetetraacetic acid
EM	ectomycorrhizal
EPR	electron paramagnetic resonance
ESI	electrospray ionisation
EtOH	ethanol
FDA	fluorescein diacetate
FPLC	fast protein liquid chromatography
gds	gram dry substrate
GFP	green fluorescence protein
HBA	4-hydroxybenzoic acid
HBT	1-hydroxybenzo-triazole
HIC	hydrophobic interaction chromatography
HOBT	1-hydroxybenzotriazole
IEF	isoelectric focusing
K_m	Michaelis-Menten constant
LC-MS/MS	liquid chromatography with mass spectrometry
LiP	lignin peroxidase
LMCO	laccase like multicopper oxidase
LMS	laccase mediator system
MBTH	3-methyl-2-benothiazolinone hydrazone hydrochloride
MCO	multicopper oxidase
MDF	medium-density fibreboards
MM	minimal medium
MnP	manganese peroxidase
MSBR	magnetic stirr bar reactor
NAD	nicotinamide adenine dinucleotide
NaF	sodium fluorid
NaN_3	sodium acid
NHAA	N-hydroxyacetanilide
NHE	normal hydrogen electrode
PAGE	polyacrylamide gel electrophoresis
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PEG	polyethylene glycol
pI	isoelectrin point
RH	relative humidity
ROI	area of interest
rpm	revolutions per minute
RT	room temperature
RuBP	ruthenium II tris (bathophenantroline disulfonate)
SDS	sodium dodecyl sulfate
SGZ	syringaldazine
SmF	submerged fermentation
SMS	spent mushroom substrate
SSF	solid state fermentation
TCA	trichloacetic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
TMP	thermomechanical pulp
TNT	2,4,6-trinitrotoluene
Tris	tris(hydroxylmethyl)aminoethan
U	international unit
VA	violuric acid
V_{max}	maximum reaction rate
VP	versatile peroxidase
YMG	yeast malt glucose
YMG/T	yeast malt glucose tryptophan medium
Å	Ångström
ε	molecular extinction coefficient
κ	kappa number

Chapter 1

Introduction

I. Laccases: sources and applications

II. Basidiomycetes: higher fungi of great economical and ecological value

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III. Aim of the thesis

IV. References

I. Laccases: sources and applications

The widespread enzyme laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) produced by many ligninolytic fungi belongs to the family of multi-copper enzymes (Messerschmidt et al. 1989) that oxidise a wide range of substrates through a one-electron oxidation reaction. In general, laccases contain four copper atoms distributed in three copper sites (T1, T2 and T3). The electron migration between the substrate and the enzyme is regulated by the T1 site, also known as blue copper site (Solomon et al. 1996), and, thus, depends on the redox potential (= reduction and oxidation potential) of this site. In fungal laccases, oxidation potentials of the blue copper can vary e.g. between 0.46 V and 0.79 V as determined for *Myceliophthora thermophila* and *Trametes versicolor*, respectively (Xu 1996; chapter 5). Electron migration in laccases takes place via a cysteine-hystidine pathway from the T1 site to the T2 and T3 sites. T2 and T3 form a trinuclear copper cluster, with one copper atom at the T2 site and two copper atoms at the T3 site, where dioxygen is reduced to water (Solomon et al. 1996, Ducros et al. 1998, Messerschmidt 1998, Hakulinen et al. 2002, Piontek et al. 2002).

Natural organic substrates, such as phenolic compounds, can be attacked directly by laccases with subsequent generation of phenoxy radicals (Fig. 1A), which can perform radical coupling or substitution reaction with other molecules (Solomon et al. 1996). In cases where physical (size) or chemical (redox-potential) properties of the laccase restrict an oxidation of the substrate, small mediating substances with higher oxidation potentials called mediators may be used (d'Acunzo et al. 2006, Fig. 1B). Mediators are activated by laccases via a monoelectronic oxidation and can afterwards react with substrates, which are not able to be degraded by laccases alone. Thus, non-phenolic substances may be degraded by laccases by usage of different kinds of natural [phenols, aniline, 4-hydroxybenzoic acid (HBA), 4-hydroxybenzyl alcohol, cysteine, methionine] and non-natural [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), N-hydroxyacetanilide (NHAA), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and violuric acid (VA)] mediators (Johannes and Majcherczyk 2000, Camarero et al. 2005). The combination of a laccase and a suitable mediator is known as laccase mediator system (LMS). Such systems are used in different industries (section 1.I.B) and may also be found in nature, where suitable mediators enable the fungal laccases to degrade recalcitrant substances (Johannes and Majcherczyk 2000).

