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

- submerged and solid state fermentation,

morphological studies of liquid cultures

and characterisation of new laccases.

Dissertation

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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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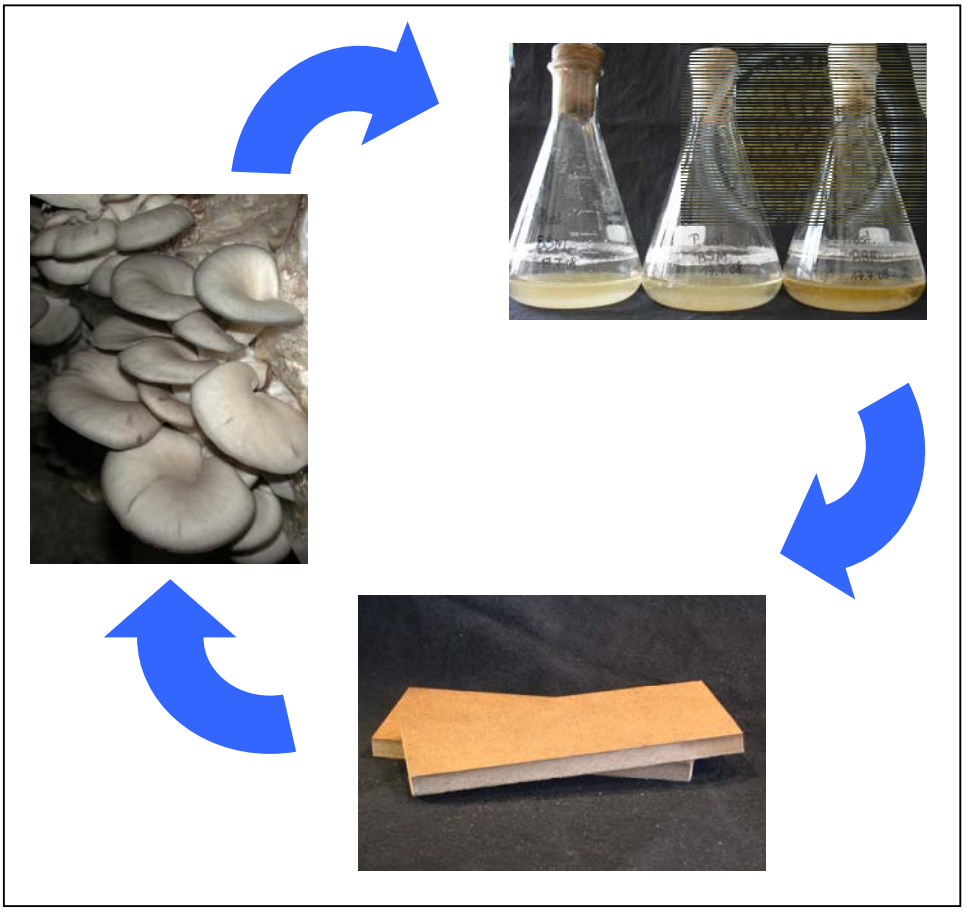
http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf

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).

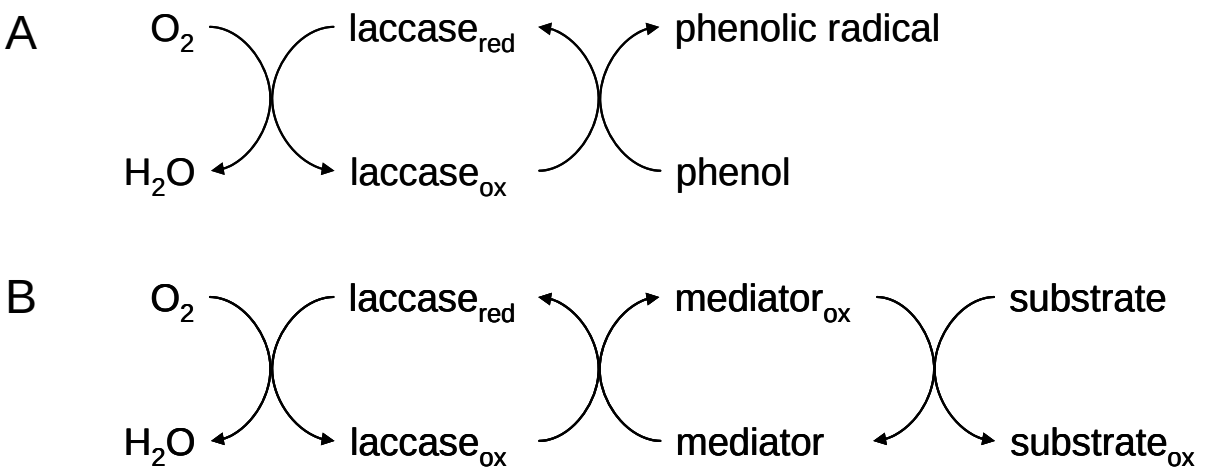


Fig. 1 Schematic reaction cascade of a laccase with a phenol (**A**) and of a laccase mediator system (**B**) after Bourbonnais et al. (1998).

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,

1994, 1997). It still seems to be unclear whether covalent bonds between the fibres or the polymerised lignin are responsible for the adhesive properties (Felby et al. 2002). Here as well, LMS systems seem to be a better choice, as indicated by results of Euring (2008) who used the Novozyme 51003 laccase product together with the mediator HBA for production of 8 mm thick MDFs.

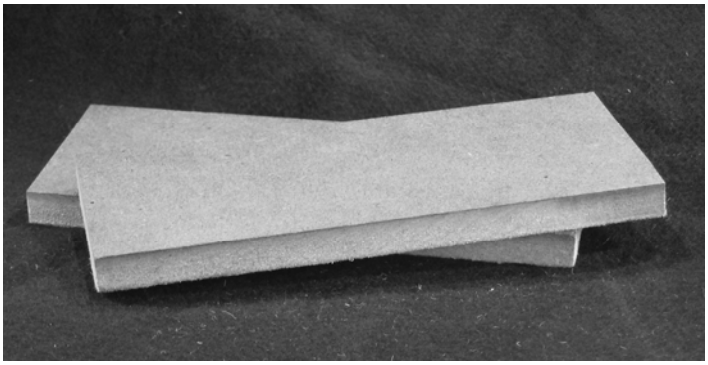


Fig. 2 MDF produced with laccase from *Pleurotus ostreatus* wheat straw cultures (section 2.II) at the pilot plant for MDFs at the Büsgen Institute, Section Molecular Wood Biotechnology and Technical Mycology

c) Alteration of wood properties with laccases

Activation of wood fibres with the help of laccase reflects a possible usage towards new materials for the paper or wood composite industry (Grönqvist et al. 2003). Grönqvist et al. (2006) used *T. hirsuta* laccase to bind tyramine onto lignin of TMP fibres: the laccase oxidises the hydroxyl groups of the phenolic compounds (lignin and tyramine) and the emerged radicals can polymerise. Another possibility to improve the properties of the wood fibres is their modification by attaching phenolic structures (like guaiacol sulphate) with oxidative coupling onto the lignin through action of the enzymes (Lund and Ragauskas 2001, Couto and Herrera 2006). For other potential applications in the forest products industry see Widsten and Kandelbauer (2008).

2. Bioremediation

Harmful compounds, such as endocrinic chemicals, munition waste, organochlorines, pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic polymers, wood preservatives, synthetic dyes and other xenobiotic compounds are contaminating soil and water (Pointing 2001, Novotny et al. 2004). Removal of these toxic substances can be performed in different ways. Besides chemical or physical detoxification or degradation, biological alternatives named bioremediation are developed (Ryan et al. 2005). In these bioremediation processes whole organisms (in vivo, see following pages) or their

metabolites (in vitro), such as oxidative enzymes, can be used. In vitro bioremediation with fungal enzymes can take place *in situ*, when the decontamination is performed at the place of its occurrence (contaminated soil, process waste water). When the contaminated material can be removed and transported somewhere else (reactor, basin, etc.) the remediation can be performed *ex situ* as well. The advantages of enzymatic bioremediation compared to in vivo bioremediation is the selective degradation of pollutants, the utilisation in environments where organisms cannot grow (salinity, pH, sludge, harmful xenobiotics) and an easier process control (Ikehata et al. 2004). Laccase and the other ligninolytic enzymes of white-rot fungi (LiP and MnP) have a very unspecific substrate range. They are thus capable of degrading recalcitrant compounds, showing homology to lignin and other phenolic and aromatic structures (Pointing 2001).

PAHs are a large group of organic pollutants with an aromatic ring structure. Degradation of these compounds by laccase alone is less efficient than a LMS, as it was shown for several PAHs oxidised by *T. versicolor* laccase and ABTS or HBT as mediators (Majcherczyk et al. 1998, Johannes et al. 1998) and *Rigidoporus lignosus* laccase with HBT, ABTS and VA (Cambria et al. 2008). For other toxic compounds from the pharmaceutical and arms industry, like the bacteriozide sulphonamides (Bialk et al. 2007) and 2,4,6-trinitrotoluene (TNT) (Durán and Esposito 2000, Nyanhongo et al. 2006), enzymatic coupling to humic substances in the soil reduces toxicity and increases retention time in the soil. Degradation of the endocrine substance bisphenol A (BPA) was achieved with laccases from *Trametes* species applied either in liquid or immobilised form to the BPA solution (Modaressi et al. 2005, Diano et al. 2007). The LMS-type is not only important for a successful degradation, but also the bioavailability of the toxic compounds towards the LMS and the stability of the enzymatic system. Additional detergents or solubiliser/solvents may help in the availability. This was shown for the herbicide dymron, which could be degraded by a LMS in which *T. versicolor* laccase was applied with either ABTS or different natural mediators (Maruyama et al. 2006). Another detergent, polyethylene glycol (PEG), was shown to protect the laccase during removal of BPA (Modaressi 2005). A kinetic description of a specific LMS towards a substrate was done by Potthast et al. (2001), who tested ABTS and HOBT (1-hydroxybenzotriazole) with *T. versicolor* laccase for oxidation of benzyl alcohols.

Besides the degradation of toxic compounds found in soil, much research was done on the treatment of waste waters from different industries, e.g. the paper and pulp and the textile industry, as well as on olive mill effluents (Call 1991, Bajpai 1999). While the aim in treating wastes from the pulp and paper industry and in olive mill effluents is to remove remaining

phenolic compounds (Attanasio et al. 2005), in the textile industry importance lies on the decolourisation of dyes and pigments, deriving from the staining process and found in the effluents. Worldwide around 10000 different dyes and pigments are produced and used in the corresponding industries (Rodriguez et al. 1999), of which 10% are lost in industrial effluents (Young and Yu 1997). Decolourisation of dyes with crude extract of fungal cultures depends mainly on the laccase activity, as it was shown for 20 different basidiomycetes tested for their decolourisation efficiency (Nozaki et al. 2008). Via a zymogram (= native polyacrylamide gel electrophoresis with a specific substrate that indicates enzymatic activity), the authors were able to show that laccase isoforms, which were detected in the zymogram with ABTS, correspond to bands of decolourised dyes. Up to now, laccases of several white-rot fungi were tested for their ability to decolourise dyes in lab-scale (Claus et al. 2002, Hou et al. 2004, Murugesan et al. 2006, Ben Younes et al. 2007, Lu et al. 2007). Nevertheless, in the study of Liu et al. (2004) azo, anthraquinone and tryphenylmethane dyes could not be decolourised by laccase from white-rot fungi alone. Only the crude supernatants, where also peroxidase activity was found, were able to perform a complete decolourisation (Liu et al. 2004). Contradictory, Claus et al. (2002) tested three different laccases (*T. versicolor*, *T. villosa* and *M. thermophila*) on the decolourisation of azo, indigo and anthraquinone dyes and showed that decolourisation is possible. The tested ascomycete laccase of *M. thermophila* showed lower efficiencies compared to the basidiomycete ones, which is possibly due to the higher redox potential of the *Trametes* laccases. However, both studies have in common that with addition of mediators a higher decolourisation rate was achieved. Therefore, studies to find new mediators for dye decolourisation are of special interest such as the work of Camarero et al. (2005) who tested several natural phenolic mediators such as acetosyringone, *p*-coumaric acid and syringaldazine (SGZ) with *P. cinnabarinus* laccase as LMS for treatment of dye effluents. In an other study, an indigo chromophore was successfully oxidised by a recombinant laccase of *M. thermophila* together with the methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid) (Galante and Formantici 2003, Riva 2006). Moldes and Sanromán (2006) used the two isoenzymes LacI and LacII from *T. versicolor* in combination with four different mediators for dye decolourisation. In this study, the aromatic mediator promazine gave the best results with LacII, whereas with HBT LacI showed higher efficiency than with promazine.

In addition to the lab scale experiments, approaches on pilot scale were performed for decolourisation of dyes. Stirred tank reactors were applied to control the decolourisation reaction at a specific temperature and pH (Soares et al. 2006). The usage of ultrasound treatment, which leads to cavitations in aqueous solution resulting in local areas with high

pressure and temperature, produces hydroxyl radicals. The hydroxyl radicals can degrade azo dyes which may form into other harmful aromatic compounds (Tauber et al. 2005). To have degradation without toxic end products, Tauber et al. (2005) combined a laccase from *Trametes modesta* with the ultrasound treatment and, thereby, obtained better results in degradation of azo dyes. Promising results with laccase immobilised on aluminium oxide indicate that an immobilisation of laccases can give better decolourisation rates than using free enzymes (Zille et al. 2003, for further details see below).

Notwithstanding certain drawbacks on the pilot scale, such as high enzyme costs and wide diversity of the dyes and other harmful compounds, remediation with laccases is an attractive and environmental friendly process, and might find its way towards commercial usage in the nearer future, when costs of this enzyme decline (own opinion and Soares et al. 2006).

3. Immobilisation of laccase for different applications

Different methods for immobilisation of enzymes exists, where enzymes can be entrapped into a polymeric matrix, covalently bonded or adsorbed onto a carrier and enclosed by a membrane. For the immobilisation of laccases, different carriers such as polystyrene (Pich et al. 2006), alignate (Brandi et al. 2006, Teerapatsakul et al. 2008), agar, glass beads, nylon membranes and many more were tested (see review by Durán et al. 2002). Immobilisation of enzymes is an important method since the enzymes can be recycled easily and reused compared to enzymes dissolved into the liquid (Kandelbauer et al. 2004). Another benefit of immobilising enzymes is a possibly higher resistance to pH changes and increased temperature stability, as reported for laccase from *T. versicolor* immobilised on polysterene (Pich et al. 2006) and on a nylon membrane (Attanasio et al. 2005). These changes in turn result in higher reaction yields, better supply towards the substrate and a broader application area.

Bioactive composites derived from cellulosic microfibres coated with laccase and urease can store the enzymatic activity and may be used for bioremediation processes, e.g. in the paper and pulp industry (Xing et al. 2007). Kandelbauer et al. (2004) immobilised *T. modesta* laccase onto γ-aluminum oxide pellets used in an "enzyme-bioreactor" to monitor the decolourisation of different industrial textile dyes inside the reaction mixture. The decolourisation rates of the tested textile dyes were alike for the immobilised and freely dispersed enzymes (Kandelbauer et al. 2004). On the other side, a reduced reaction rate was observed by Matsune et al. (2006). In their study, they observed a deterioration of the

enzymatic properties after immobilising laccase onto a ferric nanobead which was covered with silica gel (Ø 30 nm) to entrap the enzymes. Studies on the enzyme kinetics of the immobilised laccase showed a similar Michaelis-Menten constant (K_M) but a reduced maximum reaction rate (V_{max}) compared to free laccase, which is probably due to a reduced diffusion of the substrate through the polymer matrix or a deactivation of the enzyme during synthesis of the immobilisation (Matsune et al. 2006).

The implementation of biosensors is one important usage of immobilised laccase. Generally, enzymes, antibodies, organelles or whole cells in the biosensors react with chemical compounds, generating an electrical, thermical or optical signal (definition after IUPAC: International Union of Pure and Applied Chemistry). The signal is then transduced via an appropriate way, which can be an amperometric, potentiometric, optical or another measurement, depending on the type of biochemical reaction (Pemmasani 2006). Biosensors based on the reaction of laccases with polyphenols could help in easily detecting phenolic compounds in e.g. wine and olive mill waste (Gomes and Rebelo 2003, Mena et al. 2005) or low molecular phenols such as 2-amino phenol, catechol and pyrogallol, which were detected by *T. versicolor* laccase immobilised on β-cyclodextrin (low-molecular carbohydrate) (Roy et al. 2005). An amperometric detection via a graphite electrode with immobilised *T. hirsuta* laccase was positively tested on detection of lignin and can be used in the future in the paper and pulp industry and for waste waters, e.g. to detect kraft lignin and sulphate pine lignin (Shleev et al. 2006a). Gomes and Rebelo (2003) applied a laccase from *T. versicolor* (Fluka) onto a polyethersulphone membrane and could detect catechin and caffeic acid, although the sensor did not detect gallic acid, malvidin, quercetin, rutin and trans-resveratol.

Besides, several other studies reported positive detection of lignin and phenolic compounds with immobilised laccases in effluents of the paper and pulp industry or other liquid environments (Freire et al. 2002, Roy et al. 2005, Shleev et al. 2006a). Furthermore, immobilised laccases may be used in biofuel cells, where laccase acts as a catalyst at the cathode, reducing dioxygen to water (Farneth et al. 2005, Kamitaka et al. 2007).

4. Commercial laccase products

Enzymatic treatment of textiles has a positive environmental impact, as less water, energy and chemicals are needed (Schäfer et al. 2007). Novozyme (Novo Nordisk, Denmark, www.novozyme.com) established in 1996 a product called DeniLite® used for bleaching of denim textiles. In 2001, an Indian company (Zytex Pvt Ltd., Mumbai, India, www.zytex.net)

marketed a similar product called Zylite. The process of denim deinking or decolourisation is normally performed at a pH of 4-6 at 60-70 °C for 15-30 min; therefore, enzymes having optimal reaction conditions at these process requirements are favourable (Couto and Herrera 2006). Another denim processing system was launched 2007 by Genencor called IndiStar™: a LMS which is of a defined mixture of IndiStar™ Active, the laccase, and IndiStar™ Control, the mediator (www.genencor.com, Table 2).

Table 2 Laccases from different companies used in pure form and combined products in industrial applications

Product name	Source*	Application	Company	Reference
Pure laccase				
Laccase	*Trametes versicolor*	LMS	Mercian Corp. Japan	Potthast et al. (2001)
Laccase	*Trametes* sp.	Biofuel cell	Daiwa Kasei Co. (Japan)	Kamitaka et al. (2007)
	T. versicolor	Biofuel cell	Konsortium für Elektrochemische Industrie GmbH, Wacker Chemie	Farneth et al. (2005)
Laccase	Mushroom enzyme	General product	Mycoenzyme	mycoenzyme.co.il
Laccase	*Coriolus versicolor*	Used for a biosensor	FLUKA	Gomes and Rebelo (2003)
Laccase	*Pleurotus ostreatus*	Used for detoxification	Tienzyme	Keum and Li (2004)
Laccase	*Agaricus bisporus* *Rhus vernicifera* *T. versicolor*	General	Sigma-Aldrich	www.sigma-aldrich.com
Laccase	*T. versicolor*	Decolourisation of dyes and decomposition of phenolic and aromatic compounds	Jena Bioscience	www.jenabioscience.com
Laccase	*T. versicolor*	Decolourisation	Novozyme	Claus et al. (2002)
Laccase / SP504 (one isoenzyme)	*Trametes villosa*	Decolourisation / scientific wastewater treatment	Novozyme	Modaressi et al. (2005), Claus et al. (2002)
Laccase /SP850 (one isoenzyme)	*Myceliophthora thermophila*	Decolourisation / NS	Novozyme	Hager (2003) Claus et al. (2002)
Laccase	*Agaricus bisporus* *Trametes spec.*	Precipitation of phenolic compounds, enzymatic browning of foods	ASA Spezialenzyme GmbH	www.asa-enzyme.de
Laccase	*Agaricus bisporus* *Coriolus versicolor*	Organic synthesis, conversion of phenolic compounds	Jülich Fine Chemicals, now Codexis	Verkade et al. (2007) www.codexis.com
Combined product				
DeniLite®	ns	Textile industry (denim finishing)	Novozymes	Xu (2005) www.novozymes.com
Ecostone®	ns	Textile industry (denim bleaching)	AB enzymes	www.abenzymes.com
IndiStar™	ns	Textile industry (denim bleaching)	Genencor, Danisco US Inc.	www.geneencor.com
LaSOX	ns	Pulp and Paper industry (delignification and bleaching	Bioscreen e.K., Übach-Palenberg, Germany	www.bioscreenek.de
Lignozym®	ns	Pulp and Paper industry (delignification and bleaching	Lignozym GmbH, now Wacker Chemie	Call (1986, 1990), Call and Mücke (1997)

Product name	Source*	Application	Company	Reference
Novozym 51003	ns	Textile industry and lignin modification	Novozymes	Xu (2005) www.novozymes.com
NS51002	*Trametes hirsuta*	Used for Deinking	Novozyme	Hager (2003)
NS51003	*Myceliophthora thermophila*	Used for Deinking	Novozyme	
Suberase®	ns	Food industry (cork)	Novozymes	Xu (2005)
Zylite	ns	Textile industry	Zytex	Couto and Herrera (2006)

*ns: not stated.

A biodelignifcation and biobleaching process of paper pulp with a LMS was first introduced in the 1980ies (Call 1986) by the company Lignozym GmbH and seems now to be continued by Bioscreen e.K. (Table 2).
Another commercial product, Suberase®, is used in the polymersiation of cork phenols to prevent the production of the methoxybenzene 2,4,6-trichloranisole (TCA) in the cork stopper, which would cause a mouldy taste (Conrad and Sponholz 2000).

Besides the application discussed above in more detail, several other potential usages of laccases can be found in different patents. Laccase can also be used to lower the shrinkage behaviour of wool (Yoon 1998, Lantto et al. 2004). Lantto et al. (2004) demonstrated reactivity of *M. thermophila* laccase on wool with HBT and VA as mediator, but the oxidation level of the wool fibres was too low for a significantly altered surface chemistry of the fibres. In the cosmetic industry, laccases can be used within hair dyes instead of H_2O_2 as an oxidant, leading to better handling [reviewed in Couto and Herrera (2006)]. Also for the cosmetic industry, several patents are available, such as the ones submitted by the company L'Oreal (Martin et al. 1994, Lang and Cotteret 2003).

5. Other potential applications

Laccases may also be used in the food industry, e.g. for the clarification of juice or the oxidation of phenols. Other uses of laccases in this sector are given in the reviews of Kirk et al. (2002) and Minussi et al. (2002).

In the bioethanol production, laccases together with peroxidase enzymes are used for detoxification of the lignocellulosic substrates. The process leads to the degrading or oxidative polymerisation of the phenolic monomers, which are toxic to the further used organisms such as *S. cerevisiae* (Palmqvist and Hahn-Hägerdal 2000). As a result, an increase in the ethanol producitivity of 2-3fold was observed (Jonsson et al. 1998, Palmqvist

and Hahn-Hägerdal 2000). A combination of laccases with other cellulolytic enzymes, such as cellulases and xylanases, was used for delignification and saccharification of wheat straw and softwood, which might be used for further processing to bioethanol (Palonen 2004, Tabka et al. 2006).

Polymersiation of different chemical substances by laccases is another field of interest (Mattinen et al. 2005): e.g. laccase of *Trametes pubescens* was used for polymerisation of 8-hydroxyquinoline, whose polymer act as an antioxidant intercepting the radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (Ncanana and Burton 2007).

II. Basidiomycetes: higher fungi with a great potential

The fungal kingdom encompasses a very large group of species. Depending on the basis of estimation (Brock et al. 2009) it is assumed that there are between 1.5 million (Hawksworth 2001) and 3.5 million (O'Brien et al. 2005) fungal species worldwide, of which about 100000 fungal species are known today (Hawksworth 2001).

The basidioymcetes together with the ascomycetes are also referred to as higher fungi. About 30000 basidiomycete species are known, which distribute over different ecosystems, such as forest soils, wood, fresh wasters and even in marine niches. Especially the organic matter degrading basidiomycetes are important for our ecosystem making the most essential contribution to the carbon cycle (Kirk et al. 2001).

A. Applications of living basidiomycetes on lignocellulosic material

To get to the cellulose and hemicellulose in plant materials for the production of easy metabolisable carbon sources (sugars), the lignin has to be removed to a certain extent. Several studies document these processes (Jonsson et al. 1998, Palonen 2004, Schirp et al. 2006). For example, selective lignin degrading white-rot fungi were used to degrade the lignin in rice straw by incubating the fungus on this lignocellulosic waste material. For *P. ostreatus*, Taniguchi et al. (2005) showed that the total net sugar yield (calculated from hydrogen (H) and carbon (C) content) compared to untreated rice straw was increased almost 3 times, thus making the usage of the pre-treated straw interesting where lignocellulosic material with a low lignin content is needed, as in the bioethanol industry. Wastewater of the olive oil industry was treated with *Pleurotus sajor-caju* to remove phenolic compounds. Subsequent fermentation with yeast resulted in an enhancement of the ethanol production yield (Massadeh and Modallal 2008). Spent wood logs from cultivation of the mushroom *L. edodes* gave higher net sugar yields than non-inoculated wood and,

subsequently, 50% higher ethanol yields (Lee et al. 2008). Direct usage of basidiomycetes on wood was also applied for the cultivation of fine wood particles with *Panus tigrinus* to produce a natural glue (biobinder) which might be used in the wood composite production (Kondrashchenko et al. 2006).

Another biological application of basidiomycetes is their usage as biocontrol agents. Spores of *Phlebiopsis gigantea* of the Polyporaceae were thus used to hinder degradation of fresh stumps by the basidiomycete *Heterobasidion annosum* (Russulales) (Wolken et al. 2003): due to prior colonisation, *P. gigantea* makes it harder for *H. annosum* spores to colonise the tree stump (Kües et al. 2007b). Also other wood decaying fungi have been proposed as biological control agents: *Bjerkandera adusta*, *Fomitopsis pinicola* and *Hirschioporus abietinus* (Boddy 2000, Kües et al. 2007b). To hinder pathogenic fungi from attacking the roots of the trees, cord-forming saprotrophic fungi, e.g. *Hypholoma fasciculare*, *Phanerochaete velutina* and *Resinicium bicolour*, can be brought into the soil (Boddy 1993, Boddy 2000). This was shown in Australia, where *Armillaria luteobubalina* was controlled by *Hypholoma australe* and *Phanerochaete filamentosa* (Pearce et al. 1995, Boddy 2000).

B. In vivo bioremediation and dye decolourisation

In situ bioremediation of the ligninolytic enzyme laccase has already been discussed on the previous pages. However, not only pure enzymes can be used in the detoxification or decolourisation of soil and waste waters. Complete living organism (as well as crude extracts of their cultures) might be used for bioremediation. A comprehensive review on the bioremediation of toxic compounds by white-rot fungi and their ligninolytic system is given by Pointing (2001), where several substance classes (munitions waste, pesticides, PCBs, PAHs, bleaching effluents, synthetic dyes, synthetic polymers and wood preservatives) are discussed regarding their degradation by white rot fungi. Nevertheless, in the following only in vivo degradation attempts of basidiomycetes are stated, showing that this phylum of fungi is very useful.

One important parameter, when in situ decontamination of soil and water with complete micro-organism cultures should be performed, is the micro-flora of the environment which has to be remediated. As sterilisation would increase the costs and, probably, make the process uneconomical, the fungus has to colonise non-sterile substrates. Direct bioremediation in non-sterile soil was tested with added ligninolytic enzyme producing basidiomycetes, of which the white-rot fungus *P. ostreatus* and the plant pathogen

Rhizoctonia solani were the only ones showing growth in the non-sterile forest and field soil (McErlean et al. 2006). Another study in non-sterile soil demonstrated that litter degraders are able to degrade the added pollutants, whereas *Stropharia* species showed the highest degradation rates for the tested PAHs (Steffen et al. 2007b). Immobilisation of the fungi onto a carrier, either an inert or lignocellulosic based one, might help the fungus to grow in a more aerated and controllable environment. This technique is also named solid state fermentation (SSF). In case nutritive carriers are used it may be also called solid substrate fermentation (Rodriguez E et al. 2004, Compart et al. 2007 and section 2.I). SSF for bioremediation was studied with several white-rot fungi with different carriers, such as alginate, ceramics, corn cob, stainless steel, wheat straw and wood chips, for the degradation of toxic compounds (Rodriguez E et al. 2004, Compart et al. 2007) or decolourisation of dyes and other industrial effluents (Rodriguez CS et al. 2004, Dominguez et al. 2005, Boehmer et al. 2006, Pant and Adholeya 2007). Different SSF techniques for the growth of basidiomycetes with concomitant bioremediation were tested, such as fluidised bed reactor, packed bed reactor, immersion bioreactor and airlift reactor (Ryan et al. 2005, Boehmer et al. 2006, Ortega-Clemente et al. 2007 and section 2.I).

The oxidative enzymatic system of the fungi is the main factor for their ability to degrade toxic compounds. Bioremediation with *Irpex lacteus* (Novotny et al. 2004) in soil and liquid media showed that, in general, the degradation rate correlates with the secreted extracellular enzyme. To increase the efficiency of this in vivo ligninolytic system the addition of mediators helps to increase the degradation or decolourisation rate, as it was shown for *Pleurotus* species degrading phenolic and non-phenolic aromatic pollutants in SmF (submerged fermentation) and SSF cultures with HBT or ABTS as mediator (Rodriguez E et al. 2004). Another possibility besides the usage of particular produced fungal cultures for bioremediation is the application of spent mushroom compost from the mushroom industry for bioremediation of soils and waters (Eggen and Majcherczyk 1998, Lau et al. 2003, Rühl and Kües 2007).

C. Mushrooms as a valuable food

Since the Stone Age, mushrooms served humans as food, medicine and psychoactive drugs, religious symbols, and helpful tools. As a nice documentation, in the bag of the iceman Ötzi, a mummy discovered in 1991 in the Alps, remains of *Fomes fomentarius* (tinder polypore) and *Piptoporus betulinus* (birch bracket) were found. A bit further south, fragments of *Daedaleopsis tricolor* (a polypore bracket fungus) were dug up in huts of a Neolithic village

north of Roma (Peintner et al. 1998, Roussel et al. 2002, Bernicchia et al. 2006). Within native traditional populations all over the world, there is tremendous inherited knowledge on mushrooms and their potential usages (Allen and Merlin 1992, Buyck and Nzigidahera 1995, Blanchette 1997, Mahapatra and Panda 2002, Michelot and Melendez-Howell 2003, Montoya et al. 2003, Chang and Lee 2004, Zent et al. 2004, Pieroni et al. 2005, Garibay-Orijel et al. 2006, Lampman 2007) that, however, is in danger to be lost. Mushroom consumption and medical application of fungi is deep-rooted in Asian countries, also in their modern societies. In many European cultures, in contrast, mushrooms have been broadly neglected for centuries. Often, there is no practice of mushroom hunting by historical elimination of knowledge, fear of mushroom poisoning and other reasons (Benjamin 1995, de Román et al. 2006).

Traditionally, mushrooms are collected from the wild, unless a cultivation method has been established for a species. Mushroom cultivation started in China 600 years AD with artificial inoculation of twigs with *Auricularia auricula-judae* (jew's ear fungus, wood-ear mushroom), a mushroom common to the Asian kitchen. Also *L. edodes* (shiitake) was first cultivated on logs in China, around 1000 to 1100 AD (Chang 1993, Luo 2004). In Europe, mushroom cultivation came up around 300 years ago in Paris during the time of Louis XIV, when gardeners first grew *A. bisporus* (button mushroom) on beds fertilised with dung and, later on, in cellars and catacombs underneath the town (Ainsworth 1976). Until now, the 'Champignon de Paris' is the most cultivated mushroom in Europe and the world (Chang 1999, Kües and Liu 2000; Fig. 3, Table 3). *Pleurotus* ssp. (oyster mushrooms, hiratake) are currently the second most cultivated group of mushrooms (Chang 2005, Tan et al. 2005, Yamanaka 2005; Table 3). *P. ostreatus* was first artificially cultured at the beginning of the 20th century by Richard Falck (1917), who in 1910 became the first forest botany professor of the "Institut für Technische Mykologie" at the Forest Faculty in Hannoversch-Münden (Hüttermann 1987, 1991), the predecessor of the Section Molecular Wood Biotechnology at the Faculty of Forest Sciences and Forest Ecology in Göttingen. Cultivation of all other mushrooms domesticated up to 1900 (edible species: *Flammulina velutipes* - enoki mushroom, enokitake, velvet foot, *Volvariella volvacea* - paddy straw fungus, and *Tremella fuciformis* - ba mu erh, trembling fungus; medicinal mushrooms: *Poria cocos* – fu ling and *Ganoderma* spp. - reishi) happened first in China (Chang 1993).

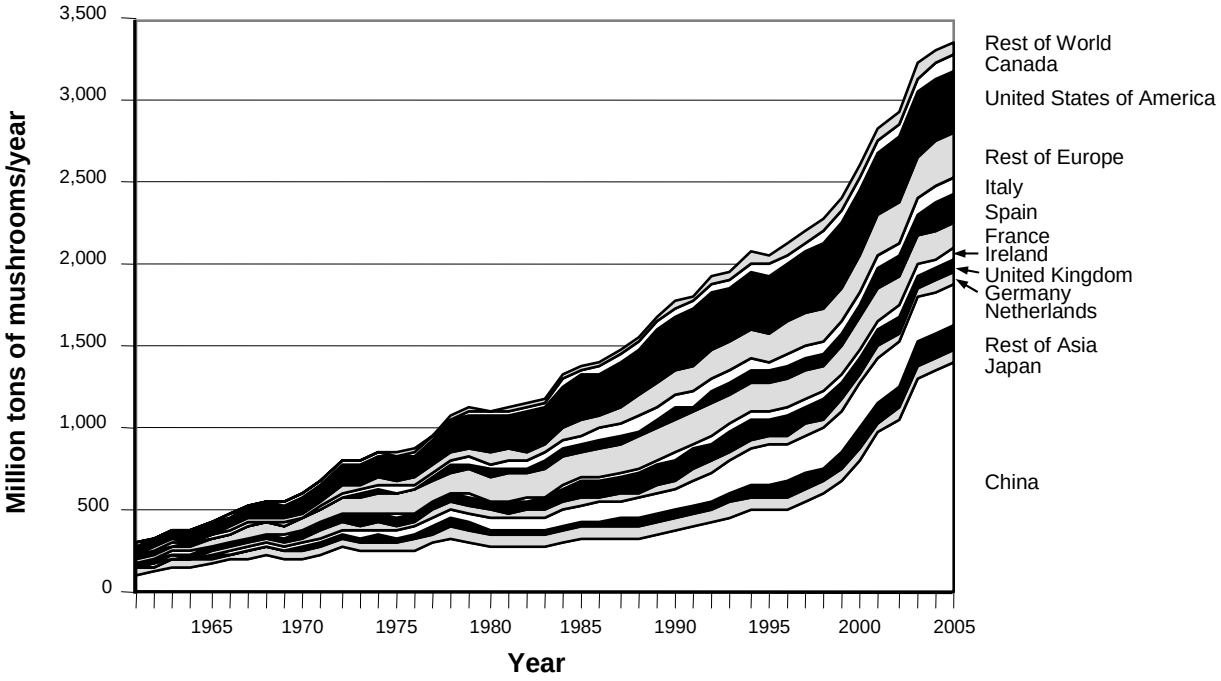


Fig. 3 Worldwide mushroom production (including interalia: *Agaricus* and *Boletus*, both basidiomycetes, and *Morchella* and *Tuber*, both ascomycetes) according to FAOSTATdata (2005). *Boletus*, *Morchella* and *Tuber* are ectomycorrhizal species that are collected from forests and plantations (Yun and Hall 2004). *Agaricus* will therefore present the majority of mushrooms in these values. This assumption is supported by the data presented in Tables 3 and 4.

Since the nineteen-eighties, mushroom production booms with more and more countries being engaged in large scale mushroom cultivation (Fig. 3) and with continually increasing numbers of new species (sometimes referred to as "speciality mushrooms") with well-established cultivation techniques (Chang 1999, Kües and Liu 2000, Chen 2004, Royse et al. 2005, Yamanaka 2005; Tables 3 and 4).