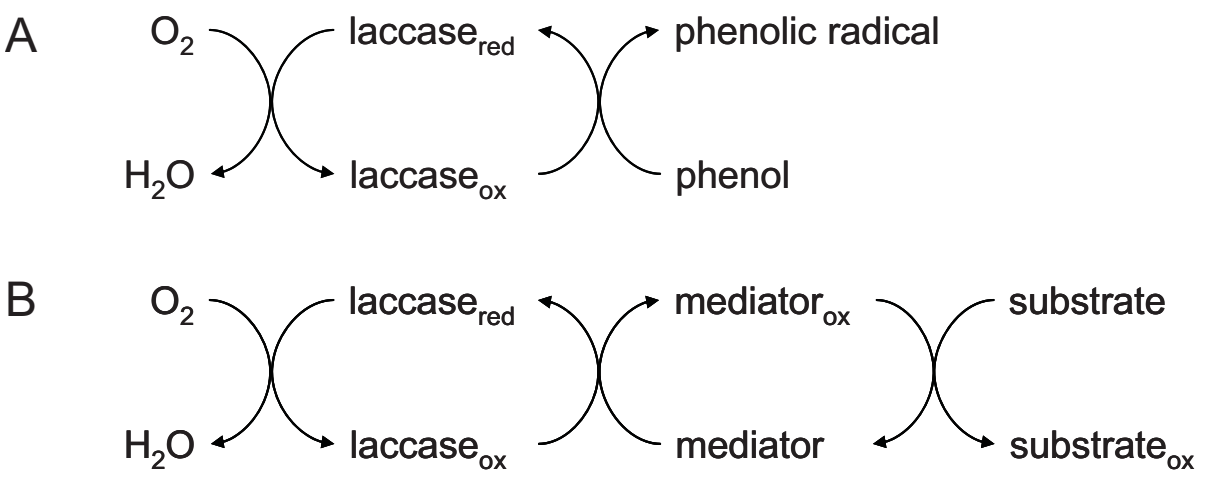

Fig. 1 Schematic reaction cascade of a laccase with a phenol (**A**) and of a laccase mediator system (**B**) after Bourbonnais et al. (1998).

A. Occurrence and functions of laccases in nature

In general, laccases are produced by various microorganisms (certain bacteria and mainly fungi) (Claus 2004), but they are also found in plants (Andreasson et al. 1976, Richardson et al. 2000, Gavnholt and Larsen 2002), insects (Sugumaran et al. 1992, Kramer et al. 2001, Arakane et al. 2005) and even crustaceans (van der Ham and Felgenhauer 2007). In the latter cases, laccase is needed for melanisation/tanning processes as well as in the immune response and wound healing (Decker et al. 2001, Lee and Soderhall 2002, Sugumaran 2002, Liu et al. 2006, Van Der Ham and Felgenhauer 2007). Insects use laccases also for tanning and for cuticle sclerotisation (Kramer et al. 2001) while in plants laccases are involved in the polymerisation of lignin (O'Malley et al. 1993).

In bacteria, laccases or laccase-like multicopper oxidases (LMCOs) were found in gram-positive bacteria, such as *Bacillus licheniformis* (Koschorreck et al. 2008), and in *Escherichia coli* (Kataoka et al. 2007), *Pseudomonas syringae* (Cha and Cooksey 1991) as well as in other gram-negative bacteria (e.g. Pseudomonadaceae) (Sharma et al. 2007). In these microorganisms, laccases might be involved in a multiplicity of processes, such as pigmentation, Cu^{2+} resistance, Mn^{2+} oxidation, sporulation and others (Sharma et al. 2007).