Table 3 Estimated worldwide mushroom production of the most important species in tons/year (Chang 1999, Kües and Liu 2000)

	1981	**1986**	**1990**	**1994**	**1997**
Agaricus bisporus	900.0	1,227.0	1,420.0	1,846.0	1,955.9
Lentinula edodes	180.0	314.0	393.0	826.2	1,564.4
Pleurotus **spp.**	35.0	169.0	900.0	797.4	875.6
Auricularia **spp.**	10.0	119.0	400.0	420.1	485.3
Volvariella volvacea	54.0	178.0	207.0	298.8	180.8
Flammulina velutipes	60.0	100.0	143.0	229.8	284.7

Nowadays, a large variety of mushrooms are cultivated all over the world. China still occupies the leading position in mushroom production and consumption, with more than 950 different known edible species in the wild of which about 50 are commercially cultivated (Chen 2004, Chang 2005, Yamanaka 2005; Table 4).

Commercial mushroom cultivation often bases on recycling of vast amounts of agro-forestry wastes. The variety of wastes reach from straw (wheat, rice, oat, …), reed grass, sawdust of different species (spruce, pine, beech, birch, pine, gum wood, …), banana and bamboo leaves, tree bark and stems, several husk types, some scrubs and others (reviewed by Poppe 2000). Cultivation of speciality mushrooms on lignocellulosic wastes represents one of the most economically and cost effective organic recycling processes (Poppe 2000, Philippoussis et al. 2001, Mandeel et al. 2005).

Table 4 The twelve mostly produced mushrooms in China: production in tons/year (Chang 2005)

	1986	**1998**	**2000**	**2001**	**2002**	**2003**
Pleurotus **spp.**	100	1020	1700	2590	2647	2488
Lentinula edodes	120	1388	2205	2072	2214	2228
Agaricus bisporus	185	426	637	743	923	1330
Auricularia **spp.**	80	491	968	1124	1242	1655
Volvariella volvacea	100	32	112	116	151	197
Flammulina velutipes	10	189	299	389	506	558
Tremella **spp.**	50	100	103	114	138	184
Hericium erinaceus	50	28	6	9,5	13	31
Hypsizygus **spp.**	-	21	84	120	190	243
Pholiota nameko	1	31	48	51	85	172
Grifola frondosa	-	10	6	15	37	25
Coprinus comatus	-	-	-	39	157	178
*Others**	-	664	470	371	464	1,100

*Others include *Pleurotus nebrodensis*, *Pleurotus eryngii*, *Agrocybe chaxinggu*, *Dictyophora* spp., *Agaricus brasiliensis*, *Ganoderma* spp., *Wolfiporia cocos*, *Lepista nuda*, *Agrocybe aegerita*, *Tricholoma gigangteum*, *Auricularia fuscossuccinea*, *Tremella cinnabarina*, *Pleurotus citrinopileatus*, *Pleurotus sapidus*, *Stropharia rugoso-annulata* and *Lentinus giganteus*

Commonly, mushrooms are understood as fruiting bodies of fungi that are edible (mushrooms) or poisonous (toadstools). Chang and Miles (1989) defined a mushroom being a macrofungus with an epigeous (above ground) or hypogeous (below ground) fruiting body, which can be seen by naked eye and picked by hand. In nature, more than 12,000 fungal species can be declared as being mushrooms, of which around 2,000 to 2,500 are estimated to be edible with a more or less savoury taste. Most of the mushrooms belong to the basidiomycetes whilst a few, including truffles and morels, are ascomycetes (Chang 1999, Yun and Hall 2004). All mushrooms being commercially produced on lignocellulosic wastes are basidiomycetes. In contrast, the black truffle, *Tuber melanosporum*, is an ascomycete

that cannot be cultivated in free culture but is fostered as an ectomycorrhizal species in combination with hazelnuts and oak trees. For further development, in vitro mycorrhized trees should be planted into fields with calcareous soils with particular properties. After 4 to 6 years, the first ascocarps (fruiting bodies of the ascomycetes) occur, but harvests start not before 10 years after plantation (Shaw et al. 1996, Olivier 2000, Yun and Hall 2004, Bonet et al. 2006, Mello et al. 2006). Nowadays, more than half of the black truffle come from commercial plantations (Hall et al. 2003). *Tuber formosanum* is a related species endemic to Taiwan that is ectomycorrhizal to the *Fagaceae Cyclobalanopsis glauca* and cultivated in man-made truffieres (Hu et al. 2005). In a few instances, fruiting bodies of ectomycorrhizal basidiomycetes have been obtained in pot-culture with a respective host (Danell and Comacho 1997, Yamada et al. 2001; see below for further information). Because mushrooms have a high content of crude fibre, crude protein (usually 20-30% of dry matter), B vitamins, low fat, and nearly no cholesterol (for example see Braaksma and Schaap 1996, Manzi et al. 1999, Chiu 2000, Mattila et al. 2001, Sanmee et al. 2003, Adejumo and Awosanya 2005, del Toro et al. 2006, de Román et al. 2006, Nwanze et al. 2006), they present a highly valuable food. Besides, mushrooms serve as medicine and for prophylaxis to keep good health. Fruiting bodies as well as fungal mycelia contain several bioactive compounds. For instance, fungal polysaccharides are recognised to have anti-tumor effects by activating various immunoresponses. Most of these bioactive polysaccharides belong to the β-glucans, like Lentinan from *L. edodes* and Schizophyllan from *S. commune* (split gill fungus), both of which are commercially available (Wasser 2002). Chang and Buswell (1996) coined the word "nutraceuticals" to indicate the dual roles of edible mushrooms being natural food and facilitators of maintaining good health. On the other hand, the term "nutriceuticals" is used for mushroom or mycelium extracts that posses both nutritional and medicinal attributes and, being incorporated into a capsule or tablet, are consumed as a dietary supplement for therapeutic purposes.

III. Aim of the thesis

Enzymes that are involved in the degradation of lignocellulosic material, such as cellulases, hemicellulases, peroxidases and laccases, are used in different industrial processes. Such enzymes may be produced with fungi either in submerged (SmF) or solid state fermentation (SSF) systems. An overview on both production systems regarding the most relevant ligninolytic enzymes for the wood industry is given in section 2.I. Production of native and recombinant laccases by selected homobasidiomycetes (*Pleurotus ostreatus*, *Coprinopsis cinerea*) is in the focus of the experimental part of this thesis. Questions of cultivation conditions, fungal morphology and development, isoenzyme patterns and characterisation of laccases are addressed.

Since basidiomycetes are filamentous fungi able to colonise lignocellulosic substrates in SSF due to their special enzymatic systems, they may be used to convert cheap substrates into a valuable product. Thus, one vital part of the study concerns the white-rot fungus *P. ostreatus* used for commercial production of mushrooms on wheat straw. *P. ostreatus* secretes ligninolytic enzymes to colonise the lignocellulosic substrate. The task was to observe the production of ligninolytic enzymes over the time in SSF on commercial wheat straw substrate as used for industrial mushroom production of *P. ostreatus*. Periods of production of ligninolytic enzymes (laccases as well as manganese dependent and versatile peroxidases) were found to alternate with fruiting body formation (section 2.II).

Although SSF has the advantage of using cheap lignocellulosic waste materials for fungal growth and conversion, technical enzymes are mainly produced in industries under submerged cultivation conditions in stirred tank reactors due to the ease to isolate the fungal products from the liquid substrates. Furthermore, liquid fermentation can be performed in closed vessel systems with genetically modified organisms. Thereby, the products of interest may change by applying different recombinant strains of an organism whilst the overall fermentation conditions for the species remain conserved. Thus, there is no need to develop a new general production procedure for a new product with a new natural host.
Currently besides bacterial hosts, yeasts and filamentous ascomycetes are mainly used as hosts for the production of recombinant proteins. However, these organisms have disadvantages (lack of proper post-translational modifications) in the production of enzymes coming from basidiomycete origin. As a consequence, newer studies employ also basidiomycetes in recombinant enzyme production (e.g. Kilaru et al. 2006). Unfortunately, there are still shortcomings in the knowledge about the behaviour in growth and morphology

of basidiomycetes in shaken and stirred cultures, although several studies on cultivation of basidiomycetes in liquid cultures can be found (section 3.I). To obtain a better insight into the morphology of higher filamentous fungi in liquid cultures, an literature overview on image analysis on determination of fungal morphology during different types of liquid cultivation is given (section 3.II) and then an image analysis system was developed (section 3.III). As a model, a recombinant laccase producing *Coprinopsis cinerea* strain was used to study its morphology and laccase production in shaken and stirred cultures at different temperatures and pH values, respectively (section 3.IV).

Previous work by other authors reported that *C. cinerea* has 17 laccase genes that in liquid culture of at least the strains FA2222 and AmutBmut are not or only poorly expressed (Kilaru 2006, Kilaru et al. 2006, Navarro-González 2008). Several other monokaryotic strains of *C. cinerea* are available in the scientific community that were analysed in this study for native production of laccases under different cultivation conditions (section 4.I). Nevertheless the fact that most strains secreted laccases into the medium, yields of native laccase production was in all cases too low for any economical industrial production. Moreover, strains differed in the type(s) of laccases produced. For industrial purposes, a cost-efficient production process of specific laccases is needed. Another task of this thesis was therefore to improve the yields of recombinant laccase production in *C. cinerea* for a possible profitable use in the industrial sector (section 4.II).

Individual laccases differ in their properties, such as in their optimal temperature of reaction, in their pH range of activity, in substrate affinity and in stability (Baldrian 2006, Kilaru 2006). *C. cinerea* laccases recombinantly produced by the strain FA2222 may have special features. Therefore, one goal was to adapt a purification protocol for production of the recombinant produced laccases in *C. cinerea* in complex cultivation medium. The main aim was the characterisation of the recombinant laccase concerning molecular, biochemical, kinetic and structural properties (chapter 5).

For an easy overview, the following list of literature and experimental tasks summarises the main aims of this thesis:

1. Review on the production of ligninolytic enzymes (section 2.I)
2. Observation of ligninolytic enzyme production in SSF of *P. ostreatus* (section 2.II)
3. Literature survey on fermentation of basidiomycetes in liquid cultures (section 3.I)
4. Literature overview on tools for observation and analysis of fungal morphology in liquid cultures (section 3.II)

5. Development of an image analysis system for morphological studies of filamentous fungi in SmF (section 3.III)
6. Determination of *C. cinerea* morphology in shake flask and stirred bioreactor cultures (section 3.IV)
7. Study of native laccase production in *C. cinerea* strains under different cultivation conditions (section 4.I)
8. Optimisation of recombinant laccase production in *C. cinerea* (section 4.II)
9. Characterisation of the recombinant produced *C. cinerea* laccases (chapter 5)

A final conclusion will discuss all results presented in the different chapters together with an emphasis on further studies as how to possibly continue the work of this thesis for improved laccase production with the host *C. cinerea* (chapter 6).

IV. References

Adejumo TO and Awosanya OB (2005) Proximate and mineral composition of four edible mushroom species from South Western Nigeria. African Journal of Biotechnology 4:1084-1088

Ainsworth GC (1976) Introduction to the history of mycology. Cambridge University Press, Cambridge, UK

Allen W and Merlin MD (1992) Psychoactive mushroom use in Koh Samui and Koh Pha-Ngan, Thailand. Journal of Ethnopharmacology 35:205-228

Anderson AJ, Kwon SI, Carnicero A and Falcon MA (2005) Two isolates of *Fusarium proliferatum* from different habitats and global locations have similar abilities to degrade lignin. FEMS Microbiology Letters 249:149-155

Andreasson LE, Branden R and Reinhammar B (1976) Kinetic studies of *Rhus vernicifera* laccase evidence for multi-electron transfer and an oxygen intermediate in reoxidation reaction. Biochimica et Biophysica Acta 438:370-379

Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR and Kramer KJ (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proceedings of the National Academy of Sciences of the United States of America 102:11337-11342

Aramayo R and Timberlake WE (1990) Sequence and molecular structure of the *Aspergillus nidulans ya* (laccase-I) gene. Nucleic Acids Research 18:3415-3415

Aramayo R and Timberlake WE (1993) The *Aspergillus nidulans ya* gene is regulated by abaa. EMBO Journal 12:2039-2048

Arora DS and Gill PK (2005) Production of ligninolytic enzymes by *Phlebia floridensis*. World Journal of Microbiology and Biotechnology 21:1021-1028

Attanasio A, Diano N, Grano V, Sicuranza S, Rossi S, Bencivenga UB, Fraconte L, Di Martino S, Canciglia P and Mita DG (2005) Nonisothermal bioreactors in the treatment of vegetation waters from olive oil: laccase versus syringic acid as bioremediation model. Biotechnology Progress 21:806-815

Bajpai P (1999) Application of enzymes in the pulp and paper industry. Biotechnology Progress 15:147-157

Baldrian P (2004) Increase of laccase activity during interspecific interactions of white-rot fungi. FEMS Microbiology Ecology 50:245-253

Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiology Reviews 30:215-242

Baldrian P and Snajdr J (2006) Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes. Enzyme and Microbial Technology 39:1023-1029

Barreca AM, Fabbrini M, Galli C, Gentili P and Ljunggren S (2003) Laccase/mediated oxidation of a lignin model for improved delignification procedures. Journal of Molecular Catalysis B-Enzymatic 26:105-110

Benjamin DR (1995) Mushrooms: poisons and panaceas. W.H. Freeman and Company, New York, NY

Ben Younes S, Mechichi T and Sayadi S (2007) Purification and characterization of the laccase secreted by the white rot fungus *Perenniporia tephropora* and its role in the decolourization of synthetic dyes. Journal of Applied Microbiology 102:1033-1042

Berka RM, Schneider P, Golightly EJ, Brown SH, Madden M, Brown KM, Halkier T, Mondorf K and Xu F (1997) Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Applied and Environmental Microbiology 63:3151-3157

Bernicchia A, Fugazzola MA, Gemelli V, Mantovani B, Lucchetti A, Cesari M and Speroni E (2006) DNA recovered and sequenced from an almost 7000 y-old Neolithic polypore, *Daedaleopsis tricolour*. Mycological Research 110:14-17

Bialk HM, Hedman C, Castillo A and Pedersen JA (2007) Laccase-mediated michael addition of N-15-sulfapyridine to a model humic constituent. Environmental Science & Technology 41:3593-3600

Blanchette RA (1997) *Haploporus odorus*: a sacred fungus in traditional Native American culture of the northern plains. Mycologia 89:233-240

Boddy L (1993) Saprotrophic cord forming fungi - warfare strategies and other ecological aspects. Mycological Research 97:641-655

Boddy L (2000) Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiology Ecology 31:185-194

Böhmer U, Suhardi SH and Bley T (2006) Decolorizing reactive textile dyes with white-rot fungi by temporary immersion cultivation. Engineering in Life Sciences 6:417-420

Bonet JA, Fischer CR and Colinas C (2006) Cultivation of lack truffle to promote reforestation and land-use stability. Agronomy for Sustainable Development 26:69-76

Bonnen AM, Anton LH and Orth AB (1994) Lignin degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. Applied and Environmental Microbiology 60:960-965

Bourbonnais R, Leech D and Paice MG (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochimica et Biophysica Acta-General Subjects 1379:381-390

Braaksma A and Schaap DJ (1996) Protein analysis of the common mushroom *Agaricus bisporus*. Postharvest Biology and Technology 7:119-127

Brandi P, D'Annibale A, Galli C, Gentili P and Pontes ASN (2006) In search for practical advantages from the immobilisation of an enzyme: the case of laccase. Journal of Molecular Catalysis B-Enzymatic 41:61-69

Brock PM, Döring H, and Bidartondo MI (2009) How to know unknown fungi: the role of a herbarium. New Phytologist 181:719-724

Bruno G and Sparapano L (2006) Effects of three esca-associated fungi on *Vitis vinifera* L.: III. Enzymes produced by the pathogens and their role in fungus-to-plant or in fungus-to-fungus interactions. Physiological and Molecular Plant Pathology 69:182-194

Buyck B and Nzigidahera B (1995) Ethnomycological notes from western Burundi. Belgian Journal of Botany 128:131-138

Call HP (1986) Verfahren zur Delignifizierung und Bleichung von lignocellulosehaltigem bzw. ligninhaltigen Material, bzw. Lignin durch enzymatische Behandlung. Patent: DE 3636208

Call HP (1990) Process of enzymatic bleaching of pulp. Patent: DE 4008893

Call HP (1991) Process for producing cellulose from lignin containing raw materials using an enzyme or microorganism while monitoring and maintaining the redox potential. Patent: US 5203964

Call HP and Mücke I (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). Journal of Biotechnology 53:163-202

Camarero S, Garcia O, Vidal T, Colom J, del Rio JC, Gutierrez A, Gras JM, Monje R, Martinez MJ and Martinez AT (2004) Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator systems. Enzyme and Microbial Technology 35:113-120

Camarero S, Ibarra D, Martinez MJ and Martinez AT (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Applied and Environmental Microbiology 71:1775-1784

Cambria MT, Minniti Z, Librando V and Cambria A (2008) Degradation of polycyclic aromatic hydrocarbons by *Rigidoporus lignosus* and its laccase in the presence of redox mediators. Applied Biochemistry and Biotechnology 149:1-8

Cha JS and Cooksey DA (1991) Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. Proceedings of the National Academy of Sciences of the United States of America 88:8915-8919

Chandra RP, Lehtonen LK and Ragauskas AJ (2004) Modification of high lignin content kraft pulps with laccase to improve paper strength properties. 1. Laccase treatment in the presence of gallic acid. Biotechnology Progress 20:255-261

Chandra RP, Saddler JN and Beatson RP (2007) Treatment of Douglas-fir heartwood thermomechanical pulp with laccases: Effect of treatment conditions on peroxide bleaching. Journal of Wood Chemistry and Technology 27:73-82

Chang S-T (1993) Mushroom biology: the impact on mushroom production and mushroom products. In: Chang, S-T, Buswell JA and Chiu SW (Eds.) Proceedings of the first international conference on mushroom biology and mushroom products. The Chinese University Press, Hong Kong, Hong Kong, 3-20

Chang S-T (1999) World production of edible and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk.) Sing. in China. International Journal of Medicinal Mushroom 1:291-301

Chang S-T (2005) Witnessing the development of the mushroom industry in China. Acta Edulis Fungi 12 (Supplement):3-19

Chang S-T and Buswell JA (1996) Mushroom nutriceuticals. World Journal of Microbiology and Biotechnology 12:473-476

Chang S-T and Miles PG (1989) Edible mushrooms and their cultivations. CRC Press Incorporation, Boca Raton, FL

Chang YS and Lee SS (2004) Utilisation of macrofungi species in Malaysia. Fungal Diversity 15:15-22

Chefetz B, Chen Y and Hadar Y (1998) Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Applied and Environmental Microbiology 64:3175-3179

Chen MM (2004) Species of edible mushrooms in China. Mushroom Science 16:155-163

Chiu SW, Law SC, Ching ML, Cheung KW and Chen MJ (2000) Themes for mushroom exploitation in the 21st century: sustainability, waste management and conservation. The Journal of General and Applied Microbiology 46:269-282

Claus H (2004) Laccases: structure, reactions, distribution. Micron 35:93-96

Claus H, Faber G and Konig H (2002) Redox-mediated decolorization of synthetic dyes by fungal laccases. Applied Microbiology and Biotechnology 59:672-678

Compart LCA, Machado KMG, Matheus DR and Cardoso AV (2007) Immobilization of *Psilocybe castanella* on ceramic (slate) supports and its potential for soil bioremediation. World Journal of Microbiology & Biotechnology 23:1479-1483

Conrad LS and Sponholz WR (2000) Treatment of cork with a phenol oxidizing enzyme. Patent: US6152966

Courty PE, Pouysegur R, Buee M and Garbaye J (2006) Laccase and phosphatase activities of the dominant ectomycorrhizal types in a lowland oak forest. Soil Biology & Biochemistry 38:1219-1222

Courty PE, Hoegger PJ, Kilaru S, Kohler A, Buée M, Garbaye J, Martin F and Kües U (2009) Phylogenetic analysis, genomic organization, and expression analysis of multi-copper oxidases in the ectomycorrhizal basidiomycete *Laccaria bicolor*. New Phytologist 182:736-750

Couto SR and Herrera JLT (2006) Industrial and biotechnological applications of laccases: A review. Biotechnology Advances 24:500-513

d'Acunzo F, Galli C, Gentili P and Sergi F (2006) Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. New Journal of Chemistry 30:583-591

D'Souza TM, Boominathan K and Reddy CA (1996) Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. Applied and Environmental Microbiology 62:3739-3744

D'Souza TM, Merritt CS and Reddy CA (1999) Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Applied and Environmental Microbiology 65:5307-5313

Danell E and Camacho FJ (1997) Successful cultivation of the golden chantarelle. Nature 385:303

Decker H, Ryan H, Jaenicke E and Terwilliger N (2001) SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum* and *Cancer magister*. Journal of Biological Chemistry 276:17796-17799

del Toro GV, Vega RC, Garin-Aguilar ME and Lara HL (2006) Biological quality of proteins from three strains of *Pleurotus* spp.. Food Chemistry 94:494-497

de Román M, Boa E and Woodward S (2006) Wild-gathered fungi for health and rural livelihoods. Proceedings of the Nutrition Society 65:190-197

De Souza CGM, Tychanowicz GK, De Souza DF and Peralta RM (2004) Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. Journal of Basic Microbiology 44:129-136

Diano N, Grano V, Fraconte L, Caputo P, Ricupito A, Attanasio A, Bianco M, Bencivenga U, Rossi S, Manco I, Mita L, Del Pozzo G and Mita DG (2007) Non-isothermal bioreactors in enzymatic remediation of waters polluted by endocrine disruptors: BPA as a model of p-ollutant. Applied Catalysis B-Environmental 69:252-261

Dominguez A, Couto SR and Sanroman MA (2005) Dye decolorization by *Trametes hirsuta* immobilized into alginate beads. World Journal of Microbiology & Biotechnology 21:405-409

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH and Davies GJ (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Ångström resolution. Nature Structural Biology 5:310-316

Durán N and Esposito E (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Applied Catalysis B-Environmental 28:83-99

Durán N, Rosa MA, D'Annibale A and Gianfreda L (2002) Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. Enzyme and Microbial Technology 31:907-931

Eggen T and Majcherczyk A (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus*. International Biodeterioration & Biodegradation 41:111-117

Eggert C, Temp U and Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Applied and Environmental Microbiology 62:1151-1158

Elegir G, Daina S, Zoia L, Bestetti G and Orlandi M (2005) Laccase mediator system: Oxidation of recalcitrant lignin model structures present in residual kraft lignin. Enzyme and Microbial Technology 37:340-346

Elissetche JP, Ferraz A, Freer J, Mendonca R and Rodriguez J (2006) Thiobarbituric acid reactive substances, Fe^{3+} reduction and enzymatic activities in cultures of *Ganoderma australe* growing on Drimys winteri wood. FEMS Microbiology Letters 260:112-118

Euring M (2008) Einsatz von Mediatoren bei der enzymatischen Aktivierung der fasereigenen Bindekräfte von enzymgebundenen, bindemittelfreien Holzwerkstoffen. Dissertation, Georg-August-Universität Göttingen, Göttingen

Fackler K, Kuncinger T, Ters T and Srebotnik E (2008) Laccase-catalyzed functionalization with 4-hydroxy-3-methoxybenzylurea significantly improves internal bond of particle boards. Holzforschung 62:223-229

Falck R (1917) Über die Waldkultur des Austernpilzes (*Agaricus ostreatus*) auf frischen Laubholzstubben. Zeitschrift für das Forst- und Jagdwesen 49:159-165

FAOSTAT data (2005) Agricultural data. Crops primary (last updated 24 January 2006). http://faostat.fao.org/

Farneth WE, Diner BA, Gierke TD and D'Amore MB (2005) Current densities from electrocatalytic oxygen reduction in laccase/ABTS solutions. Journal of Electroanalytical Chemistry 581:190-196

Felby C, Hassingboe J and Lund M (2002) Pilot-scale production of fiberboards made by laccase oxidized wood fibers: board properties and evidence for cross-linking of lignin. Enzyme and Microbial Technology 31:736-741

Frases S, Salazar A, Dadachova E and Casadevall A (2007) *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. Applied and Environmental Microbiology 73:615-621

Freire RS, Duran N and Kubota LT (2002) Development of a laccase-based flow injection electrochemical biosensor for the determination of phenolic compounds and its application for monitoring remediation of Kraft E1 paper mill effluent. Analytica Chimica Acta 463:229-238

Fu SY, Yu HS and Buswell JA (1997) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Pleurotus sajor-caju*. FEMS Microbiology Letters 147:133-137

Galante YA and Formantici C (2003) Enzyme applications in detergency and in manufacturing industries. Current Organic Chemistry 7:1399-1422

Garibay-Orijel R, Cifuentes J, Estrada-Torres A and Caballero J (2006) People using macro-fungal diversity in Oaxaca, Mexico. Fungal Diversity 21:41-67

Gavnholt B and Larsen K (2002) Molecular biology of plant laccases in relation to lignin formation. Physiologia Plantarum 116:273-280

Gomes SASS and Rebelo MJF (2003) A new laccase biosensor for polyphenols determination. Sensors 3:166-175

Goodell B, Yamamoto K, Jellison J, Nakamura M, Fujii T, Takabe K and Hayashi N (1998) Laccase immunolabelling and microanalytical analysis of wood degraded by *Lentinus edodes*. Holzforschung 52:345-350

Gramss G, Gunther T and Fritsche W (1998) Spot tests for oxidative enzymes in ectomycorrhizal, wood-, and litter decaying fungi. Mycological Research 102:67-72

Gregorio APF, Da Silva IR, Sedarati MR and Hedger JN (2006) Changes in production of lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. Mycological Research 110:161-168

Grönqvist S, Suurnakki A, Niku-Paavola ML, Kruus K, Buchert J and Viikari L (2003) Lignocellulose processing with oxidative enzymes. In: Mansfield SD and Saddler JN (Eds.) Applications of Enzymes to Lignocellulosics, ACS Symposium Series, Vol 855. American Chemical Society, Washington D.C., 46-65

Grönqvist S, Rantanen K, Alen R, Mattinen ML, Buchert J and Viikari L (2006) Laccase-catalysed functionalisation of TMP with tyramine. Holzforschung 60:503-508

Hager A (2003) Zum Effekt von Laccasen beim Altpapier Deinking. Dissertation, University Hamburg, Hamburg

Hakala TK, Lundell T, Galkin S, Maijala P, Kalkkinen N and Hatakka A (2005) Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips. Enzyme and Microbial Technology 36:461-468

Hakulinen N, Kiiskinen LL, Kruus K, Saloheimo M, Paananen A, Koivula A and Rouvinen J (2002) Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. Nature Structural Biology 9:601-605

Hall IR, Yun W and Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends in Biotechnology 21:433-438

Hao JJ, Tian XJ, Song FQ, He XB, Zhang ZJ and Zhang P (2006) Involvement of lignocellulolytic enzymes in the decomposition of leaf litter in a subtropical forest. Journal of Eukaryotic Microbiology 53:193-198

Hao JJ, Song FQ, Huang F, Yang CL, Zhang ZJ, Zheng Y and Tian XJ (2007) Production of laccase by a newly isolated deuteromycete fungus *Pestalotiopsis* sp. and its decolorization of azo dye. Journal of Industrial Microbiology & Biotechnology 34:233-240

Hatakka AI (1994) Lignin modifying enzymes from selected white-rot fungi - production and role in lignin degradation. FEMS Microbiology Reviews 13:125-135

Hatakka AI, Lundell TK, Tervilawilo ALM and Brunow G (1991) Metabolism of nonphenolic ß-O-4 lignin model compounds by the white rot fungus *Phlebia radiata*. Applied Microbiology and Biotechnology 36:270-277

Hawksworth DL (2001) Mycological research news. Mycological Research 105:641-643

Hermann TE, Kurtz MB and Champe SP (1983) Laccase localized in hulle cells and cleistothecial primordia of *Aspergillus nidulans*. Journal of Bacteriology 154:955-964

Hoegger PJ, Kilaru S, James TY, Thacker JR and Kües U (2006) Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS Journal 273:2308-2326

Hoegger PJ, Majcherczyk A, Dwivedi RC, Svobodova K, Kilaru S and Kües U (2007) Enzymes in wood degradation. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 383-432

Hou HM, Zhou JT, Wang J, Du CH and Yan B (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochemistry 39:1415-1419

Hu HT, Wang Y and Hu BY (2005) Cultivation of *Tuber formosanum* on limed soil in Taiwan. New Zealand Journal of Crop and Horticultural Science 33:363-366

Hüttermann A (1987) History of forest botany (Forstbotanik) in Germany from the beginning in 1800 until 1940 – Science in the tension field between university and professional responsibility. Berichte der Deutschen Botanischen Gesellschaft 100:107-141

Hüttermann A (1991) Richard Falck, his life and work. In: Jennings D and Bravery AF (Eds.) The dry rot fungus *Serpula lacrymans*: fundamental biology and control strategies. Wiley, New York, NY, 193-206

Iakovlev A and Stenlid J (2000) Spatiotemporal patterns of laccase activity in interacting mycelia of wood-decaying basidiomycete fungi. Microbial Ecology 39:236-245

Ibarra D, Camarero S, Romero J, Martinez MJ and Martinez AT (2006) Integrating laccase-mediator treatment into an industrial-type sequence for totally chlorine-free bleaching of eucalypt kraft pulp. Journal of Chemical Technology and Biotechnology 81:1159-1165

Ikehata K, Buchanan ID and Smith DW (2004) Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. Journal of Environmental Engineering and Science 3:1-19

Jacobson ES and Emery HS (1991) Temperature regulation of the cryptococcal phenoloxidase. Journal of Medical and Veterinary Mycology 29:121-124

Johannes C, Majcherczyk A and Hüttermann A (1998) Oxidation of acenaphthene and acenaphthylene by laccase of *Trametes versicolor* in a laccase-mediator system. Journal of Biotechnology 61:151-156

Johannes C and Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Applied and Environmental Microbiology 66:524-528

Jonsson LJ, Palmqvist E, Nilvebrant NO and Hahn-Hägerdal B (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Applied Microbiology and Biotechnology 49:691-697

Kamitaka Y, Tsujimura S, Setoyama N, Kajino T and Kano K (2007) Fructose/dioxygen biofuel cell based on direct electron transfer-type bioelectrocatalysis. Physical Chemistry Chemical Physics 9:1793-1801

Kandelbauer A, Maute O, Kessler RW, Erlacher A and Gubitz GM (2004) Study of dye decolorization in an immobilized laccase enzyme-reactor using online spectroscopy. Biotechnology and Bioengineering 87:552-563

Kataoka K, Komori H, Ueki Y, Konno Y, Kamitaka Y, Kurose S, Tsujimura S, Higuchi Y, Kano K, Seo D and Sakurai T (2007) Structure and function of the engineered multicopper oxidase CueO from *Escherichia coli* - Deletion of the methionine-rich helical region covering the substrate-binding site. Journal of Molecular Biology 373:141-152

Kellner H, Luis P and Buscot F (2007) Diversity of laccase-like multicopper oxidase genes in *Morchellaceae*: identification of genes potentially involved in extracellular activities related to plant litter decay. FEMS Microbiology Ecology 61:153-163

Keum YS and Li QX (2004) Fungal laccase-catalyzed degradation of hydroxy polychlorinated biphenyls. Chemosphere 56:23-30

Kharazipour A, Hüttermann A, Kühne G and Rong M (1993) Verfahren zur Herstellung von bindemittelfreien Formkörpern. Patent: DE4305411

Kharazipour A, Hüttermann A, Kühne G and Rong M (1994) Verfahren zum Verkleben von Holzfragmenten und nach dem Verfahren hergestellte Formkörper. Patent: EU94103099

Kharazipour A, Hüttermann A and Lüdemann HD (1997) Enzymatic activation of wood fibres as a means for the production of wood composites. Journal of Adhesion Science and Technology 11:419-427

Kilaru S (2006) Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology. Dissertation, Cuvillier Verlag, Göttingen

Kirk O, Borchert TV and Fuglsang CC (2002) Industrial enzyme applications. Current Opinion in Biotechnology 13:345-351

Kirk PM, Cannon PF, David JC and Stalpers JA (2001) In: Ainsworth & Bisby's Dictionary of the Fungi. C.A.B. International, Wallingford, UK

Kondrashchenko VI, Manukovsky NS and Kovalev VS (2006) Determination of the parameters for producing a biobinder from wood: a mathematical modelling of the transformation of lignocellulose substrate by the fungus *Panus tigrinus*. Applied Biochemistry and Microbiology 42:636-643

Koschorreck K, Richter SM, Ene AB, Roduner E, Schmid RD and Urlacher VB (2008) Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. Applied Microbiology and Biotechnology 79:217-224

Kramer KJ, Kanost MR, Hopkins TL, Jiang HB, Zhu YC, Xu RD, Kerwin JL and Turecek F (2001) Oxidative conjugation of catechols with proteins in insect skeletal systems. Tetrahedron 57:385-392

Kües U and Liu Y (2000) Fruiting body production in basidiomycetes. Applied Microbiology and Biotechnology 54:141-152

Kües U, Bohn C, Euring M, Müller C, Polle A and Kharazipour A (2007a) Enzymatically modified wood in panel board production. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 433-467

Kües U, Mai C and Militz (2007b) Biological wood protection against decay, microbial staining, fungal moulding and insect pests. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 273-294

Lampman AM (2007) Medicinal and edible mushrooms of the Tzeltal Maya of Chiapas, Mexico. International Journal of Medicinal Mushrooms 9:1-5

Lang G and Cotteret J (2003) Mixture for the oxidation tinting of keratin fibres containing a laccase and tinting method using said mixture. Patent US6576024

Lantto R, Schönberg C, Buchert J and Heine E (2004) Effects of laccase-mediator combinations on wool. Textile Research Journal 74:713-717

Larrondo LF, Salas L, Melo F, Vicuna R and Cullen D (2003) A novel extracellular multicopper oxidase from *Phanerochaete chrysosporium* with ferroxidase activity. Applied and Environmental Microbiology 69:6257-6263

Lau KL, Tsang YY and Chiu SW (2003) Use of spent mushroom compost to bioremediate PAH-contaminated samples. Chemosphere 52:1539-1546

Laufer Z, Beckett RP and Minibayeva FV (2006) Co-occurrence of the multicopper oxidases tyrosinase and laccase in lichens in sub-order *Peltigerineae*. Annals of Botany 98:1035-1042

Leatham GF and Stahmann MA (1981) Studies on the laccase of *Lentinus edodes* - Specificity, localization and association with the development of fruiting bodies. Journal of General Microbiology 125:147-157

Lee JW, Koo BW, Choi JW, Choi DH and Choi IG (2008) Evaluation of waste mushroom logs as a potential biomass resource for the production of bioethanol. Bioresource Technology 99:2736-2741

Lee SY and Soderhall K (2002) Early events in crustacean innate immunity. Fish & Shellfish Immunology 12:421-437

Lisov AV, Zavarzina AG, Zavarzin AA and Leontievsky AA (2007) Laccases produced by lichens of the order *Peltigerales*. FEMS Microbiology Letters 275:46-52

Liu GX, Yang LL, Fan TJ, Cong RS, Tang ZH, Sun WJ, Meng XH and Zhu LY (2006) Purification and characterization of phenoloxidase from crab *Charybdis japonica*. Fish & Shellfish Immunology 20:47-57

Liu WX, Chao YP, Yang XQ, Bao HB and Qian SJ (2004) Biodecolorization of azo, anthraquinonic and triphenylmethane dyes by white-rot fungi and a laccase-secreting engineered strain. Journal of Industrial Microbiology & Biotechnology 31:127-132

Lu L, Zhao M, Zhang BB, Yu SY, Bian XJ, Wang W and Wang Y (2007) Purification and characterization of laccase from *Pycnoporus sanguineus* and decolorization of an anthraquinone dye by the enzyme. Applied Microbiology and Biotechnology 74:1232-1239

Lund M and Felby C (2001) Wet strength improvement of unbleached kraft pulp through laccase catalyzed oxidation. Enzyme and Microbial Technology 28:760-765

Lund M and Ragauskas AJ (2001) Enzymatic modification of kraft lignin through oxidative coupling with water-soluble phenols. Applied Microbiology and Biotechnology 55:699-703

Lund M, Eriksson M and Felby C (2003) Reactivity of a fungal laccase towards lignin in softwood kraft pulp. Holzforschung 57:21-26

Luo X (2004) Progress in xian-gu (shiitake) cultivation in China. Mushroom Science 16:317-322

Mahapatra AK and Panda PK (2002) Ethno-pharmacological knowledge of Juang and Munda tribes of eastern India. International Journal of Sustainable Development and World Ecology 9:151-158

Maheshwari R, Bharadwaj G and Bhat MK (2000) Thermophilic fungi: their physiology and enzymes. Microbiology and Molecular Biology Reviews 64:461-488

Mai C, Kües U and Militz H (2004) Biotechnology in the wood industry. Applied Microbiology and Biotechnology 63:477-494

Majcherczyk A, Johannes C and Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme and Microbial Technology 22:335-341

Mandeel QA, Al-Laith AA and Mohamed SA (2005) Cultivation of oyster mushrooms (*Pleurotus* spp.) on various lignocellulosic wastes. World Journal of Microbiology and Biotechnology 21:601-907

Manzi P, Gambelli L, Marconi S, Vivanti V and Pizzoferrato L (1999) Nutrients in edible mushrooms: an inter-species comparative study. Food Chemistry 65:477-482

Martin R, Jumino A, Dubief C, Rosenbaum G and Audousset MP (1994) Cosmetic use of vegetable laccase or active plant extract containing laccase - in oxidative dyeing or permanent shaping of hair. Patent: FR2694018

Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D and Rokhsar D (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnology 22:695-700

Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek C, Ferreira P, Ruiz-Duenas F, Martinez A, Kersten P, Hammel K, Wymelenberg AV, Gaskell J, Lindquist E, Sabat G, Splinter BonDurant S, Larrondo L, Canessa P, Vicuna R, Yadav J, Doddapaneni H, Subramanian V, Pisabarro A, Lavin J, Oguiza J, Master E, Henrissat B, Coutinho P, Harris P, Magnuson J, Baker S, Bruno K, Kenealy W, Hoegger PJ, Kües U, Ramalya P, Lucas S, Salamov A, Shapiro H, Tu H, Chee C, Misra M, Xie G, Teter S, Yaver D, James T, Mokrejs M, Pospisek M, Grigoriev I, Brettin T, Rokhsar D, Berka R and Cullen D (2009) Genome, transcriptome,

and secretome of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. PNAS 106:1954-1959

Maruyama T, Komatsu C, Michizoe J, Ichinose H and Goto M (2006) Laccase-mediated oxidative degradation of the herbicide dymron. Biotechnology Progress 22:426-430

Massadeh MI and Modallal N (2008) Ethanol production from olive mill wastewater (OMW) pretreated with *Pleurotus sajor-caju*. Energy & Fuels 22:150-154

Matsune H, Jogasaki H, Date M, Takenaka S and Kishida M (2006) One-pot synthesis and characterization of laccase-entrapped magnetic nanobeads. Chemistry Letters 35:1356-1357

Mattila P, Konko K, Eurola M, Pihlava JM, Astola J, Vahteristo L, Hietaniemi V, Kumpulainen J, Valtonen M and Piironen V (2001) Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. Journal of Agricultural and Food Chemistry 49:2343-2348

Mattinen ML, Kruus K, Buchert J, Nielsen JH andersen HJ and Steffensen CL (2005) Laccase-catalyzed polymerization of tyrosine-containing peptides. FEBS Journal 272:3640-3650

McErlean C, Marchant R and Banat IM (2006) An evaluation of soil colonisation potential of selected fungi and their production of ligninolytic enzymes for use in soil bioremediation applications. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 90:147-158

Mello A, Murat C and Bonfante P (2006) Truffles: much more than a prized and local fungal delicacy. FEMS Microbiology Letters 260:1-8

Mena ML, Carralero V, Gonzalez-Cortes A, Yanez-Sedeno P and Pingarron JM (2005) Laccase biosensor based on N-succinimidyl-3-thiopropionate-functionalized gold electrodes. Electroanalysis 17:2147-2155

Messerschmidt A (1998) Metal sites in small blue copper proteins, blue copper oxidases and vanadium-containing enzymes. In: Metal Sites in Proteins and Models Redox Centres. Springer Verlag Berlin / Heidelberg, Berlin, 37-68

Messerschmidt A, Rossi A, Ladenstein R, Huber R, Bolognesi M, Gatti G, Marchesini A, Petruzzelli R and Finazziagro A (1989) X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini - analysis of the polypeptide fold and a model of the copper sites and ligands. Journal of Molecular Biology 206:513-529

Michelot D and Melendez-Howell LM (2003) *Amanita muscaria*: chemistry, biology, and ethnomycology. Mycological Research 107:131-146

Minussi RC, Pastore GM and Duran N (2002) Potential applications of laccase in the food industry. Trends in Food Science & Technology 13:205-216

Modaressi K, Taylor KE, Bewtra JK and Biswas N (2005) Laccase-catalyzed removal of bisphenol-A from water: Protective effect of PEG on enzyme activity. Water Research 39:4309-4316

Moldes D and Sanroman MA (2006) Amelioration of the ability to decolorize dyes by laccase: relationship between redox mediators and laccase isoenzymes in *Trametes versicolor*. World Journal of Microbiology & Biotechnology 22:1197-1204

Montoya A, Hernandez-Totomoch O, Estrada-Torres A, Kong A and Caballero J (2003) Traditional knowledge about mushrooms in a Nahua community in the state of Tlaxcala, Mexico. Mycologia 95:793-806

Moreira MT, Palma C, Feijoo G and Lema JM (1998) Strategies for the continuous production of ligninolytic enzymes in fixed and fluidised bed bioreactors. Journal of Biotechnology 66:27-39

Murugesan K, Arulmani M, Nam IH, Kim YM, Chang YS and Kalaichelvan PT (2006) Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajor-caju* under submerged culture condition and its potential in decolorization of azo dyes. Applied Microbiology and Biotechnology 72:939-946

Navarro-González M (2008) Growth, fruiting body development and laccase production of selected *Coprini*. Dissertation, Georg-August-University Göttingen, Göttingen

Ncanana S and Burton S (2007) Oxidation of 8-hydroxyquinoline catalyzed by laccase from *Trametes pubescens* yields an antioxidant aromatic polymer. Journal of Molecular Catalysis B-Enzymatic 44:66-71

Nerud F, Zouchova Z and Misurcova Z (1991) Ligninolytic properties of different white rot fungi. Biotechnology Letters 13:657-660

Novotny C, Svobodova K, Erbanova P, Cajthaml T, Kasinath A, Lang E and Sasek V (2004) Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biology & Biochemistry 36:1545-1551

Nozaki K, Beh CH, Mizuno M, Isobe T, Shiroishi M, Kanda T and Amano Y (2008) Screening and investigation of dye decolorization activities of basidiomycetes. Journal of Bioscience and Bioengineering 105:69-72

Nyanhongo GS, Couto SR and Gübitz GM (2006) Coupling of 2,4,6-trinitrotoluene (TNT) metabolites onto humic monomers by a new laccase from *Trametes modesta*. Chemosphere 64:359-370

Nwanze PI, Jatto W, Oransi S and Josiah SJ (2006) Proximate analysis of *Lentinus squarrosulus* (Mont.) Singer and *Psathyrella atroumbonata* Pegler. African Journal of Biotechnology 5:366-368

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, and Vilgalys R (2005) Fungal community analysis by large-scale sequencing of environmental samples. Applied and Environmental Microbiology 71:5544-5550

O'Malley DM, Whetten R, Bao WL, Chen CL and Sederoff RR (1993) The role of laccase in lignification. Plant Journal 4:751-757

Ohga S, Smith M, Thurston CF and Wood DA (1999) Transcriptional regulation of laccase and cellulase genes in the mycelium of *Agaricus bisporus* during fruit body development on a solid substrate. Mycological Research 103:1557-1560

Olivier JM (2000) Progress in the cultivation of truffles. Mushroom Science 15:937-942

Ortega-Clemente A, Marin-Mezo G, Ponce-Noyola MT, Montes-Horcasitas MC, Caffarel-Mendez S, Barrera-Cortes J and Poggi-Varaldo HM (2007) Comparison of two continuous fungal bioreactors for posttreatment of anaerobically pretreated weak black liquor from Kraft pulp mills. Biotechnology and Bioengineering 96:640-650

Palmqvist E and Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technology 74:17-24

Palonen H (2004) Role of lignin in the enzymatic hydrolysis of lignocellulose. VTT Publications 520:1-80

Pandey A, Soccol CR and Mitchell D (2000) New developments in solid state fermentation: I-bioprocesses and products. Process Biochemistry 35:1153-1169

Pant D and Adholeya A (2007) Enhanced production of ligninolytic enzymes and decolorization of molasses distillery wastewater by fungi under solid state fermentation. Biodegradation 18:647-659

Papinutti L and Martinez MJ (2006) Production and characterization of laccase and manganese peroxidase from the ligninolytic fungus *Fomes sclerodermeus*. Journal of Chemical Technology and Biotechnology 81:1064-1070

Pearce MH, Nelson EE and Malajczuk N (1995) Effects of the cord forming saprotrophs *Hypholoma australe* and *Phanerochaete filamentosa* and of ammonium sulfamate on establishment of *Armillaria luteobubalina* on stumps of *Eucalyptus diversicolor*. Mycological Research 99:951-956

Peintner U, Poder R and Pumpel T (1998) The iceman´s fungi. Mycological Research 102:1153-1162

Pemmasani JK (2006) Stress responses to wood and wood-derived volatiles using the yeast *Saccharomyces cerevisiae* as a model system for biological monitoring. Dissertation. Cuvillier Verlag, Göttingen

Perez J and Jeffries TW (1990) Mineralization of ^{14}C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. Applied and Environmental Microbiology 56:1806-1812

Philippoussis A, Zervakis G and Diamantopoulou P (2001) Bioconversion of agricultural lignocellulosic wastes through the cultivation of the edible mushrooms *Agrocybe aegerita*, *Volvariella volacea* and *Pleurotus* spp.. World Journal of Microbiology and Biotechnology 17:191-200

Pich A, Bhattacharya S, Adler HJP, Wage T, Taubenberger A, Li Z, van Pee KH, Böhmer U and Bley T (2006) Composite magnetic particles as carriers for laccase from *Trametes versicolor*. Macromolecular Bioscience 6:301-310

Pieroni A, Nebel S, Santoro RF and Heinrich M (2005) Food for two seasons: Culinary uses of non-cultivated local vegetables and mushrooms in a south Italian village. International Journal of Food Science and Nutrition 56:245-272

Piontek K, Antorini M and Choinowski T (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Ångström resolution containing a full complement of coppers. Journal of Biological Chemistry 277:37663-37669

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Applied Microbiology and Biotechnology 57:20-33

Poppe J (2000) Use of agricultural waste materials in the cultivation of mushrooms. Mushroom Science 15:3-23

Potthast A, Rosenau T and Fischer K (2001) Oxidation of benzyl alcohols by the laccase-mediator system (LMS) - a comprehensive kinetic description. Holzforschung 55:47-56

Richardson A, Duncan J and McDougall GJ (2000) Oxidase activity in lignifying xylem of a taxonomically diverse range of trees: identification of a conifer laccase. Tree Physiology 20:1039-1047

Riva S (2006) Laccases: blue enzymes for green chemistry. Trends in Biotechnology 24:219-226

Rodriguez CS, Sanroman MA, Hofer D and Gubitz GM (2004) Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus *Trametes hirsuta* for decolourization of textile dyes. Bioresour Technol 95:67-72

Rodriguez E, Pickard MA and Vazquez-Duhalt R (1999) Industrial dye decolorization by laccases from ligninolytic fungi. Current Microbiology 38:27-32

Rodriguez E, Nuero O, Guillen F, Martinez AT and Martinez MJ (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. Soil Biology & Biochemistry 36:909-916

Roussel B, Rapior S, Masson CL and Boutie P (2002) *Fomes fomentarius* (L.: Fr.) Fr.: a mushroom with varied uses. Cryptogamie Mycologie 23:349-366

Roy JJ, Abraham TE, Abhijith KS, Kumar PVS and Thakur MS (2005) Biosensor for the determination of phenols based on cross linked enzyme crystals (CLEC) of laccase. Biosensors & Bioelectronics 21:206-211

Royse DJ, Shen Q and Mcgarvey C (2005) Consumption and production of recently domesticated edible fungi in the United States with a projection of their potential. Acta Edulis Fungi 12 (Supplement):331-337

Rühl M and Kües U (2007) Mushroom production. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 555-586

Rühl M, Kilaru S, Navarro-González M, Hoegger PJ, Kharazipour A, and Kües U (2007) Production of laccase and other enzymes for the wood industry. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 469-507

Rühl M, Fischer C and Kües U (2008) Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate. Current Trends in Biotechnology and Pharmacy 2: 478-492

Rüttimann C, Schwember E, Salas L, Cullen D and Vicuna R (1992) Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnology and Applied Biochemistry 16:64-76

Ryan DR, Leukes WD and Burton SG (2005) Fungal bioremediation of phenolic wastewaters in an airlift reactor. Biotechnology Progress 21:1068-1074

Sanmee R, Dell B, Lumyong P, Izumori K and Lumyong S (2003) Nutritive value of popular wild edible mushrooms from northern Thailand. Food Chemistry 82:527-532

Saparrat MCN, Mocchiutti P, Liggieri CS, Aulicino MB, Caffini NO, Balatti PA and Martinez MJ (2008) Ligninolytic enzyme ability and potential biotechnology applications of the white-rot fungus *Grammothele subargentea* LPSC no. 436 strain. Process Biochemistry 43:368-375

Savoie JM, Mata G and Billette C (1998) Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. Applied Microbiology and Biotechnology 49:589-593

Schäfer T, Borchert TW, Nielsen VS, Skagerlind P, Gibson K, Wenger K, Hatzack F, Nilsson LD, Salmons S, Pedersen S, Heldt-Hansen HP, Poulsen PB, Lund H, Oxenboll KM, Wu GF, Pedersen HH and Xu H (2007) Industrial enzymes. Advances in Biochemical Engineering / Biotechnology 105:59-131

Schirp A, Loge F, Aust S, Swarier P, Turner G and Wolcott M (2006) Production and characterization of natural fiber-reinforced thermoplastic composites using wheat straw modified with the fungus *Pleurotus ostreatus*. Journal of Applied Polymer Science 102:5191-5201

Scherer M and Fischer R (1998) Purification and characterization of laccase II of *Aspergillus nidulans*. Archives of Microbiology 170:78-84

Schneider P, Caspersen MB, Mondorf K, Halkier T, Skov LK, Ostergaard PR, Brown KM, Brown SH and Xu F (1999) Characterization of a *Coprinus cinereus* laccase. Enzyme and Microbial Technology 25:502-508

Schouten A, Maksimova O, Cuesta-Arenas Y, van den Berg G and Raaijmakers JM (2008) Involvement of the ABC transporter BcAtrB and the laccase BcLCC2 in defence of *Botrytis cinerea* against the broad-spectrum antibiotic 2,4-diacetylphloroglucinol. Environmental Microbiology 10:1145-1157

Sharma P, Goel R and Capalash N (2007) Bacterial laccases. World Journal of Microbiology & Biotechnology 23:823-832

Shaw PJA, Lankey K and Jourdan A (1996) Factors affecting yield of *Tuber melanosporum* in a *Quercus ilex* plantation in southern France. Mycological Research 100:1176-1178

Shleev S, Persson P, Shumakovich G, Mazhugo Y, Yaropolov A, Ruzgas T and Gorton L (2006a) Laccase-based biosensors for monitoring lignin. Enzyme and Microbial Technology 39:835-840

Shleev S, Persson P, Shumakovich G, Mazhugo Y, Yaropolov A, Ruzgas T and Gorton L (2006b) Interaction of fungal laccases and laccase-mediator systems with lignin. Enzyme and Microbial Technology 39:841-847

Sigoillot C, Camarero S, Vidal T, Record E, Asther M, Perez-Boada M, Martinez MJ, Sigoillot JC, Asther M, Colom JF and Martinez AT (2005) Comparison of different fungal enzymes for bleaching high-quality paper pulps. Journal of Biotechnology 115:333-343

Silva CMMD, de Melo IS and de Oliveira PR (2005a) Ligninolytic enzyme production by *Ganoderma* spp. Enzyme and Microbial Technology 37:324-329

Silva EM, Machuca A and Milagres AMF (2005b) Evaluating the growth and enzyme production from *Lentinula edodes* strains. Process Biochemistry 40:161-164

Soares GMB, Amorim MTP, Lageiro M and Costa-Ferreira M (2006) Pilot-scale enzymatic decolorization of industrial dyeing process wastewater. Textile Research Journal 76:4-11

Solomon EI, Sundaram UM and Machonkin TE (1996) Multicopper oxidases and oxygenases. Chemical Reviews 96:2563-2605

Steffen KT, Cajthaml T, Snajdr J and Baldrian P (2007a) Differential degradation of oak (*Quercus petraea*) leaf litter by litter-decomposing basidiomycetes. Research in Microbiology 158:447-455

Steffen KT, Schubert S, Tuomela M, Hatakka A and Hofrichter M (2007b) Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi. Biodegradation 18:359-369

Sugumaran M (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Research 15:2-9

Sugumaran M, Giglio LB, Kundzicz H, Saul S and Semensi V (1992) Studies on the enzymes involved in puparial cuticle sclerotization in *Drosophila melanogaster*. Archives of Insect Biochemistry and Physiology 19:271-283

Tabka MG, Herpoel-Gimbert I, Monod F, Asther M and Sigoillot JC (2006) Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme and Microbial Technology 39:897-902

Tan Q, Wang Z, Cheng J, Guo Q and Guo L (2005) Cultivation of *Pleurotus* spp. in China. Acta Edulis Fungi 12 (Supplement):338-342

Tanaka H, Itakura S and Enoki A (1999) Hydroxyl radical generation and phenol oxidase activity in wood degradation by the white-rot basidiomycete *Irpex lacteus*. Material und Organismen 33:91-105

Taniguchi M, Suzuki H, Watanabe D, Sakai K, Hoshino K and Tanaka T (2005) Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. Journal of Bioscience and Bioengineering 100:637-643

Tauber MM, Guebitz GM and Rehorek A (2005) Degradation of azo dyes by laccase and ultrasound treatment. Applied and Environmental Microbiology 71:2600-2607

Teerapatsakul C, Bucke C, Parra R, Keshavarz T and Chitradon L (2008) Dye decolorisation by laccase entrapped in copper alginate. World Journal of Microbiology & Biotechnology 24:1367-1374

Thompson IA, Huber DM and Schulze DG (2006) Evidence of a multicopper oxidase in Mn oxidation by *Gaeumannomyces graminis* var. *tritici*. Phytopathology 96:130-136

Thurston CF (1994) The structure and function of fungal laccases. Microbiology-UK 140:19-26

Ullah MA, Evans CS and Hedger JN (2002) Production of ligninolytic enzymes by species assemblages of tropical higher fungi from Ecuador. In Watling R, Frankenladn JC, Ainsworth AM, Isaacs S, Robinson C (Eds.) Tropcial Mycology. Macromycetes, Vol. 1, Cambridge University Press, Cambridge, UK, 101-112

van der Ham JL and Felgenhauer BE (2007) On possible venomous effects of *Speleonectes* sp. (Remipedia). Crustaceana 80:755-765

Verkade JMM, van Hemert LJC, Quaedflieg PJLM, Schoemaker HE, Schurmann M, van Delft FL and Rutjes FPJT (2007) Laccase-mediated deprotection of *para*-methoxyphenyl (PMP)-protected amines. Advanced Synthesis & Catalysis 349:1332-1336

Wang HX and Ng TB (2006a) Purification of a laccase from fruiting bodies of the mushroom *Pleurotus eryngii*. Applied Microbiology and Biotechnology 69:521-525

Wang HX and Ng TB (2006b) A laccase from the medicinal mushroom *Ganoderma lucidum*. Applied Microbiology and Biotechnology 72:508-513

Wang JW, Wu JH, Huang WY and Tan RX (2006) Laccase production by *Monotospora* sp., an endophytic fungus in *Cynodon dactylon*. Bioresource Technology 97:786-789

Wasser SP (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology 60:258-274

White NA and Boddy L (1992) Extracellular enzyme localization during interspecific fungal interactions. FEMS Microbiology Letters 98:75-79

Widsten P and Kandelbauer A (2008) Laccase applications in the forest products industry: A review. Enzyme and Microbial Technology 42:293-307

Wolken WAM, Tramper J and van der Werf MJ (2003) What can spores do for us? Trends in Biotechnology 21:338-345

Xing Q, Eadula SR and Lvov YM (2007) Cellulose fiber-enzyme composites fabricated through layer-by-layer nanoassembly. Biomacromolecules 8:1987-1991

Xing ZT, Cheng JH, Tan Q and Pan YJ (2006) Effect of nutritional parameters on laccase production by the culinary and medicinal mushroom, *Grifola frondosa*. World Journal of Microbiology & Biotechnology 22:1215-1221

Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35:7608-7614

Xu F (2005) Applications of oxidoreductases: Recent progress. Industrial Biotechnology 1:38-50

Yamada A, Ogura T and Ohmasa M (2001) Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by *in vitro* mycorrhizal synthesis. I. Primordium and basidiocarp formation in open-pot culture. Mycorrhiza 11:59-66

Yamanaka K (2005) Cultivation of new mushroom species in East Asia. Acta Edulis Fungi 12 (Supplement):343-349

Yoon MY (1998) Process for improved shrink resistance in wool. Patent: WO9827264

Young L and Yu J (1997) Ligninase-catalysed decolorization of synthetic dyes. Water Research 31:1187-1193

Yun W and Hall IR (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. Canadian Journal of Botany 82:1063-1073

Zent EL, Zent S and Iturriaga T (2004). Knowledge and use of fungi by a mycophilic society of the Venezuelan Amazon. Economic Botany 58:214-226

Zhang SR, Hacham M, Panepinto J, Hu GW, Shin S, Zhu XD and Williamson PR (2006) The Hsp70 member, Ssa1, acts as a DNA-binding transcriptional co-activator of laccase in *Cryptococcus neoformans*. Molecular Microbiology 62:1090-1101

Zhao J and Kwan HS (1999) Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*. Applied and Environmental Microbiology 65:4908-4913

Zille A, Tzanov T, Gübitz GM and Cavaco-Paulo A (2003) Immobilized laccase for decolourization of Reactive Black 5 dyeing effluent. Biotechnology Letters 25:1473-1477

Chapter 2

Laccases and other ligninolytic enzymes of basidiomycetes

I. Production of laccase and other enzymes for the wood industry

M.Rühl, S. Kilaru, M. Navarro-González, P. J. Hoegger, A. Kharazipour and U. Kües
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II. Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate

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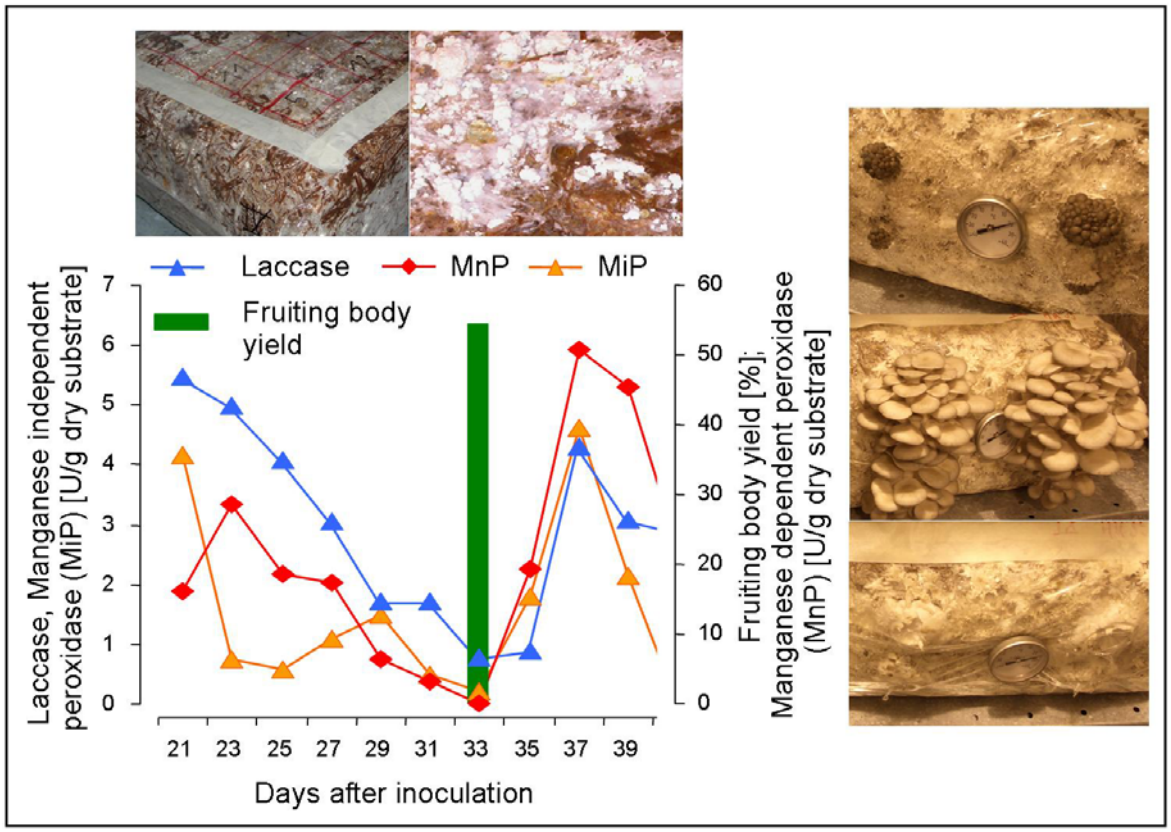

19. Production of Laccase and Other Enzymes for the Wood Industry

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Introduction

Enzymes of importance for the wood and the paper and pulp industries (see Chapter 17 of this book), in many instances, are won from fungi, either naturally or recombinantly produced. For natural enzyme production, fungal strains are selected that are most efficient in secretion of enzymes with required characteristics. Growth parameters and regulation of protein production need to be defined to establish the best conditions in the production processes, either in fermenters with a liquid medium (**submerged fermentation**, **SmF**) or in **solid state fermentation** (**SSF**) on compact moist substrates or on an inert matrix (Persson et al. 1991, Haltrich et al. 1996, Hölker et al. 2004, Hölker & Lenz 2005). Regardless of type

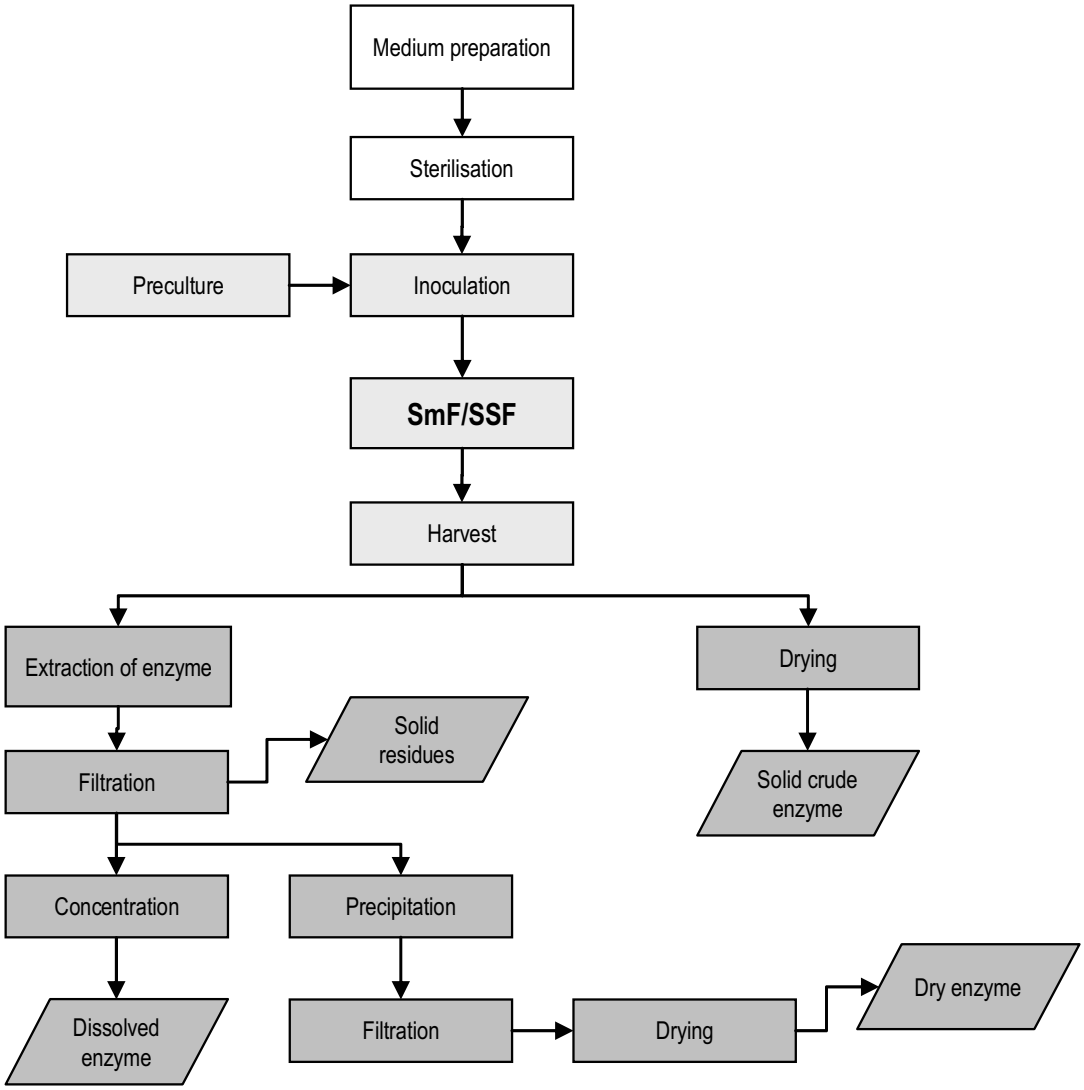

Fig. 1 General flowchart of enzyme production: steps in upstream-processing are shown in white boxes, steps of fermentation in light grey boxes, and steps in downstream-processing in darker grey boxes (adapted from Cen & Xia 1999)

of fermentation, the whole process is subdivided into three basic steps: i. upstream-processing which includes preparation of the growth substrate (most often in sterile form), ii. the fermentation process itself starting with inoculation of the production organism(s) and finishing with the harvest of the product (here enzymes), and iii. downstream-processing of the product that might be enriched and purified from the medium and production organisms and subsequently either recovered in a liquid or dried (Fig. 1).

In cases where natural strains do not yield enough products, mutagenesis and conventional genetics with appropriate screening and selection of best strains

might help to significantly improve the production rates (Parekh et al. 2000, Adrio & Demain 2006, Zhang, Y.H.P. et al. 2006). For every newly developed strain, there is further opportunity to raise the titers of products by medium modifications (Lee et al. 2005, Demain 2006). As a transgenic approach, genes for enzymes of interest are expressed under control of highly efficient promoters in homologous or in heterologous hosts, i.e., either in the species the genes came from or in a foreign species (Punt et al. 2002, Kilaru et al. 2006b). This molecular biological approach offers in addition the possibility to genetically manipulate genes in order to optimise the properties of their products to required needs (Cherry & Fidantsef 2003). Such molecular approaches, however, have also their own problems. Highly efficient promoters and an efficient gene transfer system are needed (Kilaru et al. 2006b), and problems in protein misfolding and aggregation, protein secretion, faulty protein modifications, and foreign product degradation have to be overcome (Gerngross 2004, Sørensen & Mortensen 2005, Wang et al. 2005, Guillemette et al. 2007).

In order to explain basic principles used to establish efficient enzyme production systems, we focus in this chapter on production of fungal cellulases, xylanases and, more intensively, laccases as enzymes acting on lignocellulose. However, at places, bacterial enzymes will also be incorporated. Additional aspects on production processes and regulation of expression and product degradation are discussed by Pandey et al. (1999), Beg et al. (2001), Conesa et al. (2002), Bai et al. (2003), Mach & Zeilinger (2003), Bergquist et al. (2004), Ikehata et al. (2004), Hölker et al. (2004), Aro et al. (2005), Nevalainen et al. (2005), Polizeli et al. (2005), Schügerl (2005), Sukumaran et al. (2005), Wang et al. (2005), Favela-Torres et al. (2006), Rahardjo et al. (2006), and Viniegra-Gonzalez & Favela-Torres (2006). Several of these review papers concentrate also on enzymes acting in lignocellulose degradation.

Submerged fermentation (SmF)

At the present time in Western countries, industrial production of enzymes by micro-organisms is nearly exclusively (90%) conducted in submerged fermentations in liquid media (Hölker et al. 2004). The operation of enzyme production takes place in a specific vessel, known as **fermenter** or **bioreactor**, in which micro-organisms are cultured with suitable substrates under defined process parameters. In liquid cultures, a wide variety of fermentation systems are available that distinguish by the way of oxygen input into the liquid substrate. Three main types of fermenters are available (see Fig. 2A): i. **stirred vessels** in which the liquid and the gaseous phase are mixed by stirrers, ii. **loop fermenters** in which the medium is pumped through an external circuit, and iii. **bubble columns** or **airlift reactors** without movable mechanical elements in which gas is supplied from outside and where air bubbles help in mixing (Russell et al. 1974, Schügerl 1980, Crueger &

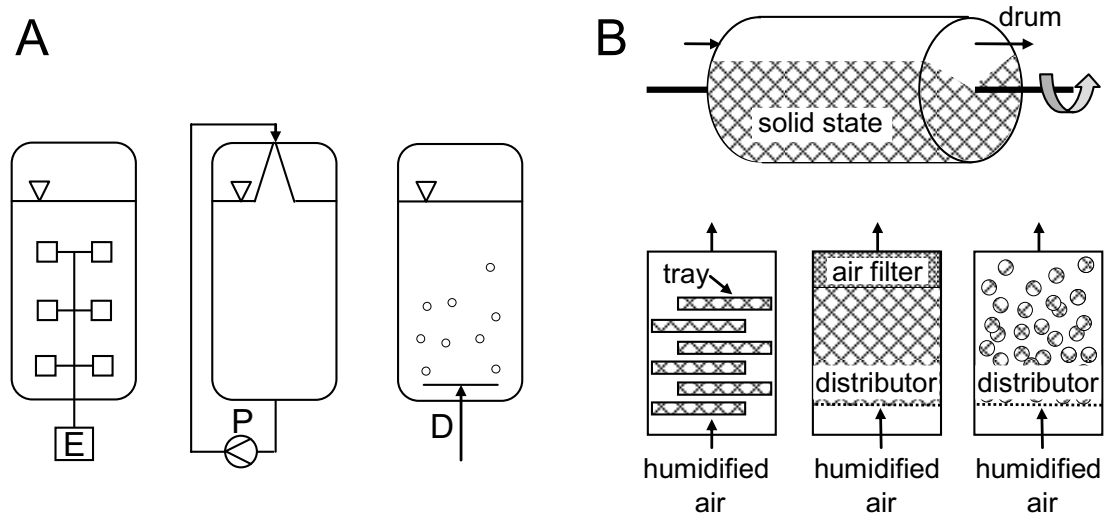

Fig. 2 Schemes of different bioreactor types. **A.** Submerged cultivation (SmF), from left to right: stirred vessel with engine (E) turning stirring blades, loop reactor with a pump (P) circulating the medium, and a bubble column with a gas dispenser (D). Note, the triangles mark the surface of the liquid medium. Modified from Crueger & Crueger (1984). **B.** Solid state fermentation (SSF): rotating drum reactor allowing mixing of the substrate (top), tray fermenter with sprinkling and circulating humidified air for humidifying the substrate on the trays (left), static column reactor or fixed-/packed-bed reactor where the solid substrate is retained on a perforated base (distributor) through which humidified air is passed through and lead into the solid substrate and an air filter to trap any dust and biological material evaporating with the air flow (middle), and fluidised bed reactor (right) used to force humidified air through a distributor into the vessel at a velocity supporting pneumatic agitation of the solid substrate particles. In each case, the solid substrate (respectively inert matrix) is indicated by the plaid pattern. Schemes were simplified from diagrams by Rodríguez Couto & Sanromán (2006b). Further explanations in Table 1

Crueger 1984, Merchuk 2003). The probably most often used reactor is the stirred vessel, both in laboratories as well as in industrial applications (Fig. 3A).