Most laccases are of fungal origin, where they are found in ascomycetes and basidiomycetes (Hoegger et al. 2006). Usually, laccases are extracellular proteins secreted by the fungi for oxidation reactions outside the cell. They occur in the soil inhabiting ascomycetous fungus *Aspergillus nidulans* during the sexual (Hermann et al. 1983, Scherer and Fischer 1998) as well as during the asexual life cycle (Aramayo and Timberlake 1990 and 1993, Scherer and

Fischer 1998). Another soil inhabiting deuteromycete fungus *Pestalotiopsis* sp. showed high natural laccase activity, which could be further increased in liquid culture (Hao et al. 2007). Laccase genes and activity are also found in the ascomycete family *Morchellaceae*, where saprotrophic, mycorrhizal-like and faint parasitism growth was observed (Kellner et al. 2007). In the ascomycetous plant pathogenic fungi *Gaeumannomyces graminis* var. *tritici* (Thompson et al. 2006) and *Fusarium proliferatum* (Anderson et al. 2005), laccase activity was detected as well. Furthermore, laccases have been found in thermophilic fungi such as the ascoymcetes *Myceliophthora thermophila* and *Chaetomium thermophilum* (Berka et al. 1997, Chefetz et al. 1998, Maheshwari et al. 2000). Laccases occur as well in fungal symbiosis such as in lichens. There, species of the sub-order *Peltigerineae* showed higher activities (mean 30fold) compared to other sub-orders, such as *Lecanorineae*, where low or no laccase activity was detected (Laufer et al. 2006). It is most probable that in these symbiotic fungal associations, laccases are involved in the metabolism of secondary phenolic compounds, like lichen acids (Lisov et al. 2007). In a pathogenic association community related to the esca syndrome, a severe illness in vine plants affecting all open plant parts, (ascomycetes: *Phaeomoniella chlamydospora* and *Togninia minima*, basidiomycete: *Fomitiporia mediterranea*) all three fungi produce laccases (Bruno and Sparapano 2006). The authors believe that the white-rot basidiomycete *F. mediterranea* laccase is involved in lignin metabolisation, whereas the ascomycetous laccases are important for the detoxifation of a polyphenolic compound (resveratrol) acting as antioxidant in grapes. Furthermore, laccase activity was detected in liquid shaken cultures of *Monotospora* sp., an endophytic fungus of the grass *Cynodon dactylon* (Wang et al. 2006).

While laccases are present throughout the fungal kingdom (Hoegger et al. 2006), basidiomycetes are the most common source for this enzyme under natural growth conditions. In ectomycorrhizal (EM) basidiomycetous fungi, noticeable laccase activity was detected in two *Lactarius* and all *Rusulla* species tested by Gramss et al. (1998). However, the highest extracellular laccase activity was detected in litter degrading species tested also in this work. The enzyme activity in the EM fungi seemed to be basically intracellular, whereas the litter degrading fungi showed predominantly extracellular activity (Gramss et al. 1998).

In another study of EM fungi, laccase activity was found on the tips of *Lactarius quietus* and *Cortinarius anomalus* in oak forests (Courty et al. 2006). Highest activities occurred during spring time, which can be ascribed to the degradation of the organic matter in the soil. This process takes place either to possibly obtain carbon (C) for the saprotrophic growth of the

fungi or to deliver nitrogen (N) for the vegetative growth of the trees (Courty et al. 2006). It is particularly interesting that the EM species *Laccaria bicolor* has 9 different laccase genes of which 3 are highly expressed in ectomycorrhizas, 1 in fruiting bodies of *L. bicolor* and 1 in mycelium of *L. bicolor* grown on glucose rich agar medium (Courty et al. 2009).