Next to classification of bioreactors, the process itself can be categorised into **batch**, **fed-batch** and **continuous cultivation**. In laboratories, enzyme production processes are usually performed in batch cultures, whereas in pilot plants and industrial applications a continuous cultivation is preferred as a complex, but more reliable and 'cheaper' cultivation technique. Batch and fed-batch operations are discontinuous fermentation processes where the culture together with the products is harvested at the end of the process (Longobardi 1994). In a batch operation, the volume of the medium is constant during the whole period; no additional nutrients are added. The fed-batch cultivation is similar to the batch cultivation

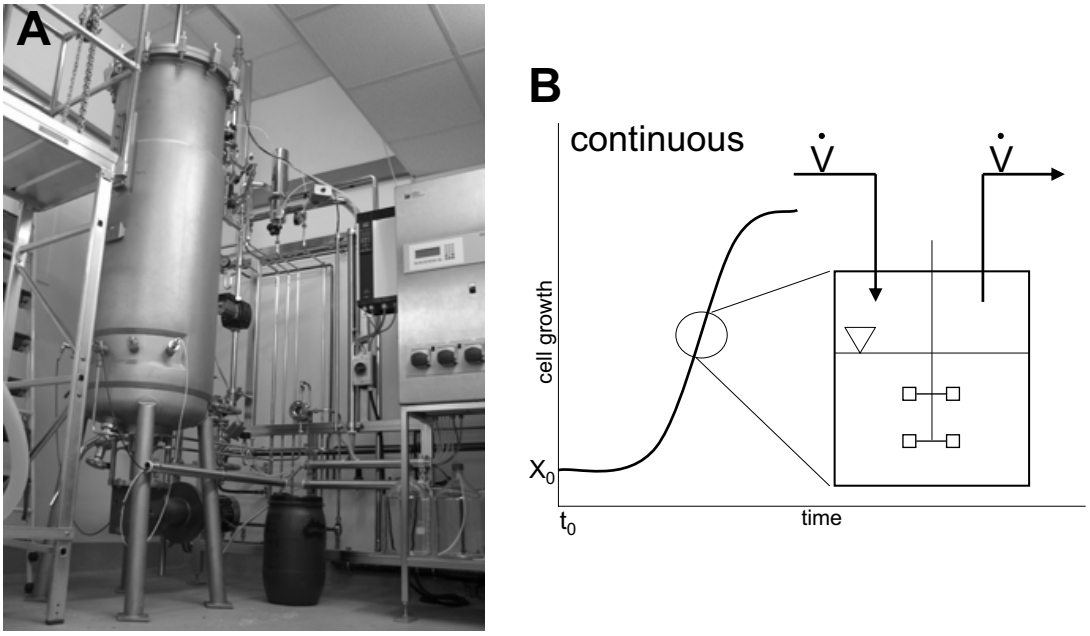

Fig. 3 500 l stirred vessel fermenter at the Institute of Forest Botany, Georg-August-University Göttingen (**A**) and scheme of a stirred tank reactor (compare Fig. 2A) at balanced operation (**B**): once after start of the process the biomass has reached an ideal density in the exponential growth phase, the inlet and the outlet volume flow of medium (indicated by the V symbols with dot) are levelled which allows continuous cultivation

until substrate limitation is reached but it offers the possibility to control the substrate concentration at a desired condition. Once the substrate is used up in fed-batch cultivation, extra substrate is added at a specific volume flow until the maximum working volume of the bioreactor is reached. The subsequent harvest of the product and/or the micro-organisms marks the end of the cultivation.

Integrated bioprocessing in which a potentially inhibitory product (and also possible toxic by-products) is continuously removed from the fermentation broth as it is produced has important advantages in improving yield and conversion relative to conventional batch and fed-batch processes. In continuous production, product yields are enhanced by both, an absolute increased product formation and an avoidance of product decomposition (Schügerl 2000, Schügerl & Hubbuch 2005). For continuous fermentation, a steady state of the process must be achieved. At this specific cultivation stage, the substrate and biomass concentration is maintained constant by simultaneously draining of culture liquid (including the product and any by-products) and entry of new substrate/medium (see Fig. 3B). Regardless of fermenter and process type used, various process parameters have to be controlled during fermentation, such as substrate composition, cell densities, biomass shape, oxygen concentration, temperature, pH, shear forces, and foam formation (Rani & Rao 1999, Papagianni 2004). By the complexity of

the systems, strategies of mathematical techniques-experimental design and optimisation are used to achieve best operating conditions for growth of micro-organisms and product formation in order to obtain optimum yields (Kennedy & Krouse 1999, Rani & Rao 1999).

Solid state fermentation (SSF)

Solid state fermentation has a long tradition in Asian food industry, e.g. in production of the protein-rich Koji for sake brewing from rice. The long-standing experiences with SSF together with the huge human capital also influenced enzyme production in Asia – cellulases from fungal *Trichoderma* strains for example are won on large scale by the more work-intense SSF (Hölker & Lenz 2005). In spite of the better established and standardised and therefore technically easier to handle SmF, a new trend towards fungal cultivation and enzyme production in SSF is seen also in Western countries (Raimbault 1998, Hölker et al. 2004, Rodríguez Couto & Sanromán 2005). Fungal morphology (cellular aggregation, mycelial pellet shape, size, etc.) very much influences production efficiencies in SmF and is often suboptimal in submerged culture of filamentous fungi that naturally prefer growth on surfaces (Papagianni 2004, Grimm et al. 2005, Kelly et al. 2006). SSF allowing the fungi to grow on surfaces therefore presents a more natural environment (Ooijkaas et al. 2000, Aguilar et al. 2004). Higher oxygen availability is guaranteed by surface growth and by the fact that in SSF the space between micro-organisms and matrix is mostly filled with gas (Durand 2003). Carbon catabolite repression (repression by fermentable sugars) of enzyme synthesis is a problem in conventional SmF but not in SSF due to different nutrient and metabolite diffusion. Also due to different diffusion, stimulation by regulator molecules (inducers) is better in SSF and the product degradation is lower (Oojikaas et al. 2000, Aguilar et al. 2004, Viniegra-Gonzalez & Favela-Torres 2006). For all these and other reasons, productivity and enzyme yields in SSF can be higher than in SmF (Díaz-Godínez et al. 2001, Viniegra-González et al. 2003, Matsumoto et al. 2004, Sandhya et al. 2005, Shah & Madamwar 2005, Hongzhang et al. 2006, Patil & Dayanand 2006). Disadvantageous in SSF is, however, the lower thermal conductivity of the air space compared to the liquid enclosing micro-organisms in SmF. The better supply of oxygen in SSF therefore leads to a reduction in heat transfer and thus to problems in process up-scaling by difficulties in temperature control. In addition, in large scale application oxygen gradients will develop (Durand 2003, Hölker et al. 2004). SSF in general is hard to control, particularly in large plants. It is labour intensive and the contamination pressure is quite high (Cen & Xia 1999, Pandey et al. 2000). Different water absorption potentials of the distinct matrices used in SSF, mechanical stress exerted on the micro-organism in certain processes (see drum reactor in Fig. 2B), the need for a sterile or non-sterile process, general process control and up-scaling can put restraints on the reactor design (Mitchell et al. 2000, Durand 2003). In contrast, cultivation of mixed organisms is more easily

Table 1 Properties of bioreactor types used in SSF (Cen & Xia 1999, Durand 2003, Rodríguez Couto & Sanromán 2006b; compare also Fig. 2B)

Fermenter type	Oxygen transfer	Advantages	Disadavantegs
Rotating drum reactor	By mixing substrate	Prevention of overheating Good oxygen/mass transfer	Clotting of substrate Low volume utilisation
Tray fermenter	By diffusion	Low investment Simple construction	Limited bed size (depths) by low oxygen transfer Up-scaling requires numerous trays and large chamber volumes
Column reactor, fixed-/packed-bed reactor	By air sparging the substrate	Relative simple construction Large bed size	Non-uniform growth Poor heat removal and difficult regulation of temperature and matrix water content
Fluidised-bed reactor	By pneumatic agitation of the substrate	Avoidance of adhesion and aggregation of substrate particles Equal temperature and humidity Continuous processes become possible	Damage to the inoculum through sheer forces

feasible in SSF which can be of advantage in production of enzyme mixtures and increasing absolute yields (Hölker et al. 2004; see below).

Also in SSF, the micro-organisms are cultured in different types of fermenters (Fig. 2B, Table 1), on a degradable (e.g. wheat bran) or non-degradable (e.g. polyurethane foam) matrix. The matrix serves the micro-organisms as anchor and, in the case of a solid substrate, also as nutrient source. Substrates for SSF can be solid by-products of agriculture and forestry such as rice grains, straw, wood chips and various other lignocellulosic wastes (Pandey et al. 2000, Rodríguez Couto & Sanromán 2006a). Costs estimated for the production of bulk enzymes by usage of such waste materials and the low budget technology for the regulation of SSF processes favor SSF over SmF. A further environmental advantage is that less waste water is generated (Aguilar et al. 2004, Hölker et al. 2004). Weighting its advantages and disadvantages (Table 1), views on whether SSF is adequate for bulk enzyme production are currently quite controversal but for special products, e.g. biopharmaceuticals, organic acids and certain enzymes, SSF might be more practical than SmF (Pandey et al. 2000). SSF is, however, not restricted to applications in productions. Instead of treating the raw materials with xylanase solutions for

biopulping and biobleaching (Daneault et al. 1994, Beg et al. 2001), fungal SSF can serve as an integral part in the process from compact lignocellulosic material to pulp and paper (Martínez-Íñigo et al. 2000, Gutíerrez et al. 2001, Helmy & El-Meligi 2002, Kang et al. 2003, Szendefy et al. 2003, van Beek et al. 2007). In order to replace petrochemicals and help to solve the problems of global warming, SSF with fungi on lignocellulosic substrates has also found high interest in biorefinery as an environmentally-friendly production system for fuels, energy, and chemicals from biomass (Tengerdy & Szakacs 2003, Watanabe 2007; see Chapters 6 and 22 of this book). Mushroom production on lignocellulosic wastes is also a form of SSF (see Chapter 22 of this book).

Cellulases

Hydrolytic enzymes cleaving β-1,4-glycosidic bonds in cellulose are known as cellulases. Three main types of cellulases are distinguished: i. endoglucanase (endo-1,4-β-glucanase, cellulase, EC 3.2.1.4), ii. exoglucanase (glucan 1,4-β-glucosidase, EC 3.2.1.74; cellulose 1,4-β-cellobiosidase, EC 3.2.1.91), and iii. β-glucosidase (cellobiase, EC 3.2.1.21). Endoglucanases cut the cellulose chain internally leaving oligosaccharides, which are cleaved by exoglucanases into cellobiose molecules (cellulose 1,4-β-cellobiohydrolase) or, glucose is chipped from their ends (glucan 1,4-β-glucosidase). β-glucosidases can react on cellobiose, thereby releasing glucose molecules (Esterbauer et al. 1991, Lynd et al. 2002; see Chapter 17 of this book).

Enzymatic systems of the various cellulolytic micro-organisms are however more diverse, acting variably on amorphous and crystalline cellulose – endoglucanase on the amorphous and exoglucanase on the micro-crystalline region of cellulose molecules (see Chapter 17 of this book). Synergistic effects between different types of cellulolytic enzymes were observed, topping in activities the sum of those of the individual enzymes (Lynd et al. 2002). In nature, cellulosic materials are processed by mixed cultures of cellulolytic organisms and in synergisms with many non-cellulolytic bacteria and fungi. The ability to decompose cellulose is widespread in bacteria in the orders Actinomycetales and Clostridiales. In fungi, it ranges from the phylum of Chytridiomycetes to the phylum of Basidiomycetes. Complex cellulase systems (known as cellulosome) bound to the cell via specific polypeptides exist in anaerobic bacteria (e.g. *Bacteroides*, *Clostridium*, *Ruminococcus*), non-complex systems of free extracellular enzymes in aerobic bacteria (e.g. *Cellulomonas*, *Thermobifida*) and also in some anaerobic bacteria (Lynd et al. 2002, Rabinovich et al. 2002, Zhang & Lynd 2004). Evidences for cellulosomes are found in some anaerobic rumen fungi such as *Neocallimastix*, *Piromyces*, and *Orpinomyces* species (Shoham et al. 1999, Harhangi et al. 2003, Steenbakkers et al. 2003, Ximenes et al. 2005) whilst aerobic fungi express mixtures of free enzymes (Lynd et al. 2002, Rabinovich et al. 2002). Bacteria and fungi are both used in batch, fed-

Table 2 Examples of cellulase production by micro-organisms in batch processes (based on Tholodur et al. 1999, Sukumaran et al. 2005)

Organism	Production conditions (process, reactor, volume)	Substrate
Bacteria		
Bacillus subtilis	SSF, column reactor, 500 ml	Soybean industry residues
Rhodothermus marinus	SmF, stirred vessel, 150 l	Carboxymethyl cellulose
Streptomyces sp. T3-1	SmF, stirred vessel, 50 l	Carboxymethyl cellulose
Ascomycetes and related anamorphic genera		
Melanocarpus albomyces	SmF, stirred vessel, 700 l	Solka-Floc (powdered cellulose)
Penicillium decumbans	SSF, tray fermentor, 50 l	Wheat straw, bran
Penicillium occitanis	SmF, stirred vessel, 20 l	Paper pulp
Thermoascus auranticus	SSF, rotating drum, 10 l	Wheat straw
Trichoderma reesei	SmF, stirred vessel, 22 l	Steam pretreated willow
(= *Hypocrea jecorina*)	SmF, stirred vessel, 5 l	Corn steep liqour + lactose + xylose
	SSF, tray fermentor, 12,000 l	Corn cob residues
Basidiomyetes		
Phanerochaete chrysosporium	SmF, stirred vessel, 100 l	Cellulose

batch and continuous processes, either in SmF or SSF, for commercial production of cellulases (Bhat & Bhat 1997, Cen & Xia 1999, Tholudur et al. 1999, Sukumaran et al. 2005; Table 2) for applications in food and beverage industries (baking, malting, brewing, extraction processes), in animal feed and pharmaceutical industry, in the wood and textile industry (Nevalainen 1994, Watanabe et al. 2000, Galante & Formantici 2003; see Chapter 18 of this book), and potentially in ethanol production from lignocellulose (Sun & Cheng 2002, Gray 2007; see Chapter 6 of this book). According to Tolan & Foody (1999), the annual consumption of cellulases in the 1990s derived from submerged cultivation comprised an amount of 23,000 t valued $US 125 million. At the time, it represented more than 10% of the total industrial enzyme market. Nevertheless, since production costs in SSF compared to SmF are low ($US 0.2/kg versus $US 20/kg at the time), crude cellulase production by SSF can have economical advantages (Tengerdy 1996).

Production of fungal cellulases

In submerged cultures in industrial production, mainly aerobic strains of the anamorphic genera *Trichoderma*, *Humicola*, *Aspergillus*, and *Penicillium* are used with volumes of up to a few hundred liters (Tolan & Foody 1999). Usually, mixtures of different enzymes are produced by the fungi (Martin et al. 2007) - an overview of different commercially available cellulases of *Trichoderma* and *Aspergillus* is given by

Nieves et al. (1998). In SmF, mostly complex media consisting of residual materials of different carbohydrazes as C-source (examples are given in Table 2) and corn steep liquor, yeast extract or peptone as N-source are used in combination with vitamins, various salts, and other minerals (Tholudor et al. 2001, Tolan & Foddy 1999, Lynd et al. 2002, Sukumaran et al. 2005; Table 2). For efficient cellulase production, fungi typically need inducers, i.e. molecules that cause an activation of promoter sequences for transcription of the genes that are controlled by these promoters. Various types of inducers of cellulase production have been described: disaccharides (cellobiose, sophorose, gentiobiose, lactose), as well as cellulose itself and its oligosaccharides, which can be degraded into soluble sugars (Tolan & Foody 1999, Aro et al. 2005, Sukumaran et al. 2005). Of the simple sugars, in industrial production only lactose in milk whey as a by-product of the diary industry is economically expedient as soluble inducer of cellulose expression. Other comparably cheap inducers are the purified powdered cellulose Solka-Floc obtained from delignification of wood and other fibre biomass, as well as spent liquors from paper mills and sugar industries containing several soluble and/or insoluble sugars (Tolan & Foody 1999, Lynd et al. 2002). To avoid hydrolysis of the natural inducers by the produced cellulases, resistant disaccharide analogs may be used where the oxygen in the *O*-glycosidic linkage is replaced by sulphur. However, such analogs are difficult to synthesise (Suto & Tomita 2001).

Cellulosic material used in SmF at higher concentrations has dual functions, serving i. as a C-source for fungal growth, and ii. as an inducer for enzmye production. Normally in fungi, there is low level constitutive expression of cellulases which leads to a release of oligosaccharides from available cellulosic material. Subsequently, these oligosaccharides act as inducers for higher levels of cellulase expression. Thereafter, the induced and secreted cellulases degrade any accessible cellulose to oligosaccharides and glucose, until larger amounts of liberated glucose cause catabolite repression of cellulase expression (Suto & Tomita 2001). Production in continuous cultivation has the advantage to reduce the catabolite repression caused by accumulation of reducing sugars (Sukumaran et al. 2005).

Because cellulases are bulk enzymes, cheap starting materials for microbial growth are preferred. In SSF, cheap solid cellulose-containing materials from agriculture and forestry are used as carbon source (Sukumaran et al. 2005). Cen & Xia (1999) list potential raw materials ranging from straw to various fibre and timber materials together with their carbohydrate, lignin, protein and ash contents. Due to the complexity, crystalline structure, and lignin content of such substrates, a problem in SSF is the slow colonisation of the raw materials by the micro-organisms. Only some feedstock (bran from various cereals, corn cobs, wastes from the pulp and paper industry) can be used directly. More complex materials need conditioning by diluted acids, lime, pretreatments by steam explosion or hydrothermal processes in order to give easier accessible substrates (Lynd et al. 2002).

Applications of fungal cellulases are various (see above and Chapter 17 of this book) and may require specific cellulases or mixtures of enzymes of different purities and properties. The choice of substrate(s) as well as the fungal organisms influences the mixtures of cellulases that will be produced (Lynd et al. 2002). Combinations of strains of different species (e.g. *Trichoderma* and *Aspergillus* strains) may in some instances be preferable (Vyas & Vyas 2005, Wen et al. 2005). By recombinant DNA technology, it is also possible to produce individual enzymes of choice in heterologous hosts, for example in the bakers yeast *Saccharomyces cerevisiae*. Cellulase genes cloned for such purposes in *S. cerevisiae* have their origin in bacteria, fungi, and plants (Lynd et al. 2002, Saloheimo 2004). Through introduction of foreign cellulose genes, it is even possible to make the yeast growing on pure cellulose (den Haan et al. 2007).

Xylanases

One of the main hemicelluloses in plants is xylan, a linear polymer of β-1,4-linked xylose, that functions in secondary cell walls as a bond between lignin and cellulose (Timell 1967, Wong et al. 1988). Depending on its origin, different substituents can be attached to the xylan backbone [for a more detailed illustration, see de Vries & Visser (2001), Beg et al. (2001) and Chapter 17 of this book]. Two main types of xylanases can be distinguished (Polizeli et al. 2005): endo-1,4-β-xylanase (EC 3.2.1.8) and xylan 1,4-β-xylosidase (EC 3.2.1.37), both cleaving the β-1,4-glycosidic bonds in xylan. Endo-1,4-β-xylanase thereby decreases the polymerisation level of the xylan backbone whereas xylan 1,4-β-xylosidase acts on smaller xylooligosaccharides and xylobiose to give β-D-xylopyranosyl residues. Other xylanolytic enzymes are acetylesterase (EC 3.1.1.6), different types of arabinase (α-*N*-arabinofuranosidase, EC 3.2.1.55; arabinan endo-1,5-α-L-arabinosidase, EC 3.2.1.99), xylan α-1,2-glucuronidase (EC 3.2.1.131) and feruloyl esterase (EC 3.1.1.73) (Beg et al. 2001, Polizeli et al. 2005; see also Chapter 17 of this book). Xylanases have a range of industrial applications (removing hemicellulosic residues in animal feed, improving dough quality in bakery, improving juice extraction from fruits and vegetables in the food industry) but are mainly used for bleaching in the pulp and paper industry (Polizeli et al. 2005; see Chapter 17 of this book). They are assumed to cleave bonds between lignin and cellulose (Paice et al. 1992, reviewed by Uffen 1997, Polizeli et al. 2005) that might help to protect against cellulolytic degradation and hinders the exposure of lignin to bleaching compounds (Viikari et al. 1994, Beg et al. 2001; see Fig. 4 in Chapter 21 of this book).

Xylanases are predominantly produced by micro-organisms (Table 3), but are also found in marine algae, protozoans, crustaceans, insects, snails, and seeds of land plants (Sunna & Antranikian 1997). As in the case of cellulases, microbial production of xylanases can take place in SmF and SSF (see Table 3), although about 80-90% of the commercial xylanases are gained through submerged fermen-

Table 3 Examples of xylanase production by micro-organisms in batch processes

Organism	Production conditions (process, substrate)	Maximum yield	Reference
Bacteria			
Bacillus circulans	SmF, sugarcane bagasse hydrolysate	8.4 U/ml	Bocchini et al. 2005
Bacillus sp.	SSF, wheat bran	720 U/g dry substrate	Gessesse & Mamo 1999
Streptomyces sp.	SSF, wheat bran	2360 U/g dry substrate	Beg et al. 2000
	SSF, *Eucalyptus* kraft pulp	1200 U/g dry substrate	
Ascomycetes and related anamorphic genera			
Aspergillus amowari	SmF, wheat bran	28.3 U/ml	Li et al. 2006
Aspergillus foetidus	SmF, oat spelt, xylan	322 U/ml	Chipeta et al. 2005
Aspergillus niger	SSF, rice straw	5070 U/g dry substrate	Kang et al. 2004
Aspergillus phoenicis	SmF, spent sulphite liquor	173 U/ml	Chipeta et al. 2005
Melanocarpus albomyces	SmF, wheat straw	172 U/ml	Saraswat & Bisaria 1997
	SSF, wheat straw	7760 U/g dry substrate	Narang et al. 2001
Paecilomyces thermophila	SSF, wheat straw	18,500 U/g dry substrate	Yang et al. 2006
Penicillium oxalicum	SmF, wheat bran	16.1 U/ml	Li et al. 2007
Trichoderma harzianum (= *Hyprocrea lixii*)	SmF, birchwood xylan	44.9 U/mg protein	Seyis & Aksoz 2005
	SSF, sugarcane bagasse	288 U/ml	Rezende et al. 2002
Trichoderma longibrachiatum	SmF, Solka-Floc	272 U/ml	Royer & Nakas 1989
	SSF, wheat bran	5.01 U/g dry substrate	Kovacs et al. 2004
Trichoderma reesei (= *Hypocrea jecorina*)	SmF, beech xylan	9 U/ml	Bailey et al. 1993
	SSF, rice straw	122 U/ml	Colina et al. 2004
Basidiomycetes			
Schizophyllum commune	SMF, microcrystalline cellulose (Avicell)	4839 U/ml	Haltrich et al. 1993
	SSF/SmF, spruce sawdust	0.37 U/ml	Paice et al. 1978
Mixed cultures			
A. phoenicis and *T. reesei*	SSF, bagasse	714 U/g dry substrate	Dueñas et al. 1995
A. niger and *T. reesei*	SSF, soymeal	2800 U/g dry substrate	Gutierrez-Correa & Tengerdy 1998

tation, usually with filamentous ascomycetes and their related anamorphic genera (Polizeli et al. 2005, Chávez et al. 2006; Table 3). Species are often the same than those used in cellulase production (compare examples in Tables 2 and 3). Fungi tend to produce mixtures of xylan-degrading enzymes, different xylanases combined with accessory xylanolytic enzymes for debranching of substituted xylans (Haltrich et al. 1996, Chávez et al. 2006) that without further downstream processing might directly be applied in biobleaching of paper pulp (Szendefy et al. 2006). Important for this is that the enzymes are as much as possible cellulase-free (Christov et al. 1999). Also for applications in the textile industry, presence of cellulases is unwanted in xylanase preparations in order not to damage the celluose (Kulkarni et al. 1999, Polizeli et al. 2005). Production conditions, in particular media components, can favour which types of enzymes are obtained from the fungi and help to avoid or reduce impurities with unwanted enzymes (Haltrich et al. 1996).

Production of fungal xylanases

Bulk substrates used for SmF and SSF are comparable to the ones used for cellulase production (Tables 2 and 3), except that in most instances cellulose is replaced by xylan and its sugar xylose in order to induce production of the required xylanases and to avoid unwanted induction of cellulase production. Various derivatives of xylan (xylose, xylobiose, xylooligosaccharides, etc.) are possible inducers of xylanases although effects on xylanase biosynthesis vary with the species used for production (Kulkarni et al. 1999). In natural production of xylanase, isolated xylan from different lignocellulosic materials (beechwood, birchwood, larchwood, oat spelt) and other agricultural and forestry residues (e.g. beet pulp, corn cobs, wheat/rice bran, straw) are often used as inducing substrate. In SmF, the concentration of the xylan substrate can be as high as 75 g/l (Haltrich et al. 1996). Measures to optimise natural xylanase production and scale up the processes are principally the same as in cellulase production. Natural production of xylanases can be improved by altering culturing conditions such as concentrations of media components (carbon, nitrogen, salts, etc.), temperature, pH, aeration, and others. Also for establishing large scale xylanase productions, modern experimental design with statistical methodologies are used to calculate best process parameters (Katapodis et al. 2006, Li et al. 2006, 2007, Azin et al. 2007). In addition, mutants with better xylanase yields are screened for (Smith & Wood 1991, Park et al. 2002, Hao et al. 2006).

In cases where pure enzymes are required and where by culture conditions it is not possible to produce pure xylanases with a filamentous fungus without cellulase contamination, fungal xylanase genes are expressed heterogously in *Escherichia coli* and, more often, in yeasts such as *Pichia pastoris* and *S. cerevisiae* (Hahn-Hägerdal et al. 2005, Jeffries 2006, Korona et al. 2006, Berrin et al. 2007, Chen et al. 2007, Tung et al. 2007). Expression in organisms well established for industrial heterologous protein production has the further advantage that fermentation conditions

are easily adapted to xylanase production (Damaso et al. 2006). However, it is also possible to enhance enzyme yields in homologous and heterologous filamentous fungi by replacing the natural xylan-inducible promoter by more efficiently working promoters underlying different regulation schemes (de Faria et al. 2002, Rose & van Zyl 2002, Levasseur et al. 2005). Recombinant protein production has another positive consequence for applications: site-directed mutagenesis and directed evolution are used in molecular gene engineering to optimise the enzyme properties (see for examples on xylanases see Xion et al. 2004, Fenel et al. 2006, Sriprang et al. 2006, Stephens et al. 2007).

Laccases

Laccases (EC 1.10.3.2) are oxidoreductases capable of oxidising phenols as well as aromatic amines (Leonowicz et al. 2001; see Chapter 17 of this book). Because of their wide substrate range, these enzymes are attractive for various biotechnological applications, for example in the food industry, the textile industry, the pulp and paper industry, in wood composite production, in soil bioremediation, and others (Hüttermann et al. 2001, Mai et al. 2004, Husain 2006, Kilaru 2006, Rodríguez Couto & Toca Herrera 2006; see also Chapters 17 and 18 of this book).

Laccases have four copper atoms as cofactors located at the catalytic centre of the protein and they belong therefore to the group of multicopper oxidases. In nature, these enzymes are produced by many fungi and plants, but also by a few bacteria and insects (Mayer & Staples 2002, Claus 2003, 2004, Baldrian 2006, Hoegger et al. 2006). Besides laccase from the lacquer tree *Rhus vernificera* (Johnson et al. 2003), commercially available laccases are usually of fungal origin (see Table 4), most likely from white-rot and saprophytic basidiomycetes such as *Trametes*, *Pleurotus* and *Agaricus* species that secrete high amounts of these enzymes (Minussi

Table 4 Examples of natural production of laccases by fungi

Organism	Production conditions* (process, reactor, volume)	Yield	Reference
Ascomycetes			
Botryosphaeria sp.	SmF, flask, 125 ml	5.4 U/ml	Vasconcelos 2000
Botrytis cinerea	SmF, stirred vessel, 10 l	28 U/ml	Fortina et al. 1996
Coniothyrium minitans	SmF, stirred vessel, 20 l	55.2 U/ml**	Dahiya et al. 1998
Monotospora sp.	SmF, flask, 250 ml	13.55 U/ml	Wang, J.W. et al. 2006

* Batch cultivation unless otherwise noted

** For better comparison, enzyme activities given in the original literature in nkat units were transformed into international units (IU) through division by a factor of 16.67, and activities given in arbitrary units (AU) by a factor of 36, respectively (see Kilaru 2006 for further explanations)

Table 4 continued

Organism	Production conditions* (process, reactor, volume)	Yield	Reference
Basidiomycetes			
Fomes sclerodermeus	SmF, flask, 2 l	20.29 U/ml	Papinutti & Martínez 2006
Funalia trogii	SmF, flask, 250 ml	4.9 U/ml	Kahraman & Gurdal 2002
Grifola frondosa	SSF, bottles, 850 ml	6 U/ml	Xing et al. 2006
Lentinus tigrinus	SSF, flask, ?	30 U/g dry substrate	Lechner & Papinutti 2006
Nematoloma frowardii	SSF, flask, ?	0.5 U/ml	Hofrichter et al. 1999
Panus tigrinus	SmF, flask, 200 ml	0.05 U/ml**	Chernykh et al. 2005
	SmF, bubble column, 3 l	4.3 U/ml	Fenice et al. 2003
	SmF, stirred vessel, 3 l	4.6 U/ml	
	SSF, rotating drum, 20 l	1.3 U/ml	
Phlebia floridensis	SmF, flask, 100 ml	10.4 U/ml	Arora & Gill 2005
Pleurotus ostreatus	SmF, flask, 250 ml	22.3 U/mg protein**	Prasad et al. 2005b
Pleurotus pulmonarius	SSF, flask, 250 ml	24.4 U/g	de Souza et al. 2006
Pycnoporus cinnabarinus	SmF, flask, 250 ml	266.6 U/ml	Lomascolo 2003
	SmF, flask, 250 ml	29 U/ml	Herpoël et al. 2000
Pycnoporus sanguineus	SSF, flask, ?	46.5 U/g dry substrate	Vikineswary 2006
Trametes gallica	SmF, flask, 250 ml	8.6 U/ml	Dong et al. 2005
Trametes hirsuta	SSF, immersion reactor***, 500 ml	2.2 U/ml	Rodríguez Couto et al. 2004b
	SSF, flask, 250 ml	5.4 U/ml**	Rosales et al. 2005
	SmF, stirred vessel, 10 l batch + fed-batch	80.7 U/ml + 83.8 U/ml	Koroleva et al. 2002
Trametes modesta	SmF, flask, 300 ml	10.7 U/ml**	Nyanhongo et al. 2002
Trametes pubescens	SmF, stirred vessel, 20 l batch + fed-batch	330 U/ml + 743 U/ml	Galhaup et al. 2002
Trametes sp.	SmF, flask, 300 ml	20.0 U/ml	Jang et al. 2002
Trametes trogii	SmF, flask, 250 ml	22.75 U/ml	Trupkin et al. 2003
Trametes versicolor	SmF, stirred vessel, 100 l	70 U/hl	Fåhraeus & Reinhammar 1967
	SmF, flask, 100 ml	5.2 U/ml	Mikiashvili et al. 2005
	SmF, bubble column, 2 l	4 U/ml	Domínguez et al. 2007
	SSF, tray, 17 l	3.5 U/ml	Rodríguez Couto et al. 2003
Ganoderma sp.	SmF, flask, 250 ml	692 U/ml	Revankar & Lele 2006

*** Immersion reactor: in such systems, micro-organisms are grown on an inert matrix or a solid substrate which are periodically immerged into culture medium (Rodríguez Couto et al. 2002b)

et al. 2002, Xu 2005). Usually, commercial laccases come from SmF fermentation (Cherry & Fidantsef 2003; Table 4).

Next to various basidiomycetes, many ascomycetes are also producers of interesting laccases (Baldrian 2006, Kilaru 2006; Table 4). Although laccases usually have a wide substrate spectrum, enzymes can differ in the range of substances they attack (Kilaru 2006, Mander et al. 2006). In consequence, enzymes may be differentially suitable for specific tasks in biotechnology. Most enzymes are (best) active in the lower pH range (pH 2-4; Baldrian 2006, Kilaru 2006) but applications may ask for reaction optima at higher pHs in the neutral or slightly alkaline range. Other catalytic properties (e.g. a high redox potential, temperature optimum) and protein stabilities play also a role in applications. In the literature, over 100 laccases have been described in more or less detail at the enzymatic level. Nevertheless, screening for new enzymes is still intensively going on in order to find enzymes better adapted to the respective biotechnological problems (Baldrian 2006, Kilaru 2006).

Production of native laccases in pure culture

The most studied genus concerning laccase production is probably *Trametes* covering numerous of the strong white-rot fungi. Several *Trametes* species have been tested for their ability to produce laccases, either in submerged or in solid state production. Although production by SmF is best established, results show that SSF also has its potential in efficient laccase production (Tables 4 and 5). As with the other enzymes, an important factor in production is the cultivation medium. Both, the sources of carbon and nitrogen as well as relative amounts of the nutrients play a role. In *T. versicolor*, cellobiose and mannitol gave better results than glucose (Mikiashvili et al. 2005) and carbon limitation (glucose depletion) acts stimulating in laccase production (Tavares et al. 2005). However, for best results

Table 5 Laccase production by *Trametes* species grown in SSF

Organism	Reactor type	Substrate/ matrix	Yield [U/ml]	Reference
Trametes hirsuta	Tray	Grape seeds	18.0*	Rodríguez Couto et al. 2006
		Nylon sponge	6.0*	
	Immersion reactor	Grape seeds	12.0*	
Trametes versicolor	Immersion reactor	Barley bran with glucose medium	0.6	Rodríguez Couto et al. 2002a
	Column reactor		0.6	
	Tray		3.5	
	Bubble column	Glucose medium	1.7	Rancaño et al. 2003

* For better comparison, nkat units were transformed into international units (IU) through division by a factor of 16.67 (see Kilaru 2006)

sugar concentrations have to be adjusted to reach a balance between fungal biomass and enzyme production (Ryan et al. 2007). In continuous cultivation, this can be achieved by constant feeding of low glucose amounts (Galhaup et al. 2002).

Plant waste material (e.g. mandarine peels, groundnut shells) and organic nitrogen sources (corn steep liquor, peptone, casein hydrolysate) perform well in cultivation with *Trametes* and also *Pleurotus* species (Dong et al. 2005, Mikiashvili et al. 2005, 2006). Nitrogen supplementation in SSF of selected agro- and forestry-wastes (saw dust, oil palm frond, wheat straw, beech tree leaves) can very much raise enzyme yields of various white-rot fungi (D´Souza et al. 1999, Kachlishvili et al. 2006, Vikineswary et al. 2006). Other studies report N-limitation to be better for laccase production in *P. ostreatus* (Hou et al. 2004) and *Pycnoporus cinnabarinus* (Eggert et al. 1996). On a superficial view, results may appear to be contradictory (Galhaup et al. 2002). Effects of nitrogen addition depend however on both strain and substrate (Kachlishvili et al. 2006). Other factors also play a role. Static cultures of *P. ostreatus* and *Trametes gallica* were reported to be superior to agitated cultures (Hou et al. 2004, Dong et al. 2005), whilst in *Irpex lacteus* no differences were seen (Kasinath et al. 2003, Shin 2004), and in *Funilia troggi* an agitated culture outperformed a static one (Birhanli & Yesilada 2006). Also culture volumes have an influence. Transfer of established conditions from laboratory to larger scales of submerged culture therefore remains a problem. Fungal morphology is likely to be different in bioreactors of increased size or different shape due to altered aeration conditions (being of special importance in fermentations where production and application of the oxygen-dependent enzymes occur simultaneously such as in waste water treatment) and due to the shearing forces applied in the different reactor types (Hess et al. 2002, Bermek et al. 2004, Ryan et al. 2005). Immobilisation of fungi on solid matrices is a very active research area to overcome problems with up-scaling in production of laccases by the various white-rot fungi. Several solid matrices have successfully been tested as support for laccase production by SSF – partially in combined procedures targeting at degradation of toxic and recalcitrant compounds in liquids: stainless steel sponge, nylon sponge, polyurethane foam and alginate beads (Rodríguez Couto et al. 1997, 2004a,b, Prasad et al. 2005a, Domínguez et al. 2007; see Table 5 for examples) and several agricultural residues, like kiwi fruit wastes (Rosales et al. 2005), barley bran (Rodríguez Couto et al. 2002a), grape seeds and stalks (Lorenzo et al. 2002, Moldes et al. 2004), and corn cab (Tychanowicz et al. 2006). Optimisation in growth and laccase production in SmF cultures is nowadays addressed by experimental design technologies considering variable parameters like nutrient concentration, inducers, agitation, pH and inoculum (Prasad et al. 2005b, Tavares et al. 2006). The pH has been shown to influence laccase yields e.g. in *Botrytis cinerea* - at pH 3.5, 20% more laccase was produced than at pH 5.0 (Fortina et al. 1996). In addition, the temperature of cultivation can have an influence (Forina et al. 1996, Koroleva et al. 2002,

Nyanhongo et al. 2002, Wang, J.W. et al. 2006). For example, in glucose-based medium, an increase from 28°C to 37°C resulted in a decrease of laccase yield in cultivation with *Trametes* sp., whereas in cellobiose medium the laccase activity in the supernatant increased more than two times with such temperature shift (Tong et al. 2007).

As with other enzymes acting in lignocellulose degradation, small amounts of extracellular laccases are often constitutively secreted (Bollag & Leonowicz 1984, Koroljova-Skorogbogat´ko et al. 1998, Scheel et al. 2000, Da Cunha et al. 2003, Zhang, M. et al. 2006). Higher yields however require induction of expression. Several inducers have been applied to increase yields of laccase: copper, phenols, 2,5-xylidine and related compounds in *T. versicolor* (Collins & Dobson 1997, Tavares et al. 2005, Kollmann et al. 2005, Domínguez et al. 2007, Ryan et al. 2007), ethanol in *P. cinnabarinus* (Lomascolo et al. 2003, Meza et al. 2006, 2007), aromatic compounds including veratryl alcohol in *Botryosphoraeria* sp. (Vasconcelos et al. 2000, Dekker & Barbosa 2001), copper and phenolic compounds in *Panus tigrinus* (Chernykh et al. 2005), copper, cotton stalk extract, dimethyl sulphoxide, and the synthetic substrate ABTS [2,2'-azino-di-(3-ethylbenzothialozin-6-sulphonic acid)] in *P. ostreatus* (Ardon et al. 1996, Palmieri et al. 2000, Hou et al. 2004, Shah et al. 2006), and copper and lignin-related compounds in *Pleurotus pulmonarius* (Souza et al. 2004, Tychanowicz et al. 2006). Complex substrates such as sugar cane bagasse and lignocellulosic material such as wood and peanut shells have also been shown to be effective in various fungi (D'Souza et al. 1999, Arora & Gill 2001, Linke et al. 2005, Makela et al. 2006). Many fungal strains produce a range of laccase isoenzymes, being either variations of differentially glycosylated laccases from the same gene or enzymes from different genes (for examples see Palmieri et al. 1997, 2000, 2003, Dong et al. 2005, D´Souza-Ticlo et al. 2006, Linke et al. 2005, Lorenzo et al. 2006; see Chapter 17 of this book). Induction can be selective on the different laccase genes as shown for example for *Trametes* sp. I-62 and *Pleurotus sajor-caju* (Soden & Dobson 2001, Terrón et al. 2004).

Inducers of laccase production might be toxic (copper, 2,5-xylidine) and/or expensive (ABTS). Laccase has been produced with *Trametes villosa* and *T. versicolor* strains in the 500 l stirred vessel shown in Fig. 3A using 0.6 mM 2,5-xylidine as an inducer in an artificial medium I (per l: 10 g glucose, 0.5 g yeast extract, 2.5 g L-asparagine, 0.15 g DL-phenylalane, salts; Shekholeslani 1991) developed for high laccase production. However, the toxic 2,5-xylidine can be replaced by kraft lignin (Indulin AT, Westvaco, Raleigh, USA). Yields of laccase obtained with medium I with the *Trametes* strains are in the range of 12 U/ml (Shekholeslami 1991; M. Euring & A. Kharazipour, unpublished). The laccase has been used in the production of MDF (medium density fibreboards) and particle boards in laboratory and technical scale at concentrations of 20 U/g raw material (dry process) and 544 U/g (wet process) (Kharazipour 1996, Kharazipour et al. 1997; further reading in Chapter 18 of this book).

Laccase production in mixed cultures

Interactions between different fungi as well as between fungi and certain prokaryotes can have an inducing effect on laccase activity, often in strain-specific manners (Iakovlev & Stenlid 2000, Baldrian 2004, Ferreira Gregorio et al. 2006, Chi et al. 2007, Kleeman 2007; Fig. 4). Such effects of co-cultivation might help individual species and microbial communities in lignin degradation but also in competition and defence of their habitats (Boddy 2000, Iakovlev & Stenlid 2000). Several white-rot and litter-degrading basidiomycetes showed higher laccase activity in cultivation with another basidiomycete species on plates and in liquid medium (Iakovlev & Stenlid 2000, Baldrian 2004, Baldrian & Šnajdr 2006, Ferreira Gregorio et al. 2006). Increases in laccase production up to 25fold have been described (Baldrian 2004). Likewise, *Trichoderma* species can stimulate laccase production of white-rot basidiomycetes in submerged and solid state cultures (Freitag & Morrell 1992, Savoie & Mata 1999, Hatvani et al. 2002, Velázquez-Cedeño et al. 2004, Zhang, H. et al. 2006). Stimulating laccase production between white-rot fungi might be useful in biopulping of wood (Wang, H.L. et al. 2006, Chi et al. 2007) and wastewater treatment (García-Mena et al. 2005). Laccase produced to larger

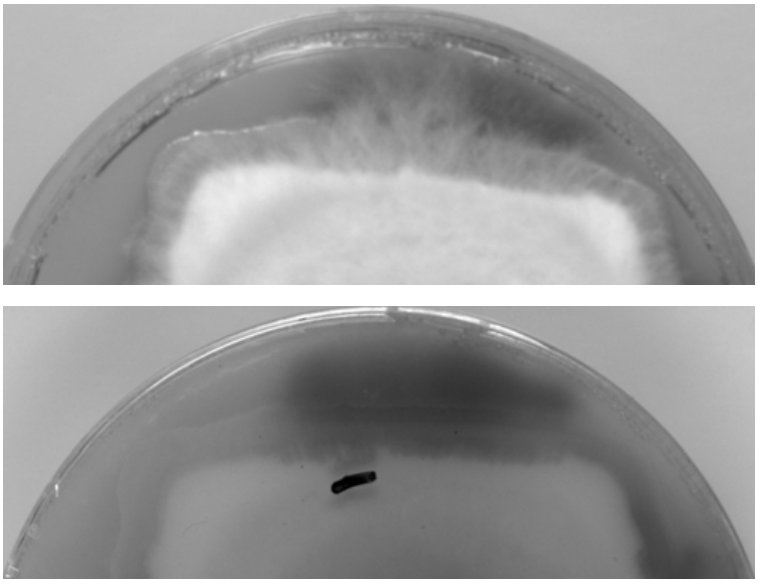

Fig. 4 Co-cultivation of *Coprinopsis cinerea* strain AmutBmut with an unknown bacterium on agar in a Petri-dish (shown from above to demonstrate the growth of the microorganisms and from below to document laccase activity by agar staining). The bacterium was streaked around the fungal mycelium (black mark = growth front at the time of bacterial inoculation) that in consequence was retarded in growth. Where the fungus managed to overcome the bacterial barrier, laccase has been produced. Laccase activity is seen by the staining of the agar through oxidation of the colourless substance ABTS converting it into a green-brown-coloured compound

amounts by mixtures of aquatic hyphomycetes can be a possible alternative in wastewater treatment (Junghanns et al. 2005). Whether co-cultivation of two species in SmF or SSF in bioreactors for the pure target of enzyme production and harvest is feasible, remains to be tested. General process management can be expected to be more complex under such circumstances.

Fungal laccase genes and their recombinant expression

Natural fungal strains can have several different laccase genes. Five non-allelic genes are currently known for the white-rots *T. villosa* and *P. sajor-caju*, four non-allelic genes in *P. ostreatus* and *T. versicolor*, three in *Lentinula edodes*, *P. cinnabarinus* and *Trametes* sp. I-62, and, in addition, three in the plant pathogen *Rhizoctonia solani* (Necochea et al. 2005, Hoegger et al. 2006). For very few of these, protein properties have been described (Table 6) - a reason for this might be the difficulty to separate the different isoenzymes coming from these genes, another that not enough enzyme is naturally produced from the genes by their hosts. Further to this, the saprophytic dung fungus *Coprinopsis cinerea* (formerly *Coprinus cinereus*) has in total seventeen different laccase genes (Kilaru et al. 2006a). In a standard nutrient-rich growth medium based on yeast- and malt-extract (Granado et al. 1997), no or only neglectable enzyme activity was encountered in *C. cinerea* cultures and only little, when copper was added (Fig. 5). 2,5-xylidine and veratryl alcohol were not effective as inducers of laccase production in this fungus (M. Navarro-Gonzá-

Table 6 Properties of laccases from known genes of white rot fungi (after Hoegger et al. 2006 and Kilaru 2006)

Organism	Laccase gene	pI	Optimal pH*				Opt. temperature [°C]
			ABTS	SGZ	DMP	Guaiacol	
Ceriporiopsis subvermispora	*lcs1*	3.6	-	-	-	-	-
Pleurotus	*pox2*	3.3	3.0	6.0	3.5	-	50-60
ostreatus	*poxa3*	4.1	3.6	6.2	5.5	-	35
Pycnoporus	*lac1*	<3.5	-	-	-	-	-
cinnabarinus	*lcc3-1*	3.7	-	-	-	4.0	-
Trametes pubescens	*lap2*	2.6	3.0	4.5	3.0	-	50-60
Trametes villosa	*lcc1*	3.5	≤ 2.7	5.0-5.5	-	-	-
	lcc2	6.2-6.8	6.0	5.0-5.5	-	-	-
Trametes sp. AH28-2	*lacA*	4.2	-	-	-	4.5	50
Trametes sp. 30	*lac1*	3.6	-	4.5-5.0	-	-	55

* substrates: ABTS = 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid), DMP = 2,6-dimethoxyphenol, SGZ = syringaldazine; -: not known

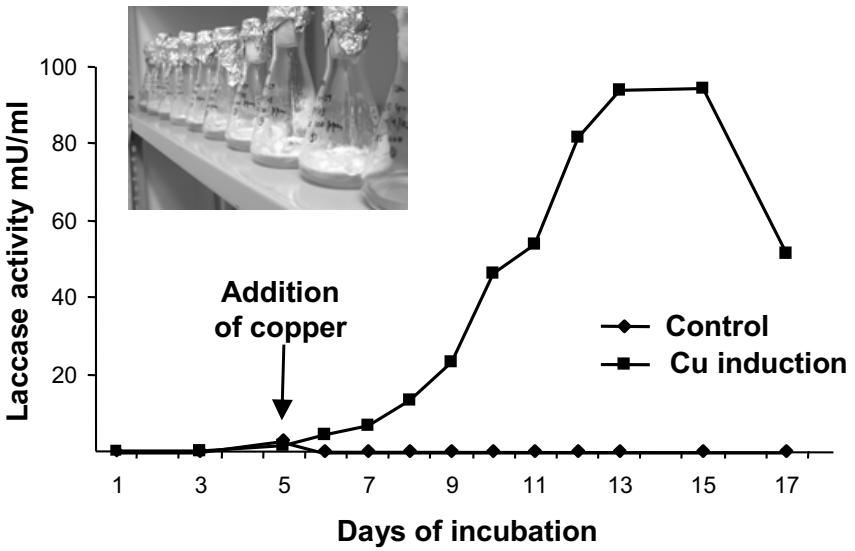

Fig. 5 Laccase production in 100 ml standing cultures of *Coprinopsis cinerea* strain AmutBmut at 37°C in yeast- and malt-extract medium with and without supplementation of 0.1 mM $CuSO_4$. Laccase activity was measured as described (Kilaru et al. 2006b)

lez, unpublished), although laccase production on protein-rich soybean-based medium has been reported (Yaver et al. 1999). Yields however were not high enough to purify and characterise the naturally produced enzyme Lcc1 beyond determining its molecular size (Schneider et al. 1999).

In order to obtain higher yields of individual laccases from basidiomycetes, several attempts have been made in the past to express selected laccase genes in ascomycetous yeasts (*Kluyveromyces lactis*, *Pichia methanolica*, *P. pastoris*, *S. cerevisiae*, *Yarrowia lipolytica*) and in filamentous ascomycetes and anamorphs (*Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *T. reseei*), species that are well established in industrial fermentation (Table 7; for a complete literature overview see Kilaru 2006). However, these attempts were usually little successful since either no or only low enzymatic activity was obtained, stabilities of enzymes were low and sometimes also enzymatic properties altered (Berka et al. 1997, Sigoillot et al. 2004, Bulter et al. 2003, Piscitelli et al. 2005). First of all, the choice of the gene had an influence on the outcome of the experiments (Piscitelli et al. 2005, Kilaru 2006, S. Kilaru et al. unpublished). Secondly, expressed proteins often had altered glycosylation patterns (Otterbein et al. 2000, Madzak et al. 2005, Piscitelli et al. 2005), a post-translational protein modification that influences the protein properties. Glycosylation modes differ between different classes of eukaryotes (Brooks 2006). Obviously, there are also fundamental differences between asco- and basidiomycetes. Newer efforts in recombinant laccase production therefore concentrate on the basidiomycetes themselves (Alves et al. 2004, Kajita et al. 2004, Kilaru et al. 2006b). In *P. cinnabarinus*, yields as high as 280 U/ml were obtained from re-

combinant enzyme production under specific culture conditions, which is nearly as high as the best natural production rate by a basidiomycete (Alves et al. 2004; compare Tables 4 and 7). In *C. cinerea*, enzyme yields of 3 U/ml in recombinant production of Lcc1 have been published (Kilaru et al. 2006b) – altering culturing conditions improved this in the meantime by a factor of 4 (Kilaru 2006, M. Rühl, unpublished; Table 7) into satisfactory levels (compare also Table 4). Currently it is unclear whether *P. cinnabarinus* is more efficient in overall laccase production than *C. cinerea*. Homologous genes were used in the expression studies with *P. cinnabarinus* (*lac1*) and *C. cinerea* (*lcc1*) and the different proteins could account for differences in yields (see below). The promoters used are possibly of higher influence – in *P. cinnabarinus* with two promoters from *Schizophyllum commune*, addition of ethanol increased laccase production by factors of more than 30. Without induction, yields of only 8-10 U/ml were achieved (Alves et al. 2004). A most recent study showed dual effects of ethanol on laccase production in *P. cinnabarinus*: increase of laccase gene transcription and inhibition of protease activities (Meza et al. 2007). Addition of ethanol to cultures of *C. cinerea* transformants unfortunately had no effect on laccase yields (Kilaru et al. 2006b).

Table 7 Recombinant expression of laccases in fungi

Laccase gene	Expression host	Yield	Reference
lcs-1 of *Ceriporiopsis subvermispora*	*Aspergillus nidulans*	0.23 U/ml	Larrondo et al. 2003
lcc1 of *Coprinopsis cincerea*	*Aspergillus oryzae*	135 mg/l	Yaver et al. 1999
	C. cinerea	3-12 U/ml	Kilaru 2006, Kilaru et al. 2006b, M. Rühl unpublished
lcc5 of *C. cinerea*	*C. cinerea*	10-30 U/ml	Kilaru 2006, M. Rühl unpublished
lac1 of *Melanocarpus albomyces*	*Trichoderma reesei*	15 U/ml*	Kiiskinen et al. 2004
MtL of *Myceliophthora thermophila*	*A. oryzae*	-	Xu et al. 1998
lac1 of Pycnoporus cinnabarinus	*Pycnoporus cinnabarinus*	280 U/ml*	Alves et al. 2004
RsL of *Rhizoctonia solani*	*A. oryzae*	-	Xu et al. 1998
lcc1 of *Trametes versicolor*	*Pichia methanolica*	12.6 U/ml	Guo et al. 2005
	Pichia pastoris	140 U/ml	Hong et al. 2002
laccase III of T. versicolor	*T. versicolor*	-	Kajita et al. 2004
lacA of *Trametes* sp.	*P. pastoris*	8.3 U/ml	Hong et al. 2006

* nkat units were divided by a factor of 16.67 into IU (see Kilaru 2006)

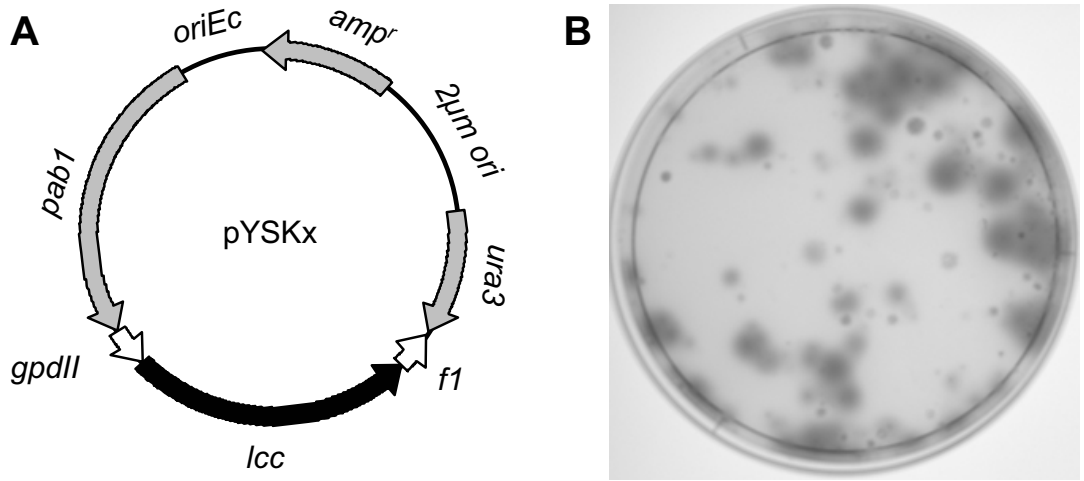

Fig. 6. Transformation of laccase genes in *Coprinopsis cinerea*. **A.** Transformation vector of the series pYSKx with a basidiomycete laccase gene *lcc* under control of the heterologous *Agaricus bisporus gpdII* promoter. *pab1* encodes *para*-aminobenzoic acid synthase and acts as a marker gene for selection in *C. cinerea pab1* auxotrophs. *oriEc* is the start point (origin) of DNA replication in *Escherichia coli*, *amp*^{r} a selection marker gene that mediates ampicillin resistance in *E. coli*, and *f1* an *E.coli* phage replication origin useful for single-strand DNA synthesis. 2µm ori is a replication origin for the yeast *Saccharomyces cerevisiae* and *ura3* a selection marker gene for orotidine 5'-phosphate decarboxylase that complements certain uracil auxtrophs of yeasts. Bacterial and yeast sequences in this vector are required for gene cloning purposes (further details in Kilaru et al. 2006b,c). **B.** Once a vector with a subcloned laccase gene (here pYSK7 with gene *lcc1* behind *gpdII* promoter) has been transformed into *C. cinerea*, positive transformants secrete functional laccase into the medium about 4 to 5 days later. ABTS added to the medium will be converted into a greenish stain, marking the positive clones by large halos that form around the colonies (larger dark spots in the photo). Note in the photo, transformed clones are just starting to grow. Transformants failing to produce laccase are seen only as small spots of pin-head size without halo

In contrast to *P. cinnabarinus* (Alves et al. 2004), *C. cinerea* is a readily transformable basidiomycete (Binninger et al. 1987, Granado et al. 1997) and therefore represents by this property an excellent choice as a host for overexpression of individual laccases, both from own genes as well as from genes of other species (Kilaru et al. 2006b,c, Grimrath 2007). Screening for suitable transformants is fast (Fig. 6) and the large numbers of transformants obtained from a single transformation experiment eases to find the best producers (Kilaru et al. 2006b). In the future, this should allow to easily analyse tailored enzymes with optimised properties produced either by targeted or by random mutagenesis. Currently, such mutagenesis screening is performed in the suboptimal host *S. cerevisiae* (Bulter et al. 2003, Alcalde et al. 2005, 2006, Madzak et al. 2006).

The *C. cinerea* laccase expression system

C. cinerea strain FA2222 is a strain with no laccase activity (Kilaru et al. 2006b) and has therefore been chosen as a production strain of individual enzymes. For efficient protein production, different promoters were tested in combination with *C. cinerea* laccase gene *lcc1* (Fig. 6A). The heterologous *Agaricus bisporus gpdII* promoter from a constitutively expressed glyceraldehyde 3-phosphate dehydrogenase gene (Kilaru & Kües 2005) was found to give highest enzyme activity (Kilaru et al. 2006b; Fig. 6). Transformed DNA integrates randomly into the genome (Granado et al. 1997). In consequence, transformants differ in quality since flanking DNA sequences can influence expression levels of inserted genes. Screening of a higher number of transformants is thus advisable. However, the coloured halos formed around colonies on ABTS-supplemented agar when expressing laccase (Fig. 6B) is an excellent pre-selection for high producers (Kilaru et al. 2006b). As expected, glycosylation of recombinantly produced protein Lcc1 was indistinguishable from the protein when produced in *C. cinerea* from its natural promoter (P.J. Hoegger unpublished).

Also the other 16 laccase genes of *C. cinerea* were transformed under the control of the *A. bisporus gpdII* promoter and at least five of these can give an active protein in *C. cinerea* but at different yields (Kilaru 2006). As far as yet known from protein analysis, the *C. cinerea* laccases differ in substrate spectrum and other enzymatic properties – enzymes with a pH optimum around pH 7.0 are of special interest (Saathoff 2005, Kilaru 2006, M. Rühl et al. unpublished). Ongoing work has proven that also laccase genes from foreign species can be expressed in *C. cinerea* (Grimrath 2007, P.J. Hoegger et al. unpublished). Since principally working, a research focus now aims on establishing best production conditions for transformants in fermenters (M. Rühl, unpublished).

Conclusions

More and more, fungal enzymes find applications in environment-friendly technologies in modern pulp and paper and wood industries as well as in ethanol production from lignocellulosic residues (Hüttermann et al. 2001, Kenealy & Jeffries 2003, Tengerdy & Szakacs 2003, Mai et al. 2004, Gray 2007, Jeffries 2006; see also Chapter 18 of this book). To make such processes economical, high quality enzymes in sufficient amounts at reasonable prices are required. Cellulases, xylanases, laccases, and other enzymes from different organisms and with different properties are commercially available, in some instances purified, more often only partially purified or crude and, therefore, they are possibly mixed with other enzymes and proteins. Often, the non-purified enzymes are sufficient for applications, as for example in wood composite production and in pulp bleaching. Active research in enzyme screening, optimising fermentation (SmF and SSF) and recombinant production is nevertheless ongoing to provide larger catalogues of easily produced enzymes with new and optimal properties as required for the various

industrial applications. Currently, lignocellulose attacking enzymes are produced predominantly by SmF, often with wildtype or mutant strains of natural enzyme producers, sometimes with recombinant strains. SSF processes, well established in Eastern cultures, now also receive higher attention in Western countries. SSF can be a particular good choice for enzyme production with fungi growing on solid lignocellulosic waste. Other promising yet not fully implemented approaches are those where different organisms are coupled in production and approaches where enzyme production and utilisation is combined. Establishing well-working recombinant production systems has the advantage that enzymes naturally produced in low amounts should become available at larger quantities. Moreover for recombinant production, enzyme properties might be optimised through genetic engineering. Using one or a few organisms in recombinant productions should furthermore eliminate the elaborative work to establish for every new natural producer and each of its interesting enzymes an own fermentation process.

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Literature

Adrio, J.L. & Demain, A.L. (2006). Genetic improvement of processes yielding microbial products. FEMS Microbiology Reviews, 30, 187-214.

Aguilar, C.N., Contreras-Esquivel, J.C., Rodríguez, R., Prado, L.A. & Octavio, L. (2004). Differences in fungal enzyme productivity in submerged and solid state cultures. Food Science and Biotechnology, 13, 109-113.

Alcalde, M., Bulter, T., Zumárraga, M., García-Arellano, H., Mencía, M., Plou, F.J. & Ballesteros, A. (2005). Screening mutant libraries of fungal laccases in the presence of organic solvents. Journal of Biomolecular Screening, 10, 624-631.

Alcalde, M., Zumárraga, M., Polaina, J., Ballesteros, A. & Plou, F.J. (2006). Combinatorial saturation mutagenesis by *in vivo* overlap extension for the engineering of fungal laccases. Combinatorial Chemistry & High Throughput Screening, 9, 719-727.

Alves, A.M.R.C., Record, E., Lomascolo, A., Scholtmeijer, K., Asther, M., Wessels, J.G.H. & Wösten, H.A.B. (2004). Highly efficient production of laccase by the basidiomycete *Pycnoporus cinnabarinus*. Applied and Environmental Microbiology, 70, 6379-6384.

Ardon, O., Kerem, Z. & Hadar, Y. (1996). Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. Journal of Biotechnology, 51, 202-207.

Aro, N., Pakula, T. & Penttilä, M. (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiological Reviews, 29, 719-739.

Arora, D.S. & Gill, P.K. (2001). Effects of various media and supplements on laccase production by some white rot fungi. Bioresource Technology, 77, 89-91.

Arora, D.S. & Gill, P.K. (2005). Production of ligninolytic enzymes by *Phlebia floridensis*. World Journal of Microbiology & Biotechnology, 21, 1021-1028.

Azin, M., Moravej, R. & Zareh, D. (2007). Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: optimization of culture condition by Taguchi method. Enzyme and Microbial Technology, 40, 801-805.

Baldrian, P. (2004). Increase of laccase activity during interspecific interactions of white-rot fungi. FEMS Microbiology Ecology, 50, 245-253.

Baldrian, P. (2006). Fungal laccases – occurence and properties. FEMS Microbiology Reviews, 30, 215-242.

Baldrian, P. & Šnajdr, J. (2006). Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes. Enzyme and Microbial Technology, 39, 1023-1029.

Bai, Z.H., Harvey, L.M. & McNeil, B. (2003). Oxidative stress in submerged cultures of fungi. Critical Reviews in Biotechnology, 23, 267-302.

Bailey, M.J., Buchert, J. & Viikari, L. (1993). Effect of pH on production of xylanase by *Trichoderma reesei* on xylan-based and cellulose-based media. Applied Microbiology and Biotechnology, 40, 224-229.

Beg, Q.K., Bhushan, B., Kapoor, M. & Hoondal, G.S. (2000). Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. Enzyme and Microbial Technology, 27, 459-466.

Beg, Q.K., Kapoor, M., Mahajan, L. & Hoondal, G.S. (2001). Microbial xylanases and their industrial applications: a review. Applied Microbiology and Biotechnology, 56, 326-338.

Bergquist, P.L., Te´o, V.S.J., Gibbs, M.D., Curach, N.C. & Nevalainen, K.M.H. (2004). Recombinant enzymes from thermophilic microorganisms expressed in fungal hosts. Biochemical Society Transactions, 32, 293-297.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K. & Xu, F. (1997). Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Applied and Environmental Microbiology, 63, 3151-3157.

Bermek, H., Gulseren, I., Li, K.C., Jung, H.C. & Tamerler, C. (2004). The effect of fungal morphology on ligninolytic enzyme production by a recently isolated wood-degrading fungus *Trichophyton rubrum* LSK-27. World Journal of Microbiology & Biotechnology, 20, 345-349.

Berrin, J.G., Ajandouz, E.H., Georis, J., Arnaut, F. & Juge, N. (2007). Substrate and product hydrolysis specificity in family 11 glycoside hydrolases: an analysis of *Penicillium funiculosum* and *Penicillium griseofulvum* xylanases. Applied Microbiology and Biotechnology, 74, 1001-1010.

Bhat, M.K. & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advances, 15, 583-620.

Binninger, D.M., Skrzynia, C., Pukkila, P.J. & Casselton, L.A. (1987). DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. The EMBO Journal, 6, 835-840.

Birhanli, E. & Yesilada, O. (2006). Increased production of laccase by pellets of *Funalia trogii* ATCC 200800 and *Trametes versicolor* ATCC 200801 in repeated-batch mode. Enzyme and Microbial Technology, 39, 1286-1293.

Bocchini, D.A., Oliveira, O.M.M.F., Gomes, E. & Da Silva, R. (2005). Use of sugarcane bagasse and grass hydrolysates as carbon sources for xylanase production by *Bacillus circulans* D1 in submerged fermentation. Process Biochemistry, 40, 3653-3659.

Boddy, L. (2000). Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiology Ecology, 31, 185-194.

Bollag, J.M. & Leonowicz, A. (1984). Comparative studies of extracellular fungal laccases. Applied and Environmental Microbiology, 48, 849-854.

Brooks, S.A. (2006). Protein glycosylation in diverse cell systems: implications for modifications and analysis of recombinant proteins. Expert Review of Proteomics, 3, 345-359.

Bulter, T., Alcalde, M., Sieber, V., Meinhold, P., Schlachtbauer, C. & Arnold, F.H. (2003). Functional expression of fungal laccase in *Saccharomyces cerevisiae* by directed evolution. Applied and Environmental Microbiology, 69, 987-995.

Cen, P. & Xia, L. (1999). Production of cellulase by solid-state fermentation. Advances in Biochemical Engineering/Biotechnology, 65, 69-92.

Chávez, R., Bull, P. & Eyzaguirre, J. (2006). The xylanolytic enzyme system from the genus *Penicillium*. Journal of Biotechnology, 123, 413-433.

Chen, X.L., Cao, Y.H., Ding, Y.H., Lu, W.Q. & Li, D.F. (2007). Cloning, functional expression and characterization of *Aspergillus sulphureus* β-mannanase in *Pichia pastoris*. Journal of Biotechnology, 128, 452-461.

Chernykh, A.M., Leont'evskii, A.A. & Golovleva, L.A. (2005). New approaches to increasing the yield of laccase from *Panus tigrinus*. Applied Biochemistry and Microbiology, 41, 508-511.

Cherry, J.R. & Fidantsef, A.L. (2003). Directed evolution of industrial enzymes: an update. Current Opinion in Biotechnology, 14, 438-443.

Chi, Y.J., Hatakka, A. & Maijala, P. (2007). Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? International Biodeterioration and Biodegradation, 59, 32-39.

Chipeta, Z.A., du Preez, J.C., Szakacs, G. & Christopher, L. (2005). Xylanase production by fungal strains on spent sulphite liquor. Applied Microbiology and Biotechnology, 69, 71-78.

Christov, L.P., Szakacs, G. & Balakrishnan, H. (1999). Production, partial characterization and use of fungal cellulose-free xylanases in pulp bleaching. Process Biochemistry, 34, 511-517.

Claus, H. (2003). Laccases and their occurrence in prokaryotes. Archives of Microbiology, 179, 145-150.

Claus, H. (2004). Laccases: structure, reactions, distribution. Micron, 35, 93-96.

Colina, A., Sulbáran-De-Ferrer, B., Aiello, C. & Ferrer, A. (2004). Xylanase production by *Trichoderma reesei* Rut C-30 on rice straw. Applied Biochemistry and Biotechnology, 105, 715-724.

Collins, P.J. & Dobson, A.D.W. (1997). Regulation of laccase gene transcription in *Trametes versicolor*. Applied and Environmental Microbiology, 63, 3444-3450.

Conesa, A., Punt, P.J. & van den Hondel, C.A.M.J.J. (2002). Fungal peroxidases: molecular aspects and applications. Journal of Biotechnology, 93, 143-158.

Crueger, W. & Crueger, A. (1984). Biotechnologie – Lehrbuch der angewandten Mikrobiologie. 2. Auflage. R. Oldenbourg Verlag, Munich, Germany.

Da Cunha, M.A.A., Barbosa, A.M., Giese, E.C. & Dekker, R.F.H. (2003). The effect of carbohydrate carbon sources on the production of constitutive and inducible laccases by *Botryosphaeria* sp.. Journal of Basic Microbiology, 43, 385-392.

Dahiya, J.S., Singh, D. & Nigam, P. (1998). Characterisation of laccase produced by *Coniothyrium minitans*. Journal of Basic Microbiology, 38, 349-359.

Damaso, M.C.T., Albano, R.M., Machado, R.C., de Castro, A.M., Almeida, O.B., Kurtenbach, E., Martins, O.B., Andrade, C.M.M.D.C. & Pereira, N. (2006). Cultivation of recombinant *Pichia pastoris* for xylanase production in a methanol-fed bioreactor. Brazilian Archives of Biology and Technology, 49, 145-151.

Daneault, C., Leduc, C. & Valade, J.L. (1994). The use of xylanases in kraft pulp bleaching – a review. TAPPI Journal, 77, 125-131.

de Faria, F.P., Te´o, V.S.J., Bergquist, P.L., Azevedo, M.O. & Nevelainen, K.M.H. (2002). Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea* var. *thermoidea* in *Trichoderma reesei*. Letters in Applied Microbiology, 34, 119-123.

Dekker, R.F.H. & Barbosa, A.M. (2001). The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete *Botryosphaeria* sp.. Enzyme and Microbial Technology, 28, 81-88.

Demain, A.L. (2006). From natural products discovery to commercialization: a success story. Journal of Industrial Microbiology & Biotechnology, 33, 486-495.

den Haan, R., Rose, S.H., Lynd, L.R. & van Zyl, W.H. (2007). Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metabolic Engineering, 9, 87-94.

de Souza, D.F., Tychanowicz, G.K., Souza, C.G.M. & Peralta, R.M. (2006). Co-production of ligninolytic enzymes by *Pleurotus pulmonarius* on wheat bran solid state. Journal of Basic Microbiology, 46, 126-134.

de Vries, R.P. & Visser, J. (2001). *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiology and Molecular Biology Reviews, 65, 497-522.

Diáz-Godínez, G., Soriano-Santos, J., Augur, C. & Viniegra-González, G. (2001). Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study. Journal of Industrial Microbiology & Biotechnology, 26, 271-275.

Domínguez, A., Gómez, J., Lorenzo, M. & Sanromán, M.Á. (2007). Enhanced production of laccase activity by *Trametes versicolor* immobilized into alginate beads by the addition of different inducers. World Journal of Microbiology & Biotechnology, 23, 367-373.

Dong, J.L., Zhang, Y.W., Zhang, R.H., Huang, W.Z. & Zhang, Y.Z. (2005). Influence of culture conditions on laccase production and isozyme patterns in the white-rot fungus *Trametes gallica*. Journal of Basic Microbiology, 45, 190-198.

Dueñas, R., Tengerdy, R.P. & Gutierrez-Correa, M. (1995). Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World Journal of Microbiology & Biotechnology, 11, 333-337.

Durand, A. (2003). Bioreactor designs for solid state fermentation. Biochemical Engineering Journal, 13, 113-125.

D´Souza, T.M., Merritt, C.S. & Reddy, C.A. (1999). Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Applied and Environmental Microbiology, 65, 5307-5313.

D´Souza-Ticlo, D., Verma, A.K., Mathew, M. & Raghukumar, C. (2006). Effect of nutrient nitrogen on laccase production, its isoenzyme pattern and effluent decolorization by the fungus NIOCC #2a, isolated from mangrove wood. Indian Journal of Marine Sciences, 35, 364-372.

Eggert, C., Temp, U. & Eriksson, K.E.L. (1996). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. Applied and Environmental Microbiology, 62, 1151-1158.