From results of a comparative study of five different basidiomycetes, of which three are litter degrading and two white-rot fungi, laccase was stated to be the most frequent ligninolytic enzyme in litter degrading fungi (Baldrian and Snajdr 2006). Contradictory, Ullah et al. (2002) tested several leaf litter decomposers and wood degraders and found that, overall, the ligninolytic enzyme manganese peroxidase MnP (EC 1.11.1.13, MnP, Mn(II):hydrogen-peroxide oxidoreductase) activity was higher in litter decomposers, whereas in wood decaying fungi the laccase activity was higher (Gregorio et al. 2006). In general, the wood degraders can be classified into brown- and white-rot fungi, of which only the latter ones are capable of degrading the lignin and, therefore, having a ligninolytic enzymatic system (Hoegger et al. 2007). Nevertheless, laccases were also found in brown-rot fungi, like *Gloeophyllum trabeum* and *Postia placenta* (D'Souza et al. 1996). A recent analysis of the established genome of *P. placenta* detected 5 MCO (multicopper oxidase) genes, of which 3 are obviously for laccases (Martinez et al. 2009). Contradictory, one typical representative of the white-rot fungi, *Phanerochaete chrysosporium*, has 4 *MCO* genes whose products are not laccases (Martinez et al. 2004). This emphasises the finding that *P. chrysosporium* is lacking classical laccase activity (Larrondo et al. 2003).

The basic ligninolytic system of fungi consists of laccase, MnP and lignin peroxidase (EC 1.11.1.14, LiP, 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol: hydrogen-peroxide oxidoreductase), which are produced in different combinations. Hatakka (1994) divided the white-rot fungi into 3 classes, according to the enzymes which are primarily involved in the degradation of lignin: LiP and MnP, MnP and laccase, and LiP and laccase. However, several studies show that certain fungi produce all three types of enzymes and other fungi exist where only laccase activity is detected (Table 1). The classification Hao et al. (2006) offers would comprise all possible groups: With the classes I, II, III and IV, respectively, producing only LiP and MnP (I), all three types of enzymes (II), laccase with either LiP or MnP (III) and solely laccase (IV). However, some white-rot fungi are difficult to classify in this system, as a lack of enzymatic activities under tested growth conditions does not indicate whether the enzymes might not be produced under altered conditions. The saprotrophic dung fungus *Coprinopsis cinerea* has 17 different laccase genes which are differently expressed under altered cultivation parameters such as media and temperature (Schneider et al. 1999,

Navarro-González 2008, section 4.I). However, the production of ligninolytic enzymes of species such as *C. cinerea* and *Ganoderma lucidum* seems also to be strain specific (Silva et al. 2005a, Table 1 and section 4.I). No MnPs or LiPs are present in *C. cinerea, L. bicolor* and *P. placenta*, but all of them seem to have another type of low redox peroxidase not closely related to MnP and LiP (Martinez et al. in 2009). Recently available genomes of the white-rot fungi *P. ostreatus* and *Schizophyllum commune* will show which genes coding for ligninolytic enzymes are present in these fungi and, thus, help to clarify the role of these enzymes in lignin degradation.

Table 1 Ligninolytic enzyme activities of a variety of basidiomycetes where laccase activity was detected

		Enzymes			
Fungal species		**MnP**	**LiP**	**Lac**	**Reference(s)**
Agaricus bisporus		+	ND*	+	Bonnen et al. 1994
Bjerkandera adusta		+	+	+	Hatakka 1994
Cerioporiopsis subvermisopra		+	-	+	Rüttimann et al. 1992
Collybia sp.		+	+	+	McErlean et al. 2006
Coriolopsis polyzona		+	-	+	Nerud et al. 1991
Dichomitus squalens		+	-	+	Nerud et al. 1991
Fomes sclerodermus		+	ND	+	Papinutti and Martinez 2006
Ganoderma australe		-	-	+	Elissetche et al. 2006
Ganoderma lucidum		+	ND*	+	D'Souza et al. 1999, Wang and Ng 2006b
Ganoderma	CB364	-	-	+	Silva et al. 2005a
spp.	GASI3.4	+	+	+	
	CCB209	+	+	+	
	GASI2	-	-	+	
Ganoderma valesiacum		+	-	+	Nerud et al. 1991
Grammothele subargentea		-	-	+	Saparrat et al. 2008
Lentinula edodes		+	+	+	Hatakka 1994, Silva et al. 2005b
Marasmiellus troyanus		+	ND	+	Gregorio et al. 2006
Marasmius quercophilus		+	ND	+	Steffen et al. 2007a
Mycena inclinata		+	ND	+	Steffen et al. 2007a
Panus tigrinus		+	-	+	Bonnen et al. 1994
Phlebia brevispora		+	+	+	Perez and Jeffries 1990
Phlebia floridensis		+	+	+	Arora and Gill 2005
Phlebia ochraceofulva		-	+	+	Hatakka 1994
Phlebia radiata		+	+	+	Hatakka et al. 1991
Phlebia tremelosa		+	+	+	Hatakka 1994
Pholiota lenta		+	ND	+	Steffen et al. 2007a
Pleurotus ostreatus		+	-	+	McErlean et al. 2006, this work section 2.II
Pleurotus sajor-caju		+	-	+	Hatakka 1994,Fu et al. 1997
Pycnoporus cinnabarinus		-	-	+	Eggert et al. 1996
Rhizoctonia solani		+	+	+	McErlean et al. 2006
Rigidoporus lignosus		+	-	+	Hatakka 1994
Stereum hirsutum		+	-	+	Nerud et al. 1991
Trametes gibbosa		+	+	+	Nerud et al. 1991
Trametes hirsuta		+	+	+	Nerud et al. 1991
Trametes versicolor		+	+	+	Tanaka et al. 1999

MnP = manganese dependent peroxidase, LiP = lignin peroxidase, Lac = laccase, + = enzyme activity was detected, - = no activity detected, ND = not determined, * LiP-like genes are present.

Besides the degradation of lignin, laccases have also other functions in basidiomycete fungi. Within the fruiting bodies of fungi, laccases may be involved in the cross-linking of the hyphal walls to guarantee a compact fruiting body structure (Leatham and Stahmann 1981, Thurston 1994, Xing et al. 2006). Laccase activities were e.g. found in the fruiting bodies of *C. cinerea* (Navarro-González 2008), *Grifola frondosa* (Xing et al. 2006), *Lentinula edodes* (Zhao and Kwan 1999), *Pleurotus eryngii* (Wang and Ng 2006a) and *Pleurotus ostreatus* (own observation).

Moreover, during cultivation of at least some of the mushroom producing fungi, laccase activity within the substrate was highest in colonised mycelium prior to fruiting. The activity declined and raised again after harvest, indicating a link of regulation of enzyme production to specific developmental processes. Such observations were made on protein level [*P. ostreatus* (Rühl et al. 2008 and section 2.II)] as well as on RNA level [*Agaricus bisporus* (Ohga et al. 1999)]. The highest expression during colonisation is probably due to the degradation of the lignocellulosic substrate (Xing et al. 2006, Rühl et al. 2008 and section 2.II).

A different function of laccase was revealed in the human pathogenic basidiomycete *Cryptococcus neoformans.* Here, laccase seems to play a role in pigmentation. In cultures of the fungus, when glucose concentration was high (>20 g/l), laccase production was inhibited and no pigmentation was observed. This effect showed that laccase production is glucose depending (Jacobson and Emery 1991, Frases et al. 2007).