Esterbauer, H., Steiner, W., Labudova, I., Hermann, A. & Hayn, M. (1991). Production of *Trichoderma* cellulase in laboratory and pilot scale. Bioresource Technology, 36, 51-65.

Fåhraeus, G. & Reinhammar, B. (1967). Large scale production and purification of laccase from cultures of fungus *Polyporus versicolor* and some properties of laccase A. Acta Chemica Scandinavica, 21, 2367-2378.

Favela-Torres, E., Volke-Sepúlveda, T. & Viniegra-González, G. (2006). Production of hydrolytic depolymerising pectinases. Food Technology and Biotechnology, 44, 221-227.

Fenel, F., Zitting, A.J. & Kantelinen, A. (2006). Increased alkali stability in *Trichoderma reesei* endo-1,4-β-xylanase II by site directed mutagenesis. Journal of Biotechnology, 121, 102-107.

Fenice, M., Giovannozzi, S.G., Federici, F. & D'Annibale, A. (2003). Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. Journal of Biotechnology, 100, 77-85.

Ferreira Gregorio, A.P., da Silva, I.R., Sedarati, M.R. & Hedger, J.N. (2006). Changes in production of lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. Mycological Research, 110, 161-168.

Fortina, M.G., Acquati, A., Rossi, P., Manachini, P.L. & DiGennaro, C. (1996). Production of laccase by *Botrytis cinerea* and fermentation studies with strain F226. Journal of Industrial Microbiology, 17, 69-72.

Freitag, M. & Morrell, J.J. (1992). Changes in selected enzyme-activities during growth of pure and mixed cultures of the white-rot decay fungus *Trametes versicolor* and the potential biocontrol fungus *Trichoderma harzianum*. Canadian Journal of Microbiology, 38, 317-323.

Galante, Y.A. & Formantici, C. (2003). Enzyme applications in detergency and in manufacturing industries. Current Organic Chemistry, 7, 1399-1422.

Galhaup, C., Wagner, H., Hinterstoisser, B. & Haltrich, D. (2002). Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme and Microbial Technology, 30, 529-536.

García-Mena, J., Cano-Ramirez, C., Garibay-Orijel, C., Ramirez-Canseco, S. & Poggi-Varaldo, H.M. (2005). A PCR method for the detection and differentiation of *Lentinus edodes* and *Trametes versicolor* in defined-mixed cultures used for wastewater treatment. Applied Microbiology and Biotechnology, 67, 524-531.

Gerngross, T.U. (2004). Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nature Biotechnology, 22, 1409-1414.

Gessesse, A. & Mamo, G. (1999). High-level xylanase production by an alkaliphilic *Bacillus* sp. by using solid-state fermentation. Enzyme and Microbial Technology, 25, 68-72.

Granado, J.D., Kertesz-Chaloupková, K., Aebi, M. & Kües, U. (1997). Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Molecular and General Genetics, 256, 28-36.

Gray, K.A. (2007). Cellulosic ethanol – state of the technology. International Sugar Journal, 109, 145-151.

Grimm, L.H., Kelly, S., Krull, R. & Hempel, D.C. (2005). Morphology and productivity of filamentous fungi. Applied Microbiology and Biotechnology, 69, 375-384.

Grimrath, A. (2007). Heterologous expression of laccase gene *lcc5* from different strains of *Trametes versicolor* in the basidiomycete *Coprinopsis cinerea*. Master thesis, Institute of Forest Botany, Faculty of Forest Science and Forest Ecology, Georg-August-University Göttingen, Göttingen, Germany.

Guillemette, T., van Peij, N.N.M.E., Goosen, T., Lanthaler, K., Robson, G.D., van den Hondel, C.A.M.J.J., Stam, H. & Archer, D.B. (2007). Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. BMC Genomics, 8, 158.

Guo, M., Lu, F., Du, L., Pu, J. & Bai, D. (2005). Optimization of the expression of a laccase gene from *Trametes versicolor* in *Pichia methanolica*. Applied Microbiology and Biotechnology, 69, 178-183.

Gutíerrez, A., del Río, J.C., Martínez, M.J. & Martínez, A.T. (2001). The biotechnological control of pitch in paper pulp manufacturing. Trends in Biotechnology, 19, 340-348.

Gutierrez-Correa, M. & Tengerdy, R.P. (1998). Xylanase production by fungal mixed culture solid substrate fermentation on sugar cane bagasse. Biotechnology Letters, 20, 45-47.

Hahn-Hägerdahl, B., Karhumaa, K., Larsson, C.U., Gorwa-Grauslund, M., Görgens, J. & van Zyl, W.H. (2005). Role of cultivation media in the development of yeast strains for large scale industrial use. Microbial Cell Factories, 4, 31, 1-16.

Haltrich, D., Preiss, M & Steiner, W. (1993). Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. Enzyme and Microbial Technology, 15, 854-860.

Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W. & Župančič, S. (1996). Production of fungal xylanases. Bioresource Technology, 58, 137-161.

Harhangi, H.R., Akhmanova, A., Steenbakkers, P.J.M., Jetten, M.S.M., van der Drift, C. & den Camp, H.J.M.O. (2003). Genomic DNA analysis of genes encoding (hemi-)cellulolytic enzymes of anaerobic fungus *Piromyces* sp. E2. Gene, 314, 73-80.

Hatvani, N., Kredics, L., Antal, Z. & Mécs, I. (2002). Changes in activity of extracellular enzymes in dual cultures of *Lentinula edodes* and mycoparasitic *Trichoderma* strains. Journal of Applied Microbiology, 92, 415-423.

Hao, X.C., Yu, X.B. & Yan, Z.L. (2006). Optimization of the medium for the production of cellulase by the mutant *Trichoderma reesei* WX-112 using response surface methodology. Food Technology and Biotechnology, 44, 89-94.

Helmy, S.M. & El-Meligi, M. (2002). Biopulping and biobleaching by white rot fungi. Journal of Scientific & Industrial Research, 61, 376-381.

Herpoël, I., Moukha, S., Lesage-Meessen, L., Sigoillot, J.C. & Asther, M. (2000). Selection of *Pycnoporus cinnabarinus* strains for laccase production. FEMS Microbiology Letters, 183, 301-306.

Hess, J., Leitner, C., Galhaup, C., Kulbe, K.D., Hinterstoisser, B., Steinwender, M. & Haltrich, D. (2002). Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes versicolor*. Applied Microbiology and Biotechnology, 98, 229-241.

Hoegger, P.J., Kilaru, S., James, T., Thacker, J. & Kües, U. (2006). Phylogenetic comparison and classification of laccase and related multi-copper oxidase protein sequences. FEBS Journal, 273, 2308-2326.

Hofrichter, M., Vares, T., Kalsi, M., Galkin, S., Scheibner, K., Fritsche, W. & Hatakka, A. (1999). Production of manganese peroxidase and organic acids and mineralization of ^{14}C-labelled lignin (^{14}C-DHP) during solid-state fermentation of wheat straw with the white rot fungus *Nematoloma frowardii*. Applied and Environmental Microbiology, 65, 1864-1870.

Hölker, U. & Lenz, J. (2005). Solid-state fermentation – are there any biotechnological advantages? Current Opinion in Microbiology, 8, 301-306.

Hölker, U., Hofer, M. & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Applied Microbiology and Biotechnology, 64, 175-186.

Hong, F., Meinander, N.Q. & Jönsson, L.J. (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. Biotechnology and Bioengineering, 79, 438–449.

Hong, Y., Xiao, Y., Zhou, H., Fang, W., Zhang, M., Wang, J., Wu, L. & Yu, Z. (2006). Expression of a laccase cDNA from *Trametes* sp. AH28-2 in *Pichia pastoris* and mutagenesis of transformants by nitrogen ion implantation. FEMS Microbiology Letters, 258, 96-101.

Hongzhang, C., Hui, W., Aijun, Z. & Zuohu, L. (2006). Alkaline protease production by solid state fermentation on polyurethane foam. Chemical and Biochemical Engineering Quarterly, 20, 93-97.

Hou, H.M., Zhou, J.T., Wang, J., Du, C.H. & Yan, B. (2004). Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochemistry, 39, 1415-1419.

Husain, Q. (2006). Potential applications of the oxidoreductive enzymes in the decoloriozation and detoxification of textile and other synthetic dyes from polluted water: a review. Critical Reviews in Biotechnology, 26, 201-221.

Hüttermann, A., Mai, C. & Kharazipour, A. (2001). Modification of lignin for the production of new compound materials. Applied Microbiology and Biotechnology, 55, 387-394.

Ikehata, K., Buchanan, I.D. & Smith, D.W. (2004). Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. Journal of Environmental Engineering and Science, 3, 1-19.

Iakovlev, A. & Stenlid, J. (2000). Spatiotemporal patterns of laccase in interacting mycelia of wood-decaying basidiomycete fungi. Microbial Ecology, 39, 236-245.

Jang, M.Y., Ryu, W.R. & Cho, M.H. (2002). Laccase production from repeated batch cultures using free mycelia of *Trametes* sp.. Enzyme and Microbial Technology, 30, 741-746.

Jeffries, T.W. (2006). Engineering yeasts for xylose metabolism. Current Opninion in Biotechnology, 17, 320-326.

Johnson, D.L., Thompson, J.L., Brinkmann, S.M., Schuller, K.A. & Martin, L.L. (2003). Electrochemical characterization of purified *Rhus vernicifera* laccase: voltammetric evidence for a sequential four-electron transfer. Biochemistry, 42, 10229-10237.

Junghanns, C., Moeder, M., Krauss, G., Martin, C. & Schlosser, D. (2005). Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases. Microbiology, 151, 45-57.

Kachlishvili, E., Penninckx, M.J., Tsiklauri, N. & Eliashvili, V. (2006). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. World Journal of Microbiology & Biotechnology, 22, 391-397.

Kahraman, S.S. & Gurdal, I.H. (2002). Effect of synthetic and natural culture media on laccase production by white rot fungi. Bioresource Technology, 82, 215-217.

Kajita, S., Sugawara, S., Miyazaki, Y., Nakamura, M., Katayama, Y., Shishido, K. & Iimura, Y. (2004). Overproduction of recombinant laccase using a homologous expression system in *Coriolus versicolor*. Applied Microbiology and Biotechnology, 66, 194-199.

Kang, K.Y., Jo, B.M., Oh, J.S. & Mansfield, S.D. (2003). The effects of biopulping on chemical and energy consumption during kraft pulping of hybrid poplar. Wood and Fiber Science, 35, 594-600.

Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I. & Kim, S.W. (2004). Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresource Technology, 91, 153-156.

Katapodis, P., Christakopoulou, V. & Christakopoulous, P. (2006). Optimization of xylanase production by *Sporotrichum thermophile* using corn cobs and response surface methodology. Engineering in Life Sciences, 6, 410-415.

Kasinath, A., Novotný, Č., Svobodová, K., Patel, K.C. & Šašek, V. (2003). Decolorization of synthetic dyes by *Irpex lacteus* in liquid cultures and packed-bed bioreactor. Enzyme and Microbial Technology, 32, 167-173.

Kelly, S., Grimm, L.H., Jonas, R., Hempel, D.C. & Krull, R. (2006). Investigations of the morphogenesis of filamentous microorganisms. Engineering in Life Sciences, 6, 475-480.

Kenealy, W.R. & Jeffries, T.W. (2003). Enzyme processes for pulp and paper: a review of recent developments. ACS Symposium Series, 845, 210-239.

Kennedy, M. & Krouse, D. (1999). Strategies for improving fermentation medium performance: a review. Journal of Industrial Microbiology and Biotechnology, 23, 456-475.

Kharazipour, A. (1996). Enzyme von Weißfäulepilzen als Grundlage für die Herstellung von Bindemittel für Holzwerkstoffe. Sauerländer´s Verlag, Frankfurt, Germany.

Kharazipour, A., Hüttermann, A. & Lüdemann, H.D. (1997). Enzymatic activation of wood fibres as a mean for the production of wood composites. Journal of Adhesion Science and Technology, 11, 419-427.

Kilaru, S. (2006). Identification of fungal multi-copper oxidase gene families: overexpression and characterization for applications in biotechnology. Cuvillier Verlag, Göttingen, Germany.

Kilaru, S. & Kües, U. (2005). Comparison of *gpd* genes and their protein products in basidiomycetes. Fungal Genetics Newsletters, 52, 18-24.

Kilaru, S., Hoegger, P.J. & Kües, U. (2006a). The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. Current Genetics, 50, 45-60.

Kilaru, S., Hoegger, P.J., Majcherzyk, A., Bruns, C., Shishido, K., Bailey, A., Foster, G.D. & Kües, U. (2006b). Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycete promoters. Applied Microbiology and Biotechnology, 71, 200-210.

Kilaru, S., Rühl, M., Saathoff, A., Dwivedi, R.C., Zomorrodi, M., Lange, K., Majcherczyk, A., Hoegger, P.J. & Kües U. (2006c). Overexpression of laccases in *Coprinopsis cinerea.* In: Pisabarro, A.G. & Ramírez, L. (Eds.) VI Genetics and cellular biology of Basidiomycetes. Universida Pública de Navarra, Pamplona, Spain, pp. 123-138.

Kiiskinen, L.L., Kruus, K., Bailey, M., Ylösmäki, M.S. & Saloheimo, M. (2004). Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology, 150, 3065-3074.

Kleemann, F. (2007). Interaktionen zwischen *Coprinopsis cinerea* und anderen Mikroorganismen: Effekte auf die Laccaseproduktion. Master thesis, Institute of Forest Botany, Faculty of Forest Science and Forest Ecology, Georg-August-University Göttingen, Göttingen, Germany.

Kollmann, A., Boyer, F.D., Ducrot, P.H., Kerhoas, L., Jolivalt, C., Touton, I., Einhorn, J. & Mougin, C. (2005). Oligomeric compounds formed from 2,5-xylidine (2,5-dimethylaniline) are potent enhancers of laccase production in *Trametes versicolor* ATCC 32745. Applied Microbiology and Biotechnology, 68, 251-258.

Koroleva, O.V., Stepanova, E.V., Gavrilova, V.P., Yakovleva, N.S., Landesman, E.O., Yavmetdinov, I.S. & Yaropolov, A.I. (2002). Laccase and Mn-peroxidase production by *Coriolus hirsutus* strain 075 in a jar fermentor. Journal of Bioscience and Bioengineering, 93, 449-455.

Koroljova-Skorobogat´ko, O.V., Stephanova, E.V., Gavrilova, V.P., Morozova, O.V., Lubimova, N.V., Dzchafarova, A.N., Jaropolov, A.I. & Makower, A. (1998). Purification and characterization of the constitutive form of laccase from the basidiomycete *Coriolus hirsutus* and effect of inducers on laccase synthesis. Biotechnology and Applied Biochemistry, 28, 47-54.

Korona, B., Korona, D. & Bielecki, S. (2006). Efficient expression and secretion of two co-produced xylanases from *Aspergillus niger* in *Pichia pastoris* directed by their native signal peptides and the *Saccharomyces cerevisiae* α-mating factor. Enzyme and Microbial Technology, 39, 683-689.

Kovacs, K., Szakacs, G., Pusztahelyi, T. & Pandey, A. (2004). Production of chitinolytic enzymes with *Trichoderma longibrachiatum* IMI 92027 in solid substrate fermentation. Applied Biochemistry and Biotechnology, 118, 189-204.

Kulkarni, N., Shendye, A. & Rao, M. (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbology Reviews, 23, 411-456.

Larrondo, L.F., Avila, M., Salas, L., Cullen, D. & Vicuña, R. (2003). Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper activated apoprotein and complex isoform patterns. Microbiology, 149, 1177-1182.

Lechner, B.E. & Papinutti, V.L. (2006). Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw. Process Biochemistry, 41, 594-598.

Lee, S.Y., Lee, D.Y. & Kim, T.Y. (2005). Systems biotechnology for strain improvement. Trends in Biotechnology, 23, 349-358.

Leonowicz, A., Cho, N., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D. & Rogalski, J. (2001). Fungal laccase: properties and activity on lignin. Journal of Basic Microbiology, 41, 185-227.

Levasseur, A., Asther, M. & Record, E. (2005). Overproduction and characterization of xylanse B from *Aspergillus niger*. Canadian Journal of Microbiology, 51, 177-183.

Li, Y., Lin, J., Meng, D.J., Lu, J., Gu, G.X. & Mao, Z.G. (2006). Effect of pH, cultivation time and substrate concentration on the endoxylanase production by *Aspergillus awamori* ZH-26 under submerged fermentation using central composite rotary design. Food Technology and Biotechnology, 44, 473-477.

Li, Y., Cui, F.J., Liu, Z.Q., Xu, Y.Y. & Zhao, H. (2007). Improvement of xylanase production by *Penicillium oxalicum* ZH-30 using response surface methodology. Enzyme and Microbial Technology, 40, 1381-1388.

Linke, D., Bouws, H., Peters, T., Nimtz, M., Berger, R.G. & Zorn, H. (2005). Laccases of *Pleurotus sapidus*: characterization and cloning. Journal of Agricultural and Food Chemistry, 53, 9498-9505.

Lomascolo, A., Record, E., Herpoël-Gimbert, I., Delattre, M., Robert, J.L., Georis, J., Dauvrin, T., Sigoillot, J.C. & Asther, M. (2003). Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. Journal of Applied Microbiology, 94, 618-624.

Longobardi, G. P. (1994). Fed-batch versus batch fermentation. Bioprocess Engineering, 10, 185-194.

Lorenzo, M., Moldes, D., Rodríguez Couto, S. & Sanromán, M.Á. (2002). Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. Bioresource Technology, 82, 109-113.

Lorenzo, M., Moldes, D. & Sanromán, M.A. (2006). Effect of heavy metals on the production of several laccase isoenzymes by *Trametes versicolor* and on the ability to decolourize dyes. Chemosphere, 63, 912-917.

Lynd, R.L., Weimer, P.J., van Zyl, W.H. & Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and Molecular Biology Reviews, 66, 506-577.

Mach, R. & Zeilinger, S. (2003). Regulation of gene expression in industrial fungi: *Trichoderma*. Applied Microbiology and Biotechnology, 60, 515-522.

Madzak, C., Otterbein, L., Chambkha, M., Moukha, S., Asther, M., Gaillardin, C. & Beckerich, J.M. (2005). Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. FEMS Yeast Research, 5, 635-646.

Madzak, C., Mimmi, M.C., Caminade, E., Brault, A., Baumberger, S., Briozzo, P., Mopugin, C. & Jolivalt, C. (2006). Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. Protein Engineering Design & Selection, 19, 77-84.

Mai, C., Kües, U. & Militz, H. (2004). Biotechnology in the wood industry. Applied Microbiology and Biotechnology, 63, 477-494.

Makela, M.R., Hilden, K.S., Hakala, T.K., Hatakka, A. & Lundell, T.K. (2006). Expression and molecular properties of a new laccase of the white rot fungus *Phlebia radiata* grown on wood. Current Genetics, 50, 323-333.

Mander, G.J., Wang, H.M., Bodie, E., Wagner, J., Vienken, K., Vinuesa, C., Foster, C., Leeder, A.C., Allen, G., Hamill, V., Janssen, G.G., Dunn-Coleman, N., Karos, M., Lemaire, H.G., Subkowski, T., Bollschweiler, C., Turner, G., Nüsslein, B. & Fischer, R. (2006). Use of laccase as a novel, versatile reporter system in filamentous fungi. Applied and Environmental Microbiology, 72, 5020-5026.

Martin, K, McDougall, B.M., McIlroy, S., Jayus, Chen, J.Z. & Seviour, R.J. (2007). Biochemistry and molecular biology of exocellular fungal β-(1,3)- and β-(1,6)-glucanases. FEMS Microbiology Reviews, 31, 168-192.

Martínez-Íñigo, M.J., Gutiérrez, A., del Río, J.C., Martínez, M.J. & Martínez, A.T. (2000). Time course of fungal removal of lipophilic extractives from *Eucalyptus globulus* wood. Journal of Biotechnology, 84, 119-126.

Matsumoto, Y., Saucedo-Castaneda, G., Revah, S. & Shirai, K. (2004). Production of β-N-acetylhexosaminidase of *Verticillium lecanii* by solid state and submerged fermentations utilizing shrimp waste silage as substrate and inducer. Process Biochemistry, 39, 665-671.

Mayer, A.M. & Staples, R.C. (2002). Laccase: new functions for an old enzyme. Phytochemistry, 60, 551-565.

Merchuk, J.C. (2003). Airlift bioreactors: review of recent advances. Canadian Journal of Chemical Engineering, 81, 324-337.

Meza, J.C., Sigoillot, J.C., Lomascolo, A., Navarro, D. & Auria, R. (2006). New process for fungal delignification of sugar-cane bagasse and simultaneous production of laccase in a vapor phase bioreactor. Journal of Agricultural and Food Chemistry, 54, 3852-3858.

Meza, J.C., Auria, R., Lomascolo, A., Sigiollot, J.C. & Casalat, L. (2007). Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. Enzyme and Microbial Technology, 41, 162-168.

Mikiashvili, N., Elisashvili, V., Wasser, S. & Nevo, E. (2005). Carbon and nitrogen sources influence the ligninolytic enzyme activity of *Trametes versicolor*. Biotechnology Letters, 27, 955-959.

Mikiashvili, N., Wasser, S.P., Nevo, P. & Elisasvili, V. (2006). Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. World Journal of Microbiology & Biotechnology, 22, 999-1002.

Mitchell, D.A., Krieger, N., Stuart, D.M. & Pandey, A. (2000). New developments in solid-state fermentation. II. Rational approaches to the design, operation and scale-up of bioreactors. Process Biochemistry, 35, 1211-1225.

Minussi, R.C., Pastore, G.M. & Duran, N. (2002). Potential applications of laccase in the food industry. Trends in Food Science & Technology, 13, 205-216.

Moldes, D., Lorenzo, M. & Sanromán, M.Á. (2004). Different proportions of laccase isoenzymes produced by submerged cultures of *Trametes versicolor* grown on lignocellulosic wastes. Biotechnology Letters, 26, 327-330.

Narang, S., Sahai, V. & Bisaria, V.S. (2001). Optimization of xylanase production by *Melanocarpus albomyces* IIS68 in solid state fermentation using response surface methodology. Journal of Bioscience and Bioengineering, 91, 425-427.

Necochea, R., Valderrama, B., Díaz-Sandoval, S., Folch-Mallol, J.L., Vázquez-Duhalt, R. & Ithrriaga, G. (2005). Phylogenetic and biochemical characterisation of a recombinant laccase from *Trametes versicolor*. FEMS Microbiology Letters, 244, 235-241.

Nevalainen, H., Suominen, P. & Taimisto, K. (1994). On the safety of *Trichoderma reseei*. Journal of Biotechnology, 37, 193-200.

Nevalainen, K.M.H., Te´o, V.S.J. & Bergquist, P.L. (2005). Heterologous protein expression in filamentous fungi. Trends in Biotechnology, 23, 468-474.

Nieves, R.A., Ehrman, C.I., Adney, W.S., Elander, R.T. & Himmel, M.E. (1998). Technical communication: survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. World Journal of Microbiology & Biotechnology, 14, 301-304.

Nyanhongo, G.S., Gomes, J., Gubitz, G., Zvauya, R., Read, J.S. & Steiner, W. (2002). Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresource Technology, 84, 259-263.

Ooijkaas, L.P., Weber, F.J., Buitelaar, R.M., Tramper, J. & Rinzema, A. (2000). Defined media and inert supports: their potential as solid-state fermentation production systems. Trends in Biotechnology, 19, 356-360.

Otterbein, L., Record, E., Longhi, S., Asther, M. & Moukha, S. (2000). Molecular cloning of the cDNA encoding laccase from *Pycnoporus cinnabarinus* I-937 and expression in *Pichia pastoris*. European Journal of Biochemistry, 267, 1619-1625.

Paice, M.G., Jurasek, L., Carpenter, M.R. & Smillie, L. (1978). Production, characterization, and partial amino acid sequence of xylanase A from *Schizophyllum commune*. Applied and Environmental Microbiology, 36, 802-808.

Paice M.G., Gurnagul N., Page D.H. & Jurasek, L. (1992). Mechanism of hemicellulose-directed prebleaching of kraft pulps. Enzyme Microbiological Technology, 14, 272-276.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capassoi, A. & Sannia, G. (1997). A novel white laccase from *Pleurotus ostreatus*. The Journal of Biological Chemistry, 272, 31301-31307.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. & Sannia, G. (2000). Copper induction of laccase isoenzymes in the lignolytic fungus *Pleurotus ostreatus*. Applied and Environmental Microbiology, 66, 920-924.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G. & Giardina, P. (2003). Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. Enzyme and Microbial Technology, 33, 220-230.

Pandey, A., Selvakumar, P., Soccol, C.R. & Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. Current Science, 77, 149-162.

Pandey, A., Soccol, C.R. & Mitchell, D. (2000). New developments in solid state fermentation: I-bioprocesses and products. Process Biochemistry, 35, 1153-1169.

Papagianni, M. (2004). Fungal morphology and metabolit production in submerged mycelial processes. Biotechnology Advances, 22, 189-259.

Papinutti, L. & Martínez, M.J. (2006). Production and characterization of laccase and manganese peroxidase from the lignolytic fungus *Fomes sclerodermus*. Journal of Chemical Technology and Biotechnology, 81, 1064-1070.

Parekh, S., Vinci, V.A. & Strobel, R.J. (2000). Improvement of microbial strains and fermentation processes. Applied Microbiology and Biotechnology, 54, 287-301.

Park, Y.S., Kang, S.W., Lee, J.S., Hong, S.L. & Kim, S.W. (2002). Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs. Applied Microbiology and Biotechnology, 58, 761-766.

Patil, A. & Dayanand, A. (2006). Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. Bioresource Technology, 97, 2054-2058.

Persson, I., Tjerneld, F. & Hahnhagerdal, B. (1991). Fungal cellulolytic enzyme production – a review. Process Biochemsitry, 26, 65-74.

Piscitelli, A., Giardina, P., Mazzoni, C. & Sannia, G. (2005). Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology, 69, 428-439.

Polizeli, M.L.T.M., Rizatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. & Amorim, D.S. (2005). Xylanases from fungi: properties and industrial applications. Applied Microbiology and Biotechnology, 67, 577-591.

Prasad, K.K., Mohan, S.V., Bhaskar, Y.V., Ramanaiah, S.V., Babu, V.L., Pati, B.R. & Sarma, P.N. (2005a). Laccase production using *Pleurotus ostreatus* 1804 immobilized on PUF cubes in batch and packed bed reactors: influence of culture conditions. Journal of Microbiology, 43, 301-307.

Prasad, K.K., Mohan, S.V., Rao, R.S., Pati, B.R. & Sarma, P.N. (2005b). Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochemical Engineering Journal, 24, 17-26.

Punt, P.J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J. & van den Hondel, C.A.M.J.J. (2002). Filamentous fungi as cell factories for heterologous protein production. Trends in Biotechnology, 20, 200-206.

Rabinovich, M.L., Melnik, M.S. & Bolobova, A.V. (2002). Microbial cellulases (review). Applied Biochemistry and Microbiology, 38, 305-321.

Rahardjo, Y.S.P., Tramper, J. & Rinzena, A. (2006). Modeling conversion and transport phenomena in solid-state fermentation: a review and perspectives. Biotechnology Advances, 24, 161-177.

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. Electronical Journal of Biotechnology, 1, 174-188.

Rancaño, G., Lorenzo, M., Molares, N., Rodríguez Couto, S. & Sanromán, M.Á. (2003). Production of laccase by *Trametes versicolor* in an airlift fermenter. Process Biochemistry, 39, 467-473.

Rani, K.Y. & Rao, V.S.R. (1999). Control of fermenters – a review. Bioprocess Engineering, 21, 77-88.

Revankar, M.S. & Lele, S.S. (2006). Enhanced production of laccase using a new isolate of white rot fungus WR-1. Process Biochemistry, 41, 581-588.

Rezende, M.I., Barbosa, A.D., Vasconcelos, A.F.D. & Endo, A.S. (2002). Xylanase production by *Trichoderma harzianum* Rifai by solid state fermentation on sugarcane bagasse. Brazilian Journal of Microbiology, 33, 67-72.

Rodríguez Couto, S. & Sanromán, M.Á. (2005). Application of solid-state fermentation to lignolytic enzyme production. Biochemical Engineering Journal, 22, 211-219.

Rodríguez Couto, S. & Sanromán, M.Á. (2006a). Effect of two wastes from groundnut processing on laccase production and dye decolourization ability. Journal of Food Engineering, 73, 388-393.

Rodríguez Couto, S. & Sanromán, M.Á. (2006b). Application of solid-state fermentation to food industry – a review. Journal of Food Engineering, 76, 291-302.

Rodríguez Couto, S. & Toca Herrera, J.L. (2006). Industrial and biotechnological applications of laccases: A review. Biotechnology Advances, 24, 500–513.

Rodríguez Couto, S., Santoro, R., Cameselle, C. & Sanromán, M.Á. (1997). Laccase production in semi-solid cultures of *Phanerochaete chrysosporium*. Biotechnology Letters, 19, 995–998.

Rodríguez Couto, S., Gundín, M., Lorenzo, M. & Sanromán, M.Á. (2002a). Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid-state conditions. Process Biochemistry, 38, 249-255.

Rodríguez Couto, S., Barreiro, M., Rivela, I., Longo, M.A. & Sanromán, M.Á. (2002b). Performance of a solid-state immersion bioreactor for ligninolytic enzyme production: evaluation of different operational variables. Process Biochemistry, 38, 219-227.

Rodríguez Couto , S., Moldes, D., Liebanas, A. & Sanromán, M.Á. (2003). Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions. Biochemical Engineering Journal, 15, 21-26.

Rodríguez Couto, S., Sanromán, M.Á., Hofer, D. & Gubitz, G.M. (2004a). Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus *Trametes hirsuta* for decolourization of textile dyes. Bioresource Technology, 95, 67-72.

Rodríguez Couto, S., Sanromán, M.Á., Hofer, D. & Gubitz, G.M. (2004b). Production of laccase by *Trametes hirsuta* grown in an immersion bioreactor and its application in the decolorization of dyes from a leather factory. Engineering in Life Sciences, 4, 233-238.

Rodríguez Couto, S., López, E. & Sanromán, M.Á. (2006). Utilisation of grape seeds for laccase production in solid-state fermentors. Journal of Food Engineering, 74, 263-267.

Rosales, E., Rodríguez Couto, S. & Sanromán, M.Á. (2005). Reutilisation of food processing wastes for production of relevant metabolites: application to laccase production by *Trametes hirsuta*. Journal of Food Engineering, 66, 419-423.

Rose, S.H. & van Zyl, W.H. (2002). Constitutive expression of the *Trichoderma reesei* β-1,4-xylana se gene (*xyn2*) and the β-1,4-endoglucanase gene (*egl*) in *Aspergillus niger* in molasses and defined glucose media. Applied Microbiology and Biotechnology, 58, 461-468.

Royer, J.C. & Nakas, J.P. (1989). Xylanase production by *Trichoderma longibrachiatum*. Enzyme and Microbial Technology, 11, 405-410.

Russell, T.W.F., Dunn, I.J. & Blanch, H.W. (1974). Tubular loop batch fermentor – basic concepts. Biotechnology and Bioengineering, 16, 1261-1272.

Ryan, D.R., Leukes, W.D. & Burton, S.G. (2005). Fungal bioremediation of phenolic wastewaters in an airlift reactor. Biotechnology Progress, 21, 1068-1074.

Ryan, D., Leukes, W. & Burton, S. (2007). Improving the bioremediation of phenolic wastewaters by *Trametes versicolor*. Bioresource Technology, 98, 579-587.

Saathoff, A. (2005). Production and characterisation of the recombinant laccase Lcc5 out of *C. cinerea* and its use in the wood fiber industry. Master thesis, Institute of Forest Botany, Faculty of Forest Science and Forest Ecology, Georg-August-University Göttingen, Göttingen, Germany.

Saloheimo, A. (2004). Yeast *Saccharomyces cerevisiae* as a tool in cloning and analysis of fungal genes. Applications for biomass hydrolysis and utilisation. VTT Publications, 541, 1-84.

Sandhya, C., Sumantha, A., Szakacs, G. & Pandey, A. (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. Process Biochemistry, 40, 2689-2694.

Saraswat, V. & Bisaria, V.S. (1997). Biosynthesis of xylanolytic and xylan-debranching enzymes in *Melanocarpus albomyces* IIS 68. Journal of Fermentation and Bioengineering, 83, 352-357.

Savoie, J.M. & Mata, G. (1999). The antagonistic action of *Trichoderma* sp. hyphae to *Lentinula edodes* hyphae changes lignocellulotytic activities during cultivation in wheat straw. World Journal of Microbiology & Biotechnology, 15, 369-373.

Scheel, T., Hofer, M., Ludwig, S. & Hölker, U. (2000). Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. Applied Microbiology and Biotechnology, 54, 686-691.

Schneider, P., Caspersen, M.B., Mondorf, K., Halkier, T., Skov, L.K., Østergaard, P.R., Brown, K.M., Brown, S.H. & Xu, F. (1999). Characterization of a *Coprinus cinereus* laccase. Enzyme and Microbial Technology, 25, 502-508.

Schügerl, K. (1980). New bioreactors for aerobic processes. Chemie Ingenieur Technik, 52, 951-965.

Schügerl, K. (2000). Integrated processing of biotechnology products. Biotechnology Advances, 18, 581-599.

Schügerl, K. (2005). Process development in biotechnology – a re-evaluation. Engineering in Life Sciences, 5, 15-28.

Schügerl, K. & Hubbuch, J. (2005). Integrated bioprocesses. Current Opinion in Microbiology, 8, 294-300.

Seyis, I. & Aksoz, N. (2005). Xylanase production from *Trichoderma harzianum* 1073 D3 with alternative carbon and nitrogen sources. Food Technology and Biotechnology, 43, 37-40.

Shah, A. & Madamwar, D. (2005). Xylanase production under solid-state fermentation and its characterization by an isolated strain of *Aspergillus foetidus* in India. World Journal of Microbiology & Biotechnology, 21, 233-243.

Shah, V., Baldrian, P., Eichlerova, I., Dave, R., Madamwar, D., Nerud, F. & Gross, R. (2006). Influence of dimethyl sulfoxide on extracellular enzyme production by *Pleurotus ostreatus*. Biotechnology Letters, 28, 651-655.

Shekholeslani, M. (1991). Optimierung der Enzymproduktion von Weißfäulepilzen für den Einsatz bei der biotechnologischen Erzeugung von Bindemittel. PhD thesis, Georg-August-University Göttingen, Göttingen, Germany.

Shin, K.W. (2004). The role of enzymes produced by white-rot fungus *Irpex lacteus* in the decolorization of the textile industry effluent. Journal of Microbiology, 42, 37-41.

Shoham, Y., Lamed, R. & Bayer, E.A. (1999). The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends in Microbiology, 7, 275-281.

Sigoillot, C., Record, E., Belle, V., Robert, J.L., Levasseur, A., Punt, P.J., van den Hondel, C.A.M.J.J., Fournel, A., Sigoillot, J.C. & Asther, M. (2004). Natural and recombinant fungal laccases for paper pulp bleaching. Applied Microbiology and Biotechnology, 64, 346-352.

Smith, D.C. & Wood, T.M. (1991). Xylanase production by *Aspergillus awamori* – development of a medium and optimization of the fermentation parameters for the production of extracellular xylanase and β-xylosidase while maintaining low protease production. Biotechnology & Bioengineering, 38, 883-890.

Soden, D.M. & Dobson, A.D.W. (2001). Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. Microbiology, 147, 1755-1763.

Sørensen, H.P. & Mortensen, K.K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. Journal of Biotechnology, 115, 113-128.

Souza, C.G.M., Tychanowicz, G.K., de Souza, D.F. & Peralta, R.M. (2004). Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. Journal of Basic Microbiology, 44, 129-136.

Sriprang, R., Asano, K., Gobsuk, J., Tanapongpipat, S., Champreda, V. & Eurwilaichtr, L. (2006). Improvement of thermostability of fungal xylanases by site-dirrected mutagenesis. Journal of Biotechnology, 126, 454-462.

Steenbakkers, P.J.M., Harhangi, H.R., Bosscher, M.W., van der Hooft, M.M.C., Keltjens, J.T., van der Drift, C., Vogels, G.D. & den Camp, H.J.M.O. (2003). β-glucosidase in cellulosome of the anearobic fungus *Piromyces* sp. strain E2 is a family 3 glycoside hydrolase. Biochemical Journal, 370, 963-970.

Stephens, D.E., Rumbold, K., Permaul, K., Prior, B.A. & Singh, S. (2007). Directed evolution of thermostable xylanase from *Thermomyces lanuginosus*. Journal of Biotechnology, 127, 348-354.

Sukumaran, R.K., Singhanai, P.R. & Pandey, A. (2005). Microbial cellulases – production, applications and challenges. Journal of Scientific & Industrial Research, 64, 832-844.

Sunna, A. & Antranikian G. (1997). Xylanolytic enzymes from fungi and bacteria. Critical Review in Biotechnology, 17, 39-67.

Sun, Y. & Cheng, J.Y. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 83, 1-11.

Suto, M. & Tomita, F. (2001). Induction and catabolite repression mechanisms of cellulase in fungi. Journal of Bioscience and Bioengineering, 92, 305-311.

Szendefy, J., Szakacs, G., Kemeny, S. & Christov, L. (2003). *In situ* solid-state fermentation and utilization of xylanase in pulp bleaching. ACS Symposium Series, 855, 255-284.

Szendefy, J., Szakacs, G. & Christopher, L. (2006). Potential of solid-state fermentation enzymes of *Aspergillus oryzae* in biobleaching of paper pulp. Enzyme and Microbial Technology, 39, 1354-1360.

Tavares, A.P.M., Coelho, M.A.Z., Coutinho, J.A.P. & Xavier, A.M.R.B. (2005). Laccase improvement in submerged cultivation: induced production and kinetic modelling. Journal of Chemical Technology and Biotechnology, 80, 669-676.

Tavares, A.P.M., Coelho, M.A.Z., Agapito, M.S.M., Coutinho, J.A.P. & Xavier, A.M.R.B. (2006). Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. Applied Biochemistry and Biotechnology, 134, 233-248.

Tengerdy, R.P. (1996). Cellulase production by solid substrate fermentation. Journal of Scientific and Industrial Research, 55, 313-316.

Tengerdy, R.P. & Szakacs, G. (2003). Bioconversion of lignocellulose in solid substrate fermentation. Biochemical Engineering Journal, 13, 169-179.

Terrón, M.C., González, T., Carbajo, J.M., Yagüe, S., Arana-Cuenca, A., Téllez, A., Dobson, A.D.W. & González, A.E. (2004). Structural close-related aromatic compounds have different effects on laccase activity and on *lcc* gene expression in the ligninolytic fungus *Trametes* sp. I-62. Fungal Genetics and Biology, 41, 954-962.

Tholudur, A., Ramirez, W.F. & McMillan, J. (1999). Mathematical modeling and optimization of cellulase protein production using *Trichoderma reesei* RL-P37. Biotechnology and Bioengineering, 66, 1-16.

Timell, T.E. (1967). Recent progress in the chemistry of wood hemicelluloses. Wood Science and Technology, 1, 45-70.

Tolan, J.S. & Foody, B. (1999). Cellulase from submerged fermentation. Advances in Biochemical Engineering/Biotechnology, 65, 41-57.

Tong, P.G., Hing, Y.Z., Xiao, Y.Z., Zhang, M., Tu, X.M. & Cui, T.J. (2007). High production of laccase by a new basidiomycete, *Trametes* sp.. Biotechnology Letters, 29, 295-301.

Trupkin, S., Levin, L., Forchiassin, F. & Viale, A. (2003). Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology. Journal of Industrial Microbiology and Biotechnology, 30, 682-690.

Tung, M.Y., Chang, C.T. & Chung, Y.C. (2007). Biochemical properties of genetic recombinant xylanase II. Applied Biochemistry and Biotechnology, 136, 1-16.

Tychanowicz, G.K., de Souza, D.F., Souza, C.G.M., Kadowaki, M.K. & Peralta, R.M. (2006). Copper improves the production of laccase by the white-rot fungus *Pleurotus pulmonarius* in solid state fermentation. Brazilian Archives of Biology and Technology, 49, 699-704.

Uffen, R.L. (1997). Xylan degradation: a glimpse at microbial diversity. Journal of Industrial Microbiology and Biotechnology, 19, 1-6.

van Beek, T.A., Kuster, B., Claassen, F.W., Tienvieri, T., Bertaud, F., Lenon, G., Petit-Conil, M. & Sierra-Alvarez, R. (2007). Fungal bio-treatment of spruce wood with *Trametes versicolor* for pitch control: influence on extractive contents, pulping process parameters, paper quality and efflux toxicity. Bioresource Technology, 98, 302-311.

Vasconcelos, A.F.D., Barbosa, A.M., Dekker, R.F.H., Scarminio, I.S. & Rezende, M.I. (2000). Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. Process Biochemistry, 35, 1131-1138.

Velázquez-Cedeño, M.A., Farnet, A.M., Ferré, E. & Savoie, J.M. (2004). Variations of lignocellulosic activities in dual cultures of *Pleurotus ostreatus* and *Trichoderma longibrachiatum* on unsterilized wheat straw. Mycologia, 96, 712-719.

Viikari, L., Kantelinen A., Sundquist, J. & Linko, M. (1994). Xylanases in bleaching, from an idea to the industry. FEMS Microbiological Reviews, 13, 335-350.

Vikineswary, S., Abdullah, N., Renuvathani, M., Sekaran, M., Pandey, A. & Jones, E.B.G. (2006). Productivity of laccase in solid substrate fermentation of selected agro-residues by *Pycnoporus sanguineus*. Bioresource Technology, 97, 171-177.

Viniegra-González, G. & Favela-Torres, E. (2006). Why solid-state fermentation seems to be resistant to catabolic repression? Food Technology and Biotechnology, 44, 397-406.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Rómero-Gomez, S.D., Díaz-Godínez, G. & Augur, C. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal, 13, 157-167.

Vyas, A. & Vyas, D. (2005). Production of fungal cellulases by solid state bioprocessing of groundnut shell wastes. Journal of Scientific & Industrial Research, 64, 767-770.

Wang, H.L., Liu, G.S., Li, P. & Pan, F. (2006). The effect of bioaugmentation on the performance of sequencing batch reactor and sludge characteristics in the treatment of papermaking wastewater. Bioprocess and Biosystems Engineering, 29, 283-289.

Wang, J.W., Wu, J.H., Huang, W.Y. & Tan, R.X. (2006). Laccase production by *Monotospora* sp., an endophytic fungus in *Cynodon dactylon*. Bioresource Technology, 97, 786-789.

Wang, L.P., Ridgway, D., Gu, T.Y. & Moo-Young, M. (2005). Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnological Advances, 23, 115-129.

Watanabe, M., Watanabe, J., Sonoyama, K. & Tanabe, S. (2000). Novel method for producing hypoallergenic wheat flour by enzymatic fragmentation of the constituent allergens and its application to food processing. Bioscience Biotechnology and Biochemistry, 64, 2663-2667.

Watanabe, T. (2007). Trends in biorefinery and pretreatments of lignocellulosics by white rot fungi. Mokuzai Gakkaishi, 53, 1-13.

Wen, Z.Y., Liao, W. & Chen, S.L. (2005). Production of cellulose/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on diary manure. Process Biochemistry, 40, 3087-3094.

Wong, K.K.Y., Tan, L.U.L. & Saddler, J.N. (1988). Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiology Reviews, 52, 305-317.

Ximenes, E.A., Chen, H.Z., Kataeva, I.A., Cotta, M.A., Felix, C.R., Ljungdahl, L.G. & Li, X.L. (2005). A mannase, ManA, of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2 has carbohydrate binding and docking modules. Canadian Journal of Microbiology, 51, 559-568.

Xing, Z.T., Cheng, J.H., Tan, Q. & Pan, Y.J. (2006). Effect of nutritional parameters on laccase production by the culinary and medicinal mushroom, *Grifola frondosa*. World Journal of Microbiology & Biotechnology, 22, 1215-1221.

Xion, H.R., Fenel, F., Leisola, M. & Turunen, O. (2004). Engineering the thermostability of *Trichoderma reesei* endo-1,4-β-xylanase II by combination of disulphide bridges. Extremophiles, 8, 393-400.

Xu, F. (2005). Applications of oxidoreductases: recent progress. Industrial Biotechnology, 1, 38-50.

Xu, F., Berka, R.M., Wahleithner, J.A., Nelson, B.A., Shuster, J.R., Brown, S.H., Palmer, A.E. & Solomon, E.I. (1998) Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. Biochemical Journal, 334, 63–70.

Yaver, D.S., Overjero, M.D., Xu, F., Nelson, B.A., Brown, K.M., Halkier, T., Bernauer, S., Brown, S.H. & Kauppinen, S. (1999). Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase Lcc1. Applied and Environmental Microbiology, 65, 4943-4948.

Yang, S.Q., Yan, Q.J., Jiang, Z.Q., Li, L.T., Tian, H.M. & Wang, Y.Z. (2006). High-level of xylanase production by the thermophilic *Paecilomyces thermophila* J18 on wheat straw in solid-state fermentation. Bioresource Technology, 97, 1794-1800.

Zhang, H., Hong, Y.Z., Xiao, Y.Z., Yuan, J., Tu, X.M. & Zhang, X.Q. (2006). Efficient production of laccases by *Trametes* sp. AH28-2 in cocultivation with a *Trichoderma* strain. Applied Microbiology and Biotechnology, 73, 89-94.

Zhang, M., Wu, F., Wei, Z.Y., Xiao, Y.Z. & Gong, W.M. (2006). Characterization and decolorization ability of a laccase from *Panus rudis*. Enzyme and Microbial Technology, 39, 92-97.

Zhang, Y.H.P. & Lynd, L.R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnology and Bioengineering, 88, 797-824.

Zhang, Y.H.P., Himmel, M.E. & Mielenz, J.R. (2006). Outlook for cellulase improvement: screening and selection strategies. Biotechnological Advances, 24, 452-481.

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Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate

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Abstract

Two commercial *Pleurotus ostreatus* strains (030 and K12) were cultivated under industrial conditions on wheat straw blocks 17-18 kg in weight. Within 10 weeks of cultivation, three flushes of fruiting body production were obtained with total yields of 4.2 and 4.1 kg mushrooms/substrate block and a biological efficiency (BE = total yield of fresh mushrooms per dry weight of substrate) of about 95%, respectively. Ligninolytic activities were followed up within the substrate over the cultivation periods. Laccase, manganese peroxidase (MnP) and versatile (manganese-independent) peroxidase (VP) activities were high in vegetative growth phases within the substrate and dropped upon initiation of fruiting body formation to be hardly detectable at the harvest of the mature mushrooms. Directly after harvest at all observed flushes, there was a sharp increase in all three enzymatic activities in the substrate for both strains (maximum activities for laccase 1.1-1.3 U/ml, for MnP 15-16 U/ml and for VP 1.1-1.4 U/ml). Enzymes can be extracted by pressing the spent mushroom substrates (SMS). Potential applications for the press juice or the enzymes in the SMS are discussed.

Key words

Laccase, peroxidase, fruiting bodies, spent mushroom substrate (SMS), solid state fermentation (SSF)

Introduction

Worldwide, with constantly increasing production rates, *Pleurotus* species are currently the second most produced edible mushrooms (1, 2). Alone in China, the annual production in 2003 was about 2.5 million t (3). *Pleurotus ostreatus* (Jacq.) P. Kumm. 1871, known as hiratake in Japan and as oyster mushroom in Western countries, is a saprophytic white-rot basidiomycete growing in forests on hardwoods and less often on softwoods (4). For commercial production of the fruiting bodies, mainly straw from wheat (Western countries) and rice (Asia) is used as cheap basic substrate, but saw dust and wood chips and other agricultural wastes may also be used (5). To facilitate growth on such lignocellulosic substrates, white rot-fungi secrete different types of oxidative enzymes for lignin degradation (6, 7). *P. ostreatus* produces different types of peroxidases (MnP: manganese dependent peroxidase and VP: versatile peroxidase; synonym: manganese-independent peroxidase MiP) as well as laccases (8, 9).

Ligninolytic enzymes have also repeatedly been linked to fruiting body production in higher basidiomycetes but species appear to differ at timings of high enzyme production and there is no clear-cut picture on functional relevance of the enzymes in fruiting (1, 10). From small scale experiments in *P. ostreatus*, it has previously been reported that production of ligninolytic enzymes increases with vegetative biomass production on

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solid growth substrates and that it drops during the sexual fruiting stage (11 - 13). Here, we present a study on the ligninolytic activities of two commercial *P. ostreatus* strains during substrate colonisation and over three flushes of fruiting body production under industrial conditions. High yields of laccases and peroxidases were detected in the substrate blocks shortly after mushroom harvests offering the possibility to harvest from spent mushroom substrate (SMS) enzymes for biotechnological applications as an extra benefit from mushroom cultivation.

Methods

Cultivation of *Pleurotus ostreatus* on solid substrate

Industrial produced pasteurised wheat-straw-based substrate originally prepared and inoculated with a fungus by Substratbetriejf Horst (Horst, Netherlands) was provided by the commercial mushroom grower druid Austernpilze (Immichenhain, Germany). Two different production strains of *P. ostreatus* were used, 030 (Le Champignon, Loches, France) and K12 (Sylvan Inc., Horst, Netherlands). Commercial substrate blocks (50 x 40 x 17 cm^3, around 17-18 kg each) wrapped in plastic foil were placed in a mushroom cultivation chamber.

During vegetative growth or for regenerative growth after mushroom harvest of the first flush, the temperature in the chamber was kept at 20-25 °C and the relative humidity (RH) at 90-95%. Once fruiting body primordia (sized 1 to 2 mm in Ø) became visible for the naked eye, the temperature was lowered to 16 to 18 °C and RH was increased to 95-98% as done in commercial mushroom production to improve fruiting body development and quality of the mushrooms (5). The lower temperature was hold up to the harvest of the mature mushrooms. Upon the first flush, the temperature was again raised for regenerative growth but not upon the second flush. The actual temperature setting throughout the cultivation was influenced by internal substrate conditions. During the whole cultivation periods, the internal temperature of substrate blocks was measured. The internal values were kept around 20 °C (20-21 ± 1 °C) in vegetative growth phases and around 19 °C (19 ± 2 °C) in mushroom production phases. Deviations from these internal values were compensated by smaller adjustments in temperature in the cultivation chambers (± 5 °C during mycelial growth phases, ± 2 °C during mushroom production phases). The moisture content of the substrate blocks was also controlled throughout the length of the cultivation period and found to be relatively constant (76 to 78%) at a level as wanted for mushroom cultivation (14).

Determination of mushroom yields

Fruiting bodies were harvested at maturation and weighted. Total mushroom yields were calculated as biological efficiency (BE) in percentage of the weight of fresh fruiting bodies per weight of dried substrate (5, 15).

Enzyme detection in the substrate

Every second day from day 21 after inoculation onwards when the substrate was fully colonised, samples of about 50 g (± 10 g) throughout colonised substrate were taken by cutting approximately 4 x 4 x 2 cm^3 sized cuboids from the substrate blocks. The samples with the submerged mycelium were weighted and subsequently squeezed by a self-made hand press (contact surface 25 x 30 cm^2) in order to extract all free liquid from the substrate. The residual substrate was dried at 80 °C for 48 h and the substrate moisture content was calculated from subtracting the dry weight (gram dried substrate [gds]) from the wet weight at sample harvest.

The liquids pressed from substrate samples were collected and measured in volume and their

pHs were determined. Upon centrifugation in order to eliminate any smaller solid parts from the liquids, aliquots were taken for enzyme measurements. Laccase activity was determined in 120 mM sodium acetate buffer (pH 5.6) with ABTS (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonate]) as a substrate and the oxidation was determined at 420 nm according to Matsumura et al.(16). Laccase activities were calculated using an extinction coefficient of ε_{420} = 36000 $M^{-1}cm^{-1}$ (17). Peroxidase activities were analysed by oxidation of phenol red under presence and absence of Mn^{2+} for determining MnP and VP activities, respectively, according to the protocol of Kuwahara et al. (18). Peroxidase activities were calculated using an extinction coefficient of ε_{610} = 4460 M^{-1} cm^{-1} (19). Enzyme activities are given in units (U) defined as the amount of ABTS, respectively phenol red in µmol transformed per min.

Results

Laccase and mushroom production alternate during cultivation of *P. ostreatus* 030

In a first experiment, laccase activities were followed up within three commercial substrate blocks inoculated with *P. ostreatus* strain 030. Once the first primordia were observed (day 4 after fully colonising the substrate blocks), the temperature was set at 16-18 °C and RH to 95-98%. In all three substrate blocks, laccase activities were comparably low during subsequent primordia and fruiting body development (approximately 0.3-0.5 U/ml press juice) and at harvest of the mature fruiting bodies (approximately 0.1-0.2 U/ml press juice). 3.0 ± 0.4 kg mushrooms per substrate block were collected at the first flush lasting four days. The day after harvest, there was a sudden increase in enzyme activity with an average laccase activity of 0.6 U/ml press juice. For regenerative mycelial growth, two days later the temperature for cultivation was raised to 20-25 °C and there was a further increase in laccase activity to about 1.5 U/ml hold in this range over the following seven days. Afterwards, laccase activities in the substrate blocks gradually dropped to about 0.8 U/ml press juice for the next seven days. At this point, primordia of the second flush became visible and the temperature was lowered to 18°C. Laccase activities declined further in the next days of incubation. Mature fruiting bodies of the second, slightly prolonged flush were first harvested after one week (2.0 ± 0.7 kg mushrooms per substrate block; 125% BE in total calculated from the yields of the first and the second flush) when laccase activities were barely detectable. During the three subsequent days of flush of the second harvest, there was again a dramatic increase in laccase activity to about 0.8 U/ml at day 3 of harvest (last day of harvest) and about 1.4 U/ml press juice two days later. The observations suggested an inverted correlation between laccase activity and fruiting body maturation with the minimum of activity occurring when the mushrooms were mature.

Mushroom production in a large scale experiment with two different *P. ostreatus* strains

To follow this observation up in more detail, commercial wheat straw blocks inoculated with either *P. ostreatus* 030 or with *P. ostreatus* K12 were incubated in a cultivation chamber under conditions of industrial mushroom production. Per strain, six substrate blocks were analysed. All substrate blocks were completely colonised 21 days after inoculation (Fig. 1A). After three further days, when small primordia appeared (Fig. 1B), the temperature was lowered for fruiting body production (Fig. 1C) until mushrooms were harvested (Fig. 1D) upon which the temperature was raised for regenerative growth (Fig. 1E). Two further flushes of mushroom production were followed up (not

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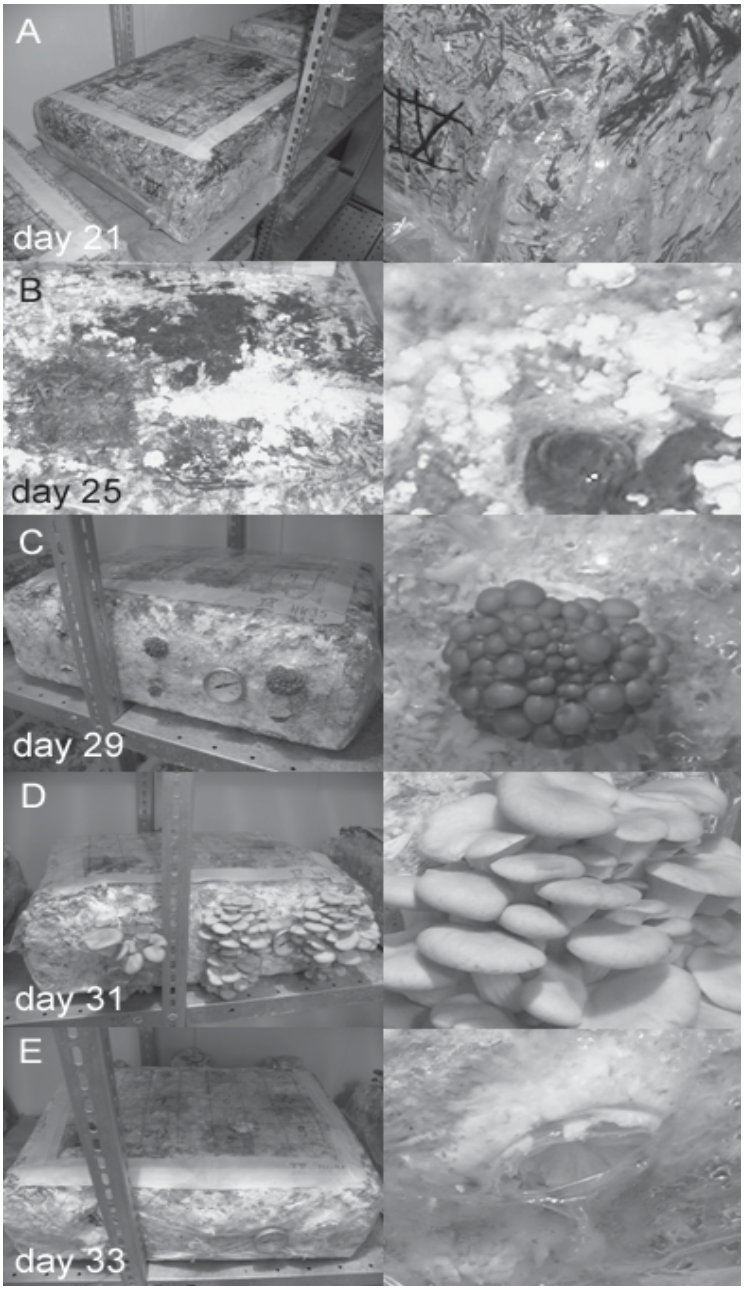

Fig. 1 Wheat straw substrate blocks at different stages of cultivation with *P. ostreatus* strain 030 (2nd experiment) shown in full sight (left panels) and representative detailed views with fungal structures (right panels). Days indicate the age of the substrate block after inoculation with the strain. **A.** A substrate block at day 21 when the whole substrate was colonised by the fungus. **B.** A substrate block at day 25 when the young primordia appeared. **C.** A substrate block at day 29 with maturating fruiting bodies. **D.** A substrate block at day 31 with mature sporulating fruiting bodies. **E.** A substrate block at day 33, after mushroom harvest.

documented by photographs). For both strains, the three flushes occured synchronised in weeks 5, 8 and 10 of cultivation with only slight time shifts between *P. ostreatus* strain 030 and K12 (Fig. 2A and Fig. 3A). Mushroom yields declined with the three flushes from 2.2 kg to 0.5 kg per substrate block (Table 1). Whilst the first flush was confined in both strains to one day, the second flush was spread over a few days and the third flush with one day was short (Fig. 2A, Fig. 3A) with little yield (Table 1). The total mushroom yield (in average 4.1 and 4.2 kg per substrate block) and the total BE (95%) did not differ significantly between the two strains (Table 1).

Table 1: Mushroom yields per substrate block (n=6)

Strain	Fruiting bodies per substrate block				
	1st harvest	2nd harvest	3rd harvest	Total harvest	Total BE(%)
030	2.2 ± 0.4 kg	1.4 ± 0.8 kg	0.5 ± 0.3 kg	4.1 ± 0.8 kg	95 ± 16%
K12	2.2 ± 0.2 kg	1.5 ± 0.6 kg	0.5 ± 0.3 kg	4.2 ± 0.6 kg	95 ± 19%

Ligninolytic enzymes in the large scale experiment

The water content of the substrate blocks was relatively constant over the whole cultivation periods with values between 76% and 78% and the pH of the press juice from the substrate was stable at pH 5.0 ± 0.2, regardless of the *P. ostreatus* strain used. Activities of three types of enzymes (laccase, MnP, VP) were measured in eluates obtained from pressing representative samples of the substrate blocks. The activity of all the ligninolytic enzymes showed a periodical alteration with mushroom production (Fig. 2 and Fig. 3). For both strains, activities of all three enzymes gradually declined with progress of vegetative growth and primordia production to be lowest at the point of mushroom harvest. Also for both strains, there was a sharp increase in activities of all three enzymes shortly after each mushroom harvest. Over the time, the activities curves of all three enzymes in both strains followed similar patterns with maxima and minima occurring at comparable periods. However, highest laccase activities of 1.4 U and 1.1 U per ml press juice (5.45 U/gds and 4.30 U/gds) and 0.9 U and 1.1 U per ml press juice (3.55 U/gds and 3.88 U/gds) were reached for *P. ostreatus* strains 030 and K12, respectively, prior and after the first flush at vegetative growth phases where primordia were not yet formed. At mushroom harvests, laccase activities in the substrate were in the range of 0.2 to 0.5 U/ml (Fig. 2B, Fig. 3B). Maximum MnP and VP activities were observed shortly after the second and the third flush in regenerative phases prior to primordia formation. MnP activities were always about 10 times higher than VP activities (compare Fig. 2C and D, Fig. 3C and D). Maximum MnP activities were 15.0 U/ml and 16.0 U/ml (50.63 U/gds and 55.31 U/gds) (Fig. 2C, Fig. 3C), and maximum VP activities 1.9 U/ml and 1.5 U/ml (5.91 U/gds and 5.58 U/gds) for strains 030 and K12, respectively (Fig. 2D, Fig. 3D). At times of harvest, when all enzyme activities were lowest, minimum values between 0.0 U/ml to 4.5 U/ml were measured for MnP activities (Fig. 2C, Fig. 3C) and minimum values between 0.0 U/ml to 0.3 U/ml for VP activities (Fig. 2D, Fig 3D), respectively. Parallel to the prolonged harvest time of the second flush (Fig. 2A, Fig. 3A), the period of low level enzymatic activities in the substrate was longer at the second flush than at the first flush (Fig. 2B-C, Fig. 3B-D). Since temperature shifts

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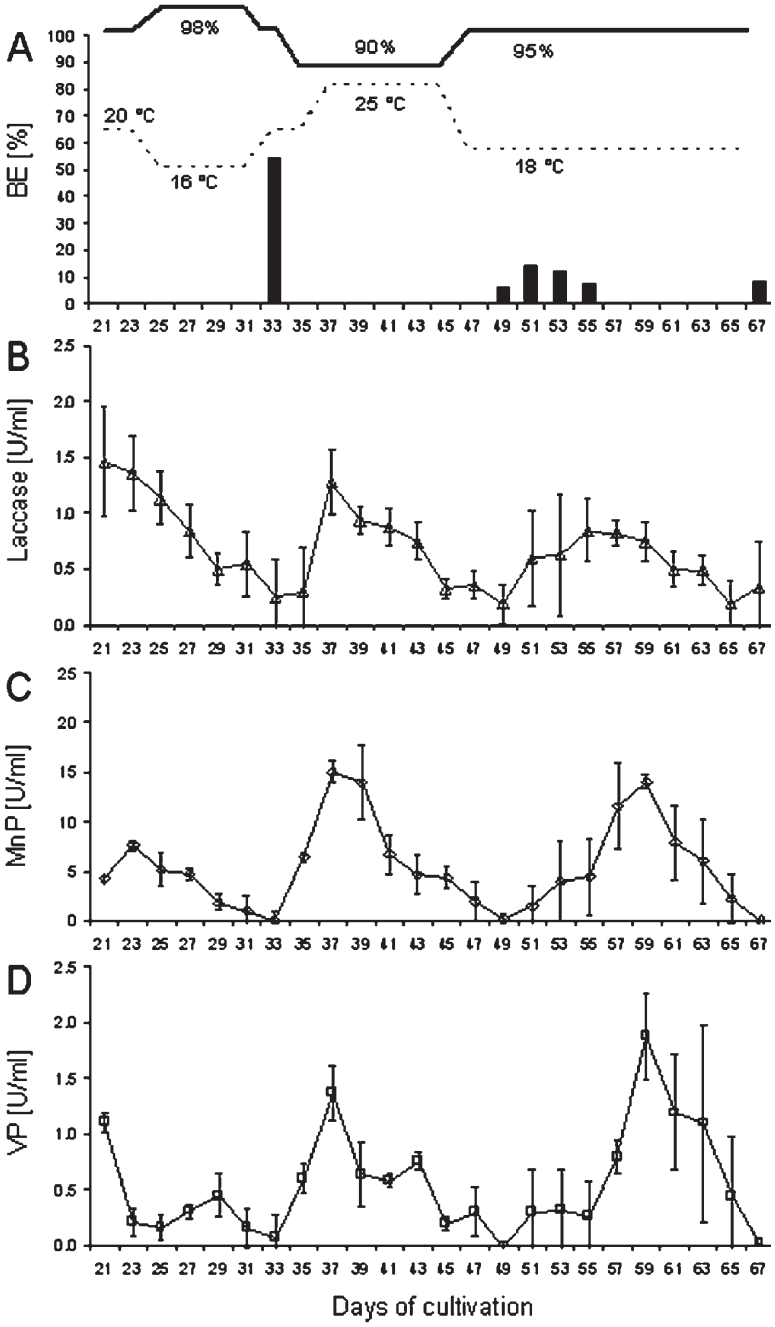

Fig. 2 Fruiting body production (**A**) and ligninolytic enzyme activities (**B.** laccase, **C.** manganese peroxidase = MnP, **D.** versatile peroxidase = VP) during cultivation of *P. ostreatus* strain 030 on industrial wheat straw substrate blocks. Values are calculated averages and standard deviations from six substrate blocks. In **A.**, the environmental settings in the climate chamber (relative humidity: solid line; temperature: dashed line) are given in addition to the biological efficiency (BE) of fruiting body production.

applied in the culturing regime were always done after a drastic change in enzyme activities, respectively not done after the second flush (Fig. 2A, Fig. 3A), we can conclude that changes in enzymatic activities were influenced by the internal physiological conditions of the fungus related to synchronisation within the mycelium of fruiting and non-fruiting periods. Some influence on the absolute level of enzymatic activities by the temperature of the air cannot be excluded but substrate temperature, moisture and pH did not differ significantly by the different settings of environmental conditions in the climate chamber (see above). The microclimate within the substrate might be expected to be more decisive for the enzymatic activities of the submerged mycelium. However, communication with the surface mycelium and the developing fruiting structures must exist in order to regulate enzymatic activities in the submerged mycelium appropriately up or down as required in the cycle of vegetative mycelial growth and sexual reproduction.

Discussion

Various types of oxidative enzymes are produced by white rot fungi in order to make use of lignocellulosic substrates for nutrition (6, 7). In this study, we found commercial *P. ostreatus* strains to produce laccase, MnP and VP in solid state fermentation on wheat straw as described previously for the species in the literature (12, 20, 21). Under conditions of industrial mushroom cultivation using 17-18 kg blocks of wheat straw, enzyme activities in the substrate blocks alternated with periods of mushroom production (Fig. 2, Fig. 3). Mata et al. (11) and Elisashvili et al. (13) reported a similar diametrical behaviour in enzymatic activities (laccase and MnP) and fruiting body production when strains were cultivated on other lignocellulosic substrates (sugarcane bagasse, tree leaves). Mata et al. (11) tested however only the first flush on 0.5 kg blocks with sugar cane bagasse as substrate and Elisashvili et al. (13) apparently also but on 2 kg substrate blocks of either tree leaves or wheat straw. In our study, we followed up the enzymatic behaviour in the substrate over three flushes on large industrial substrate blocks. As in mushroom industry (5), we obtained two flushes with reasonable yields whilst the output of the third flush dropped to a third of the yield of the second flush (Table 1). In industrial production, a third flush is usually not waited for since the earnings expected from a third flush do not outweigh the costs of the prolonged cultivation under a controlled temperature and humidity regime (5). Our total yields in both series of experiments were comparable to standard yields in commercial production (5).

The diametrical cycling pattern of enzymatic activities in the substrate and fruiting body production suggests a clear physiological distinction within cultures of *P. ostreatus* between phases of vegetative growth and phases of sexual reproduction. Lignocellolytic enzymes appear in *P. ostreatus* to be produced for regenerative vegetative hyphal growth but not for fruiting body development. Similarly, high enzymatic activities were observed in lignocellulosic substrates during growth phases in the white-rotting basidiomycetes *Lentinus tigrinus* [laccase and peroxidase (22)], *Lentinula edodes* [laccase and peroxidase (23)] and *Grifola frondosa* [laccase (24)] with drastic reductions in enzyme activities during the period of fruiting body formation. For these species, it is very likely that the enzymes are produced for lignocellulosic substrate degradation in order to provide nutrients for the growing organism. In contrast, in cultures of the compost fungus *Agaricus bisporus*, laccase and peroxidase activities increase in the substrate from vegetative growth to early stages of fruiting body development and drop strongly during fruiting body maturation (25, 26). Furthermore, laccase activities increase greatly in substrates

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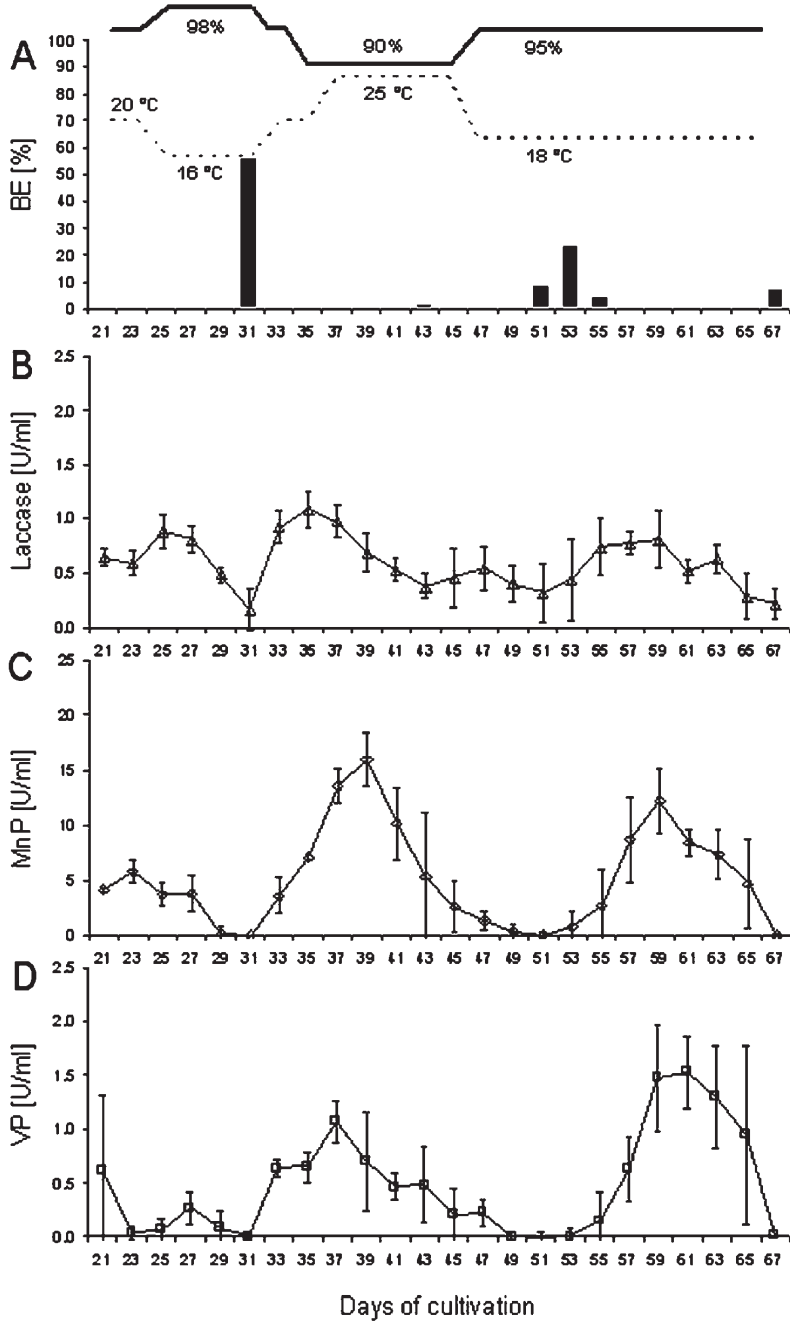

Fig. 3 Fruiting body production (**A**) and ligninolytic enzyme activities (**B.** laccase, **C.** manganese peroxidase = MnP, **D.** versatile peroxidase = VP) during cultivation of *P. ostreatus* strain K12 on industrial wheat straw substrate blocks. Values are calculated averages and standard deviations from six substrate blocks. In **A.**, the environmental settings in the climate chamber (relative humidity: solid line; temperature: dashed line) are given in addition to the biological efficiency (BE) of fruiting body production.

after vegetative growth at fruiting body initiation of cultures of the straw fungus *Volvariella volvacea* and they stay at high level until the fruiting bodies matured (27). If not also for nutritional reasons produced under an alternative regulation regime, enzyme production might be oxidative stress-related (28, 29) and/or the enzymes might have functions in fruiting body morphogenesis and pigmentation (1, 10, 27). The discussion on biological functions of laccases and peroxidases in relation to fruiting body development of basidiomycetes remains still controversial (1, 10).