In nature, laccase activities were found to accumulate also in interaction zones where white-rot fungi are confronted with other micro-organisms (Iakovlev and Stenlid 2000, Baldrian 2004), e.g. bacteria (Baldrian 2004), basidiomycetes (White and Boddy 1992, Gregorio et al. 2006) and ascomycetes (Savoie et al. 1998). Comparable observations have been reported from laboratory experiments (Rühl et al. 2007 and section 2.I). In laboratory experiments with two basidiomycetes that compete for the same biotope in nature, laccase production in liquid culture of *Marasmiellus troyanus* was induced by the culture filtrate of *Marasmius pallescens* (Gregrio et al. 2006). The authors hypothesised that phenolic compounds, which are present in the culture filtrate of *M. pallescencs*, induced the laccase production. As phenolics are produced in nature to defeat foreign organisms, the reason for higher laccase titres in interacting fungal colonies was explained by Gregorio et al. (2006) as a defensive mechanism. Also other authors stated that phenolic compounds, such as ferulic acid, gallic acid, guaiacol, p-coumaric acid, syringic acid, vanillin and more, which are able to induce laccase production and secretion in fungi, may be converted and, when dangerous, thereby detoxificated by laccases (Thurston 1994, De Souza et al. 2004). A proof that

laccase is involved in detoxification of phenolic compounds was also shown for the plant pathogenic fungus *Botrytis cinerea* (Schouten et al. 2008). Although, laccase from *B. cinerea* cultures could not detoxify the phenolic antibiotic (2,4-diacetylphloroglucinol) produced by an antagonistic microorganism alone, addition of tannic acid resulted in a complete degradation of the antibiotic. Contradictory to the defence mechanisms laccases are involved in, the enzyme also plays a role in infection mechanisms. In a human pathogenic fungus, the yeast *C. neoformans*, laccase was found to act as a virulence factor by generating immunomodulatory products during infection of the host (Zhang et al. 2006).

B. Scientific and commercial applications

The wide substrate range of laccases makes these proteins all-round enzymes qualified for several uses in industries and ecology. Of the potential applications, only a few are commercialised for the use in industry down to the present day. But several reviews about potential usage of laccases in industrial applications were published (Minussi et al. 2002, Couto and Herrera 2006, Riva 2006). In addition, laccases are discussed in more general reviews on industrial enzymes (Kirk et al. 2002, Schäfer et al. 2007). An overview on different potential applications of laccases is given on the following pages with respect to application in connection to their natural occurrence (lignin modification, bioremediation) and possible transfer of the reaction mechanism on artificial substrates (dye decolourisation). Strategies of immobilisation of the enzymes are described for uses as biosensors and other possible applications.

1. Lignin modification: degradation and polymerisation

The reactivity of laccases on lignin can be used in three different ways: a) the treatment of pulp to degrade the brown lignin, b) the modification of wood fibre to produce fibreboards and c) the improvement of the properties of wood (Couto and Herrera 2006).

a) Laccases and laccase mediator systems (LMS) in pulp processing

The usage of laccases in the pulp and paper industry is one of the most studied applications. For paper production, the lignin in the kraft pulp (pulp from wood defibration) has to be separated or degraded from the cellulosic fibres so that the brownish colour diminishes (Moreira et al. 1998, Pandey et al. 2000, Hakala et al. 2005, Ibarra et al. 2006). Chandra et al. (2007) tested several laccases from *Trametes* and *Aspergillus* species for their ability to

bleach the Douglas-fir heartwood thermomechanical pulp (TMP). No differences in action of the tested laccases were observed. In both cases, additional supplementation of pure oxygen did not increase the brightness of the TMP. In several other studies of industrial and laboratory scale, different laccases were tested in the treatment of paper pulps (Sigoillot et al. 2005). Bajpai (1999), who reviewed enzyme usage in the pulp and paper industry, concluded that laccase alone as a bleaching agent did not give good results, as a higher polymerisation rate is achieved in vitro in contrast to the required level of depolymerisation and degradation. A LMS can support the process as it contributes to the reactions in nature, where helper molecules (mediators) are secreted by the fungus or arise from degradation processes (Bajpai 1999). Laccase alone though is less adequate than a LMS, since the protein molecule is too large to penetrate into the fibre of wood, unlike the activated mediator (Goodell et al. 1998).

The first LMS used in the pulp industry developed by Lignozym GmbH (now Wacker Chemie AG, Munich, Germany) is a commercialised and patented application (Call 1986, Call 1990). By applying a *Coriolus versicolor* (current used name is *Trametes versicolor*) laccase together with the mediator 1-hydroxybenzotriazole (HBT), Call and Mücke (1997) were able to decrease the kappa number (ISO 302:2004: The kappa number (κ) is an indication of the lignin content or bleachability of pulp and used as an indication for the brightness of the pulp) down to 50-70%. A decrease in the kappa number was also achieved with laccase from *Trametes villosa* and VA as a mediator (Barreca et al. 2003). *Pycnoporus cinnabarinus* laccase together with HBT as mediator decreased also the kappa number and increased the brightness of flax pulp (Sigoillot et al. 2005). A further improvement of the LMS can be achieved when using surfactants such as Tween. In a study where *Ceriporiopsis subvermispora* and *Trametes hirsuta* laccases were used in combination with the mediators HBT and ABTS as LMS, a hard to oxidise lignin model compound was only degraded when Tween20 or Tween80 were added to the reaction mixture (Elegir et al. 2005). This shows that research on the combination of specific enzymes and mediators can contribute to improve LMSs.

Besides the optimisation of the LMS itself by testing different enzyme mediator combinations, the right point of treatment during the bleaching process can be important as well. This was shown by Camarero et al. (2004), who used *P. cinnabarinus* laccase together with HBT as a mediator in an industrial bleaching process of eucalyptus kraft pulp (Ibarra et al. 2006). Although the HBT radical seems to inactivate the *P. cinnabarinus* laccase by reacting with its aromatic aminoacids tyrosine and tryptophan, another study on *T. versicolor* laccases in

degradation of dyes showed that LMS with HBT gave better results than most other mediators (Moldes and Sanromán 2006).
A special effect of the LMS-treatment was reported by Lund and co-workers (2001, 2003): Paper made from kraft pulp treated with *T. villosa* laccase from Novozyme and different mediators, such as ABTS and HBT, had an increased wet strength. This increase in strength of the paper sheets was explained by covalent bonds that emerged in the lignocellulosic matrix from radicals generated by the LMS (Lund and Felby 2001). Also Chandra et al. (2004) using *T. villosa* laccase from Novozyme reported an increased strength of paper derived from kraft pulp when incubation with laccase with and without gallic acid was performed. Coupling of gallic acid to the pulp fibres as well as phenoxy radical coupling between fibres within the paper sheet was stated to be the reason for the increased strength, compared to the control where no laccase and gallic acid was used (Chandra et al. 2004).

For further information on the usage of LMS in the modification of lignin, the reader might refer to the following reviews and the articles stated therein: Call and Mücke (1997), Bajpai (1999) and Shleev et al. (2006b).

b) Laccase in the wood composite industry

For the manufacturing of particle boards or medium-density fibreboards (MDF) laccase were applied in order to utilise the self-bonding properties of the fibre surface – the lignin (Kharazipour et al. 1997, Felby et al. 2002, Mai et al. 2004, Fackler et al. 2008). The self-bonding process of wood composites with the help of phenoloxidases or other oxidising enzymes can be divided into an one-component and a two-component system (Mai et al. 2004). The first one uses the auto-adhesive properties of the wood (lignin) for bonding the fibres, whereas in the latter one the lignin is being activated by ligninolytic enzymes and then used as a binder in the production of wood composites (reviewed by Mai et al. 2004 and Kües et al. 2007a). An example for MDF produced after the one-component system, where culture broth of *P. ostreatus* wheat straw cultures was used, can be seen in Fig. 2.
For both systems, higher temperatures for pressing as well as increased pressing times (in laccase-bonded MDFs compared to UF-resin-bonded boards) are needed. This is necessary to reach the glass transition point of lignin inside the boards, as lignin seems to be the main compound in mediating the bonding between fibres (Felby et al. 2002). The laccases depolymerise the phenolic lignin structure, forming radicals on the fibre surface. These radicals can react which each other, building oligo- or polymers (Kharazipour et al. 1993,