The anti-cyclic pattern of enzyme production and fruiting body development as observed in our study for *P. ostreatus* is however of a potential biotechnological interest. Industrial enzymes from fungi are mostly produced in bioreactors in liquid cultures but production by solid state fermentation (SSF) finds increasing attention (30-32). *P. ostreatus* has been tested in SSF with wheat straw, canola meal, sawdust based SMS, grapewine sawdust, sugarcane bagasse and tree leaves as substrates in production of phenol oxidases and/or also cellulases (9,11,13,33-37). In small scale production [using either 16 g substrate with grapevine sawdust (9), 500 g bagasse substrate (11), 10 g dry canola meal (35), or 16 g substrate of tree leaves (37)], yields between 0.12-32.2 U/gds and 18.0 U/gds were reported for laccases and yields between 0.37-6.9 U/gds and 1820 U/gds for MnP although values might not all be directly comparable due to the use of different substrates (Table 2). Only Stajic et al. (9) reported also VP activities (measured with DMP = 2,6-dimethoxyphenol). With values between 2.2 U/l to 38 U/l (0.03 – 0.58 U/gds), the VP activities were around 10% of the measured MnP activities. In our studies, with the drastic increase in enzymatic activities two to four days after mushroom harvests, we obtained enzymatic yields of up to 2.9 U/gds for laccase, 45 U/gds for MnP and 5.9 U/gds VP. Compared to the other studies where the same substrates were used as in our study (ABTS, phenol red, Table 2), our enzyme yields are thus evenly good or even better.

Trials of obtaining the free liquid from whole industrial wheat straw substrate blocks with a larger hydraulic press resulted in volumes of about 5-6 l (not shown). Taken at optimum enzymatic activities within the substrate, this volume of press juice should then contain a calculated total amount of about 5.5-6.6 kU of laccase, of about 75-80 kU of MnP and of 7.5-11.4 kU of VP. The raw liquid has however a brown colour, likely caused by a high content of phenolic compounds. Unfortunately, concentrating the enzyme activities by ammonium precipitation did not result in purification of the enzymes from these compounds since they co-precipitated (not shown). Potential applications for the raw enzyme solutions or of concentrated solutions are therefore restricted to processes where a brown coloration does not matter or were presence of extra phenolic compounds might even be of advantage. An example for such case can be the production of wood and paper composites by a two-component system in which the glue consists of ligninolytic enzymes (usually laccase) and phenolic compounds (e.g. lignosulphonate) [reviewed by (38, 39)]. Matcham and Wood (40), Singh et al. (34) and Ko et al. (36) published previously protocols as how to wash out enzymes with tap water and various buffers from SMS of various types of mushrooms (*A. bisporus*, *Flammulina velutipes*, *Hericium erinaceum*, *L. edodes*, *P. ostreatus*). However, procedures were only tested on small scale such as 100 ml and it remains to be shown whether these procedures will be economic enough for obtaining larger volumes of purified enzymes needed for other commercial applications such as in the food and in the pharmacy industries (41, 42).

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Table 2: Laccase and MnP activities of *Pleurotus ostreatus* cultures on different lignocellulosic substrates in SSF

Substrate	Enzymatic yields*				Reference
	Laccase		MnP		
	Value in paper	U/gds	Value in paper	U/gds	
Wheat straw	1.1-1.4 U/ml (ABTS)	3.9-5.5	15-16 U/ml (phenol red)	50.6-55.3	This paper
Grapevine sawdust	8–2145 U/l (SGZ)	0.12-32.2[a]	25-459 U/l (DMP)	0.37–6.9[a]	9
Sugarcane bagasse	0.04 U/g substrate (SGZ)	0.16[b]	455 U/g substrate (DMAB+MBTH)	1820[b]	11
Canola meal	300 nkat/gds (SGZ)	18.0[c]	nd	-	35
Tree leaves	15 U/flask (ABTS)	3.8[a]	7.9 U/flask (phenol red)	2.0[a]	37

* U/gds were calculated from a) the weight of dry substrate and the measured activity given in the paper, b) the weight of the substrate deduced by the known moisture content and the measured activity given in the paper, and c) the conversion of nkat into international units as described in (32). Substrates used in enzymatic tests are given in brackets; ABTS = 2,2‘-azino-bis [3-ethylbenzthiazoline-6-sulfonate], SGZ = syringaldazine, DMP = 2,6-dimethoxyphenol, DMAB = 3-dimethylaminobenzoic acid, MBTH = 3-methyl-2-benzothiazolinone hydrazone hydrochloride. nd = not determined.

Alternatives for beneficial usage of the high enzymatic levels within substrates shortly after mushroom harvest are to apply the SMS or the enzymatic crude extract of SMS in bioremediation of contaminated soils and water. The used up the spent substrate or the crude extract might be mixed with the soil or the water for promoting the degradation of phenolic and non-phenolic aromatic pollutants (43 - 50). For mushroom growers, it might therefore be useful to wait a few days after the final mushroom harvest before disposal of the SMS in order to recycle it in a best possible way at a stage of high enzymatic activities. Currently, schemes for a broad re-use of the mushroom cultivation residues have been established only in China which produces more than 4 million tons of SMS per year (51). In other countries, SMS is still considered as waste but recent changes in legislation in favour of ecological-friendly SMS disposal altered this view in a number of European countries. In a topical review on alternative uses of SMS, Oei et al. judge the extraction of specific proteins as one of the most beneficial potential applications of SMS (52).

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References

1. Kües, U. and Liu, Y. (2000). Fruiting body production in basidiomycetes. Applied Microbiology and Biotechnology, 54: 141-152.

2. Yamanaka, K. (2005). Cultivation of new mushroom species in East Asia. Acta Edulis Fungi, 12 (Supplement): 343-349.

3. Chang, S.T. (2005). Witnessing the development of the mushroom industry in China. Acta Edulis Fungi, 12 (Supplement): 3-19.

4. Breitenbach, J. and Kränzlin, F. (1991). Fungi of Switzerland, Vol 3. Mykologia Luzern, Luzern, Switzerland.

5. Rühl, M. and Kües, U. (2007). Mushroom production. In: Kües, U. (Ed.), Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, pp. 555-586.

6. Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N.S., Hofrichter, M. and Rogalski, J. (1999). Biodegradation of lignin by white rot fungi. Fungal Genetics and Biology, 27: 175-185.

7. Hoegger, P.J., Majcherczyk, A., Dwivedi, R.C., Svobodová, K., Kilaru, S. and Kües, U. (2007). Enzymes in wood degradation. In: Kües, U. (Ed.), Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, pp. 389-438.

8. Cohen, R., Persky, L. and Hadar, Y. (2002). Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. Applied Microbiology and Biotechnology, 58: 582-594.

9. Stajic, M., Persky, L., Cohen, E., Hadar, Y., Brceski, I., Wasser, S.P. and Nevo, E. (2004). Screening of laccase, manganese peroxidase, and versatile peroxidase activities of the genus *Pleurotus* in media with some raw plant materials as carbon sources. Applied Biochemistry and Biotechnology, 117: 155-164.

10. Wösten, H.A.B. and Wessels, J.G.H. (2006). The emergence of fruiting bodies in basidiomycetes. In: Kües, U, Fischer, R. (Eds.), The Mycota, Vol. I, 2nd Ed. Growth, Differentiation and Sexuality. Springer, Berlin, Heidelberg, Germany, pp. 393-414.

11. Mata, G., Cortes, E. and Salmones, D. (2007). Mycelial growth of three *Pleurotus* (Jacq.: Fr.) P. Kumm. species on sugarcane bagasse: Production of hydrolytic and oxidative enzymes. International Journal of Medicinal Mushrooms, 9: 385-394.

12. Savoie, J.-M., Salmones, D. and Mata, G. (2007). Hydrogen peroxide concentration measured in cultivation substrates during growth and fruiting of the mushrooms *Agaricus bisporus* and *Pleurotus* spp. Journal of the Science of Food and Agriculture, 87: 1337-1344.

Current Trends in Biotechnology and Pharmacy, Vol. 2 (4) 478 -492 (2008)
ISSN 0973-8916

13. Elisashvili, V., Kachlishvili, E. and Penninckx, M.J. (2008). Lignocellulolytic enzymes profile during growth and fruiting of *Pleurotus ostreatus* on wheat straw and tree leaves. Acta Microbiologica et Immunologica Hungaria, 55: 157-168.

14. Lelley, J. (1991). Pilzanbau. Biotechnologie der Kulturspeisepilze. Handbuch des Erwerbsgärtners, 2nd Edition. Verlag Eugen Ulmer, Stuttgart, Germany.

15. Royse, D.J. (1992). Recycling of spent shiitake substrate for production of the oyster mushroom, *Pleurotus sajor-caju*. Applied Microbiology and Biotechnology, 38: 179-182.

16. Matsumura, E., Yamamoto, E., Numata, A., Kawano, T., Shin, T. and Murao, S. (1986). Structures of the laccase-catalyzed oxidation-products of hydroxy-benzoic acids in the presence of ABTS (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)). Agricultural and Biological Chemistry, 50: 1355-1357.

17. Childs, R.E. and Bardsley, W.G. (1975). Steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromogen. Biochemical Journal, 145: 93-103.

18. Kuwahara, M., Glenn, J.K., Morgan, M.A. and Gold, M.H. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Letters, 169: 247-250.

19. Michel, F.C., Dass, S.B., Grulke, E.A. and Reddy, C.A. (1991). Role of manganese peroxidases and lignin peroxidases of *Phanerochaete chrysosporium* in the decolorization of Kraft bleach plant effluent. Applied and Environmental Microbiology, 57: 2368-2375.

20. in der Wiesche, C., Wolter, M., Zadrazil, F. and Aksu, S. (2000). Activities of ligninolytic enzymes as a means of for monitoring the colonization of straw substrate pretreated at different temperatures by *Pleurotus ostreatus*. In: van Griensven, L.J.L.D. (Ed.), Science and Cultivation of Edible Fungi. Balkema, Rotterdam, The Netherlands, pp. 391-398.

21. Eichlerova, I., Homolka, L., Nerud, F., Zadrazil, F., Baldrian, P. and Gabriel, J. (2000). Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates. Biodegradation 11, 279-287.

22. Lechner, B.E. and Papinutti,V.L. (2006). Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw. Process Biochemistry, 41: 594-598.

23. Mata, G. and Savoie, J.M. (1998). Extracellular enzyme activities in six *Lentinula edodes* strains during cultivation in wheat straw. World Journal of Microbiology and Biotechnology, 14: 513-519.

24. Xing, Z.T., Cheng, J.H., Tan, Q. and Pan, Y.J. (2006). Effect of nutritional parameters on laccase production by the culinary and medicinal mushroom, *Grifola frondosa*. World Journal of Microbiology and Biotechnology, 22: 1215-1221.

25. Wood, D.A. and Goodenough, P.W. (1977). Fruiting of *Agaricus bisporus* - Changes in extracellular enzyme-activities during

growth and fruiting. Archives of Microbiology, 114: 161-165.

26. Bonnen, A.M., Anton, L.H. and Orth, A.B. (1994). Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. Applied and Environmental Microbiology, 60: 960-965.

27. Chen, S.C., Ma, D.B. and Buswell, J.A. (2003). Induction of laccase activity in the edible straw mushroom, *Volvariella volvacea*. FEMS Microbiology Letters, 218: 143-148.

28. Savoie, J.-M., Minvielle, N. and Largeteau, M. (2008). Radical scavenging properties of extracts from the white button mushroom *Agaricus bisporus*. Journal of Agricultural and Food Chemistry, 88: 970-975.

29. Savoie, J.-M. (2008). Reactive oxygen species and the strategy of antioxidant defence in mushrooms. In: Lelley, J.I. and Buswell, J.A. (Eds.), Mushroom Biology and Mushroom Products. Proceedings of the Sixth International Conference on Mushroom Biology and Mushroom Products 2008. GAMU GmbH, Institut für Pilzforschung, Krefeld, Germany, pp. 8-20.

30. Hölker, U., Hofer, M. and Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Applied Microbiology and Biotechnology, 64: 175-186.

31. Hölker, U. and Lenz, J. (2005). Solid-state fermentation – are there any biotechnological advantages? Current Opinion in Microbiology, 8: 301-306.

32. Rühl, M., Kilaru, S., Navarro-Gonzaléz, M., Hoegger, P.J., Kharazipour, A., Kües, U. (2007). Production of laccase and other enzymes for the wood industry. In: Kües, U. (Ed.), Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, pp. 469-507.

33. Lang, E., Nerud, F., Novotná, E., Zadrazil, F. and Martens, R. (1996). Production of ligninolytic exoenzymes and ^{14}C-pyrene mineralization by *Pleurotus* sp. in lignocellulose substrate. Folia Microbiologica, 41: 489-493.

34. Singh, A., Abdullah, N. and Vikineswary, S. (2003). Optimization of extraction of bulk enzymes from spent mushroom compost. Journal of Chemical Technology and Biotechnology, 78: 743-752.

35. Hu, J. and Duvnjak, Z. (2004). The production of a laccase and the decrease of the phenolic content in canola meal during the growth of the fungus *Pleurotus ostreatus* in solid state fermentation processes. Engineering in Life Sciences, 4: 50-55.

36. Ko, H.G., Park, S.H., Kim, S.H., Park, H.G. and Park, W.M. (2005). Detection and recovery of hydrolytic enzymes from spent compost of four mushroom species. Folia Microbiologica, 50: 103-106.

37. Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T. and Kvesitadze, G. (2008). *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in

Current Trends in Biotechnology and Pharmacy, Vol. 2 (4) 478 -492 (2008) 491
ISSN 0973-8916

submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresource Technology, 99: 457-462.

38. Mai, C., Kües, U. and Militz, H. (2004). Biotechnology in the wood industry. Applied Microbiology and Biotechnology, 63: 477-494.

39. Kües, U., Bohn, C., Euring, M., Müller, C., Polle, A. and Kharazipour, A. (2007). Enzymatic modified wood in panel board production. In: Kües, U. (Ed.), Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, pp. 433-467.

40. Matcham, S.E. and Wood, D.A. (1992). Purification of *Agaricus bisporus* extracellular laccase from mushroom compost. Biotechnology Letters, 14: 297-300.

41. Minussi, R.C., Pastore, G.M. and Duran, N. (2002). Potential applications of laccase in the food industry. Trends in Food Science and Technology, 13: 205-216.

42. Couto, S.R. and Herrera, J.L.T. (2006). Industrial and biotechnological applications of laccase: A review. Biotechnological Advances, 24: 500-513.

43. Eggen, T. and Majcherczyk, A. (1998). Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus*. International Biodeterioration & Biodegradation, 41: 111-117.

44. Lau, K.L., Tsang, Y.Y. and Chiu, S.W. (2003). Use of spent mushroom compost to bioremediate PAH-contaminated samples. Chemosphere, 52 : 1539-1546.

45. Law, W.M., Lau, W.N., Lo, K.L., Wai, L.M. and Chiu, S.W. (2003). Removal of biocide pentachlorophenol in water system by the spent mushroom compost of *Pleurotus pulmonarius*. Chemosphere, 52: 1531-1537.

46. Rodriguez, E., Nuero, O., Guillen, F., Martinéz, A.T. and Martinéz, M.J. (2004). Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. Soil Biology & Biochemistry, 36: 909-916.

47. Polcaro, C.M., Brancaleoni, E., Donati, E., Frattoni, M., Galli, E., Migliore, L. and Rapanà, P. (2008). Fungal bioremediation of creosote-treated wood: a laboratory scale study on creosote components degradartion by *Pleurotus ostreatus* mycelium. Bulletin of Environmental Contamination and Toxicology, 81: 180-184.

48. Rodriguez, E., Pickard, M.A. and Vazquez-Duhalt, R. (1999). Industrial dye decolorization by laccases from ligninolytic fungi. Current Microbiology, 38: 27-32.

49. De Souza, C.G.M., Zilly, A. and Peralta, R.M. (2002). Production of laccase as the sole phenoloxidase by a Brazilian strain of *Pleurotus pulmonarius* in solid state fermentation. Journal of Basic Microbiology, 42: 83-90.

50. Dellamatrice, P.M., Monteiro, R.T.R., Kamida, H.M., Nogueira, N.L., Rossi, M.L. and Blaise, C. (2005). Decolourization of municipal effluent and sludge by *Pleurotus sajor-caju* and *Pleurotus ostreatus*. World Journal of Microbiology and Biotechnology, 21: 1363-1369.

51. Li, X.M. (2003). Development and utilization of residues of edible fungi. Journal of Henan Agricultural Sciences, 5: 40-42.

52. Oei, P., Zeng, H., Liao, J., Dai, J., Chen, M. and Cheng, Y. (2008). Alternative uses of spent mushroom compost. In: Lelley, J.I. and Buswell, J.A. (Eds.), Mushroom Biology and Mushroom Products. Proceedings of the Sixth International Conference on Mushroom Biology and Mushroom Products 2008. GAMU GmbH, Institut für Pilzforschung, Krefeld, Germany, pp. 231-245.

Chapter 3

Morphology of *Coprinopsis cinerea* in submerged cultures

I. Introduction: Fermentation of basidiomycetes in liquid cultures

II. Tools for analysis

III. Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete *Coprinopsis cinerea*

M. Rühl and U. Kües
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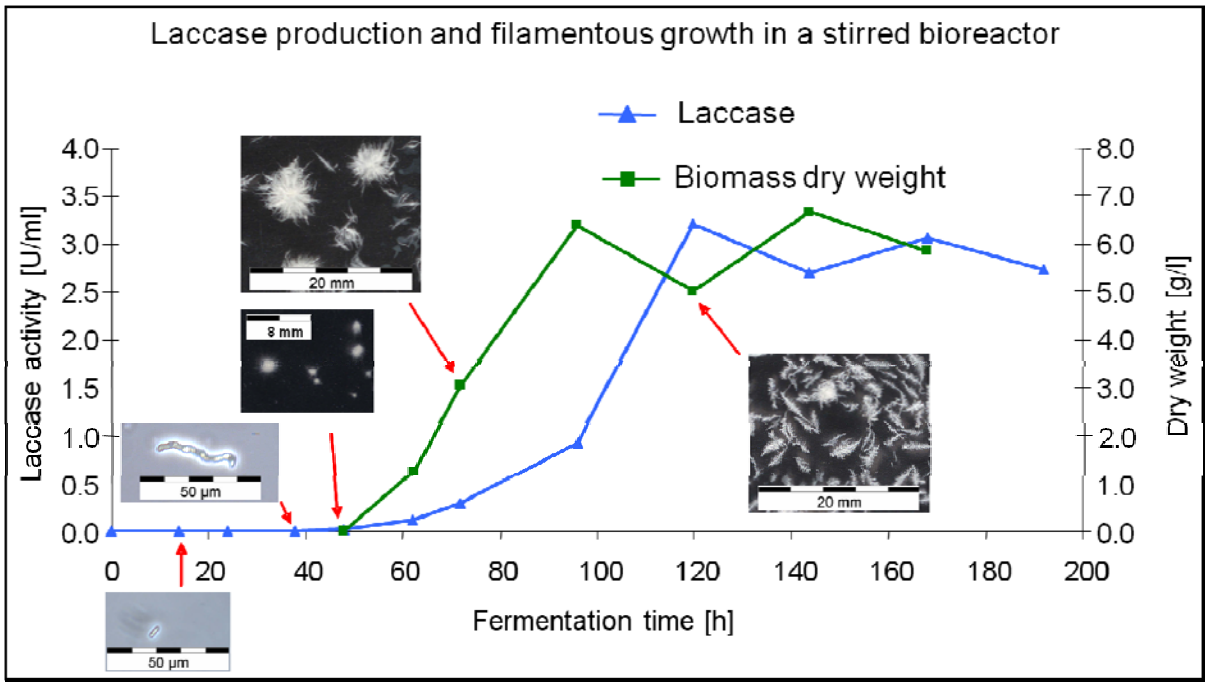
IV. Fermentation of *Coprinopsis cinerea* in shake flasks and bioreactors

V. References



I. Introduction: Fermentation of basidiomycetes in liquid cultures

Filamentous fungi, to which many of the basidiomycetes belong, can grow in liquid culture either in a free form of loose mycelial hyphae or in an aggregated form, called mycelial aggregates or mycelial pellets (Braun and Vecht-Lifshitz 1991, Paul and Thomas 1998, Cui et al. 1998). Normally, on the laboratory scale, flasks are used for the liquid cultivation of fungi either as standing or shaken cultures. On the technical and industrial scale, the microorganisms are grown in vessels called bioreactors (fermenters) which can differ in their mode of mixing. Accordingly, they are referred to as stirring fermenters, airlift reactors or bubble columns (section 2.I). The advantage of the agitated systems over the standing culture is the higher aeration rate and, therefore, better availability of oxygen. In standing cultures, mycelial mats do form at the upper surface of the liquid medium due to a lack of mixing, which would bring the mycelium into the liquid. In comparison, in agitated cultures different mycelial forms can occur. Several factors have an influence on the type of growth in the agitated cultures, such as strain, growth rate, inoculum size, cell-wall composition, nutrition, C/N-ratio, Ca^{2+} concentrations, pH, temperature, dissolved oxygen, solid particles inside the culture broth, fermentation time, surface-active agents, aeration, agitation or stirring speed, respectively shear forces, bioreactor construction and parameters related to the fermenter design, such as foaming (Braun and Vecht-Lifshitz 1991, Cui et al. 1998, Papagianni 2004, Fazenda et al. 2008).
Some factors such as inoculum, oxygen, vessel and impeller type, as well as modes of cultivation will be discussed in the following regarding their effect on fungal biomass, the morphology of the cultivated fungi and yields of different metabolic products (enzymes, acids).

Generally, the production of secondary metabolites (i.e. citric acid, antibiotics) and recombinant production of proteins (i.e. glucoamylase, human interleukin 6) is of great interest. Liquid cultivation studies on fungi concentrate on ascomycetes capable of high product yields for natural production of secondary metabolites and recombinant proteins (Radzio and Kück 1997, Punt et al. 2002, Papagianni 2004, Grimm et al. 2005). Thus, most of the research on fungal morphology was done for filamentous ascomycetes, such as *Aspergillus* and *Penicillium* species either in liquid cultivation in a laboratory scale in shake flask cultures or in a more technical scale in fermenters (Vanhoutte et al. 1995, Cui et al. 1997, Agger et al. 1998, McIntyre et al. 2001, Kelly et al. 2006, Lecault et al. 2007).

Due to the low growth rates and their special requirements for liquid cultivation, basidiomycetes are rarely described in the literature in terms of morphology in liquid cultures. Nevertheless, also basidiomycetes are applied in liquid cultures for production of glucans, peroxidases or laccases (Fåhraeus and Reinhammar 1967, Fenice et al. 2003, Boh and Berovic 2007, chapter 1, section 2.I). Production of native laccases by different basidiomycetes was already listed in this work (Table 4 in section 2.I), but more information on parameters in liquid fermentation in bioreactors is given below (Table 1). Herein, the probably most important parameters on fungal morphology are the stirring speed of the impeller in the stirred vessel reactor, the aeration amount, the pH of the medium and the cultivation temperature (Braun and Vecht-Lifshitz 1991, Cui et al. 1997, Papagianni 2004, Fazenda et al. 2008).

Table 1 Parameters of laccase production with basidiomycete fungi grown in submerged industrial scale bioreactor cultures

		Parameters[3]							
Fungus[1]	**Bioreactor type, size[2]**	**Revolutions per minute**	**Aeration [vvm]**	**pH**	**T [°C]**	**Inoculum [v/v] or [g/l]**	**Working volume [%], (medium vol.)**	**Laccase yield[4]**	**Reference**
Lentinus tigrinus	SV, 3 l	250-500	1.0	-	28	0.9 g/l	66% (2 l)	4.6 U/ml	Fenice et al. 2003
	ALR, 3 l	ns	1.0	-	28	0.9 g/l	83% (2.5 l)	4.3 U/ml	
Pleurotus ostreatus	SV, 10 l	150	1.0	-	28	-	-	874 U/ml	Mazumder et al. 2008
Trametes hirsuta	ALR, 2 l	ns	1.0 l/min	nc	30	**	-	1.0 U/ml	Dominguez et al. 2005
	SV, 10 l	160	0.88	-	28	15%	60% (6 l)	80 AU/ml	Koroleva et al. 2002
Trametes pubesence	ALR, 3.5 l	ns	2.2 l/min	nc	28	10%	-	11.8 U/ml	Ryan et al. 2005
	SV, 20 l	100	0.1-1.25 *	nc	25	10%	-	743 U/ml	Galhaup et al. 2002
Trametes versicolor	SV, 100 l	120	0.1	nc	25	8.3%	60% (60 l)	70 AU	Fåhraeus and Reinhammar 1967
	ALR, 2 l	ns	1.0 l/min	nc	30	**	-	4.0 U/ml	Dominguez et al. 2007
	SV, -	200-350	0.2-1.0	4.5-5.5	22 ± 2	8%	60% (-)	-	Thiruchelvam and Ramsay 2007
	MSR, 5 l	-	-	nc		8%	25% (1.3 l)	1.4 U/ml	

1) corresponding names given in the references: *Panus tigrinus*, *Coriolus hirsuta* and *Polyporus versicolor*
2) ALR: airlift-reactor; MSR: magnetic stirr bar reactor; SV: stirred vessel
3) vvm: gas volume per liquid medium volume per minute; v/v: volume of preculture per volume of fermentation medium; working volume: volume of medium per bioreactor size; nc: not controlled; ns: no stirring (ALR); -: not mentioned in the reference; * controlled to obtain 40% air saturation; ** immobilised in alginate
4) AU: arbitrary units defined in the reference.

Generally, shake flask cultures are used for optimisation of culture conditions, such as media composition, cultivation temperature and pH, prior to technical scale cultivation. The information given in the available literature references only on pellet sizes and, so far, effects by the morphology of the fungi were in the studies mostly neglected (Gehrig et al. 1998, Fang and Zhong 2002, Žmak et al. 2006).

An area in which basidiomycetes play an important role is the solid-state fermentation (SSF). Most basidiomycetes possess a complex enzymatic system for the degradation of lignocellulosic waste materials which may serve as solid substrates (section 2.II). Therefore, SSF provides filamentous organisms with a more natural growth environment and higher oxygen availability (for advantages and disadvantages of SSF see section 2.I). With regard to recombinant protein production, liquid cultivation might be more favourable due to a more easy process of protein purification compared to SSF with lignocellulosic materials, where phenolic substances and other degradation products contaminate the culture broth (section 2.II).

This chapter intends to give a literature overview on liquid cultivation of basidiomycete fungi regarding laccase production in bioreactors (Table 1), morphological structures of the mycelium and parameters affecting the filamentous structure.

A. Important parameters of fermentation on fungal morphology and on production yields of enzymes and other metabolites

1. Inoculum

Cultivation of any microorganism starts with an inoculum. The type and amount of this inoculum for liquid cultures can vary, especially when filamentous organisms growing in multicellular hyphal forms are used. Submerged cultures of filamentous fungi can be inoculated with spores if available (Vecht-Lifshitz et al. 1990, Tucker and Thomas 1992) or with mycelium, which might be available in loose filamentous (Tavares et al. 2005) or pelleted form (Carmichael and Pickard 1989, Thiruchelvam and Ramsay 2007). These forms, normally derived from a pre-culture, can be used directly without homogenisation or after smashing the mycelial agglomerates into smaller fragments. When spores are used, the inoculum level depends on the spore stock preparation, germination of the spores (time and viability) and altering conditions influencing the germination of the spores, like medium, pH and aeration (Tucker and Thomas 1992, Paul et al. 1993).

Advantages of spore inoculi are the countability of the inoculum and, therefore, the reproducibility. On the other hand, the lag phase is longer in cultures where spores are used and a higher contamination danger exists. As most basidiomycetes do not produce asexual spores, filamentous inoculi are usually used. Exceptions, where asexual spores from basidiomycetes were used in liquid cultivation, are *Coprinopsis cinerea* (this study), *Phanerochaete chrysosporium* (Leisola et al. 1985, Jiménez-Tobon et al. 2003, Žmak et al. 2006) and *Punctularia atropurpurascens* (Böker 1990).

2. Oxygen

Oxygen is a limiting factor for growth. When the fungus grows in a pelleted form, a reduction of O_2 inside the pellet can occur which leads to oxygen starvation and, therefore, to cell death and lower yields of enzymes or other metabolites (Michel et al. 1992). To balance this problem, a reduction of the pellet size or an increase of oxygen concentration can be applied. The latter one can be achieved when using pure oxygen instead of compressed air. Kirk et al. (1978) obtained higher lignin degradation activity on synthetic ^{14}C-lignin at 100% oxygen saturation compared to air saturation (21% O_2) in liquid *Phanerochaete chrysosporium* cultures in basal medium (defined medium with glucose as a C-course). Similar observations were made by Dosoretz et al. (1990) who reported an increase in ligninolytic enzyme yield in submerged *P. chrysosporium* cultures when aerating with pure oxygen. However, there was a concomitant higher proteolytic activity and, in consequence, a faster degradation rate of the ligninolytic activity. In their oxygenated cultures, Dosoretz et al. (1990) observed that the *P. chrysosporium* pellets had a dark colour and a smoother surface compared to the pellets from cultures with "normal" aeration showing hairy-white pellets. To clarify the factor for higher ligninolytic enzyme yields in oxygenated cultures of *P. chrysosporium*, Michel et al. (1990) determined the oxygen concentration within fungal pellets. The authors used pellets derived from liquid shaken cultures, which were flushed daily with pure oxygen. The obtained data were used for generating a mathematic model, which allowed predictions of respiration kinetics in liquid cultures on the basis of pellet size and oxygen concentration inside the medium. The authors stated by applying their model on different studies with *P. chrysosporium* that oxygen limitations inside *P. chrysosporium* pellets can be compensated when using pure oxygen.

Table 2 Fungal morphology of basidiomycetes grown in liquid cultures

	Cultivation parameters[1]					Pellet data				
Fungus	**Inoculum: [%] or [spores/ml]**	**T [°C]**	**Revolutions per minute [rpm]**	**Bioreactor type, size**	**Wokring volume [%], (medium vol.)**	**Diameter [mm]**	**Number per volume [ml]**	**Density [kg/m³]**	**Focus of study**	**Reference**
Agaricus campestris	Sc, 0.5 cm[2]	24	150	FL, 250 ml	40 (0.1 l)	up to 6.0[3],[4]	-	-	Pellet production in different media	Martin and Bailey 1985
	Mp, 5%	24	100 200	ALR, SV, 1 l	50 (0.5 l)		-	-	in FL and SV at different rpm	
Coprinopsis cinerea	Mp, 5%	25 37	120	FL, 500 ml	21 (0.15 l)	1 to 3	15 to 45	-	Laccase production and	this study section 3.IV
	Cs, 1.0 x 10^6	37	120	SV, 5 l	90 (0.45 l)	-	-	-	morphological characteristics	
Cyathus striatus	Mp, 2%	25	200	SV, 20 l	-	Up to 6.0	-	-	Striatal production	Gehrig et al. 1998
Daedalea quercina	Mp, -	25	40 60	FL, 1000 ml	10 (0.1 l)	4 to 5 2 to 3	-	-	EPS production	Manzoni and Rollini 2001
Ganoderma lucidum	Mp, 10%	30	120	FL, 250 ml	20 (50 ml)	1.2 to 1.6[5]	-	-	Ganoderic acid production	Fang and Zhong 2002
Ganoderma resinaceum	Mp 4%	30	50 150 300	SV, 5l	60 (3 l)	max 2.4 max 2.1 max 0.5	-	-	EPS production	Kim et al. 2006
Phanerochaete chrysosporium	Mp 10%	39	100 - 260	FL, 125 ml	36 (45 ml)	6.6 to 1.3[4]	0.4 to 36.5	-	LiP production	Michel et al. 1990
	Mp 10%	37	173	FL, 250 ml	34 (85 ml)	1.5 to 2.0	7.4	65	Oxygen limitation and kinetics	Michel et al. 1992
	Cs, 1.0 x 10^4	30	150	FL, 500 ml	32 (0.16 l)	3.5	-	-	Immunolabelling for MnP and chlamydospore like cells	Jiménez-Tobon et al. 2003
	Cs, 3.4 x 10^3	-	150	FL, 1000 ml	30 (0.3 l)	1.0 to 1.2	-	-	Several flask sizes	Leisola et al. 1985
	Cs, 4.5 x 10^6	32	120	FL, 500 ml[2]	20 (0.1 l)	max 2.0	12.0	45	Testing of different vessels	Žmak et al. 2006
Phelinus baumi *Phelinus gilvus* *Phelinus linteus*	Mp 5%	30	150	SV, 5 l	60 (0.3 l)	max 5.2 max 1.9 max 1.1	-	-	EPS production	Hwang et al. 2004
Pleurotus ostreatus	Mp, 5%	26	200 300 400	SV, 14 l	71 (10 l)	3.5 3.2 2.8	-	-	Growth characteristics in liquid culture	Márquez-Rocha et al. 1999
	Mp, -	28	120	FL, 500 ml	50 (0.25 l)	1.0 to 2.0 or 10.0	-	-	MnP production	Ha et al. 2001
Trametes versicolor	Mp, 8%	22 ± 2	200 - 350	SV, -	-	0.5 to 3.0	-	-	Laccase production	Thiruchelvam and Ramsay 2007
Tremella fuciformis	Mp, 2%	28	200	SV, AL 5L	60 (0.3 l)	-[6]			EPS production	Cho et al. 2006

1) AL: airlift reactor; Cs: conidiospores as inoculum (spores/ml); EPS: exopolysaccharides; FL: shake flasks; Mp: mycelial preculture as inoculum (v/v: volume of preculture per volume of fermentation medium); Sc: stock culture from agar slants as a direct inoculum w/o preculture; SV: stirred vessel; -: not mentioned in the reference; 2) comparable to 10 cm flask diameter; 3) morphology cluster into three categories: filamentous growth, ≤ 2 mm, 3 – 6 mm; 4) depends on the rpm value; 5) pellets clustered into three categories: <1.2 mm, 1.2 – 1.6 mm and > 1.6 mm; 6) no filaments, yeast like growth form.

In *Pleurotus ostreatus* shake flask cultures, additional oxygen supply had an impact on pellet size (Ha et al. 2001). A shift in aeration at day 5 of cultivation from compressed air to pure oxygen resulted in a pellet size of 1-2 mm and laccase activities of 5 U/ml. In contrast, in aeration experiments with pure oxygen from the beginning of the liquid culture, pellets of 5-10 mm in diameter and laccase activities of 2 U/ml were achieved. Increased enzyme activity in cultures with smaller pellets seems to be due to a better oxygen supply inside the pellet (Ha et al. 2001) whilst in larger pellets oxygen starvation might still happen, due to the enlarged diameter increasing the length of the diffusion ways (Michel et al. 1992).

3. Vessel and impeller

The aeration of fungal cultures depends not only on the flow rate of compressed air, pure oxygen or other gases, but also on the quality of mixing of the broth (section 2.I). This dispersion is guaranteed by the design of the bioreactor and the used impellers, which are also affecting the mycelial morphology (Cui et al. 1998). On the other side, high biomass concentrations in the bioreactor vessel lead to non-Newtonian fluids (the viscosity of the fluid is not stable), lower oxygen supply and bad heat transfer within the bioreactor. Therefore, an adequate type of mixing is required for optimal fermentation conditions. However, with higher stirring and agitation rates an increased shear stress towards the hyphae, either in pelleted or filamentous form, emerges and, thus, is critical on fungal morphology.

Next to the agitation speed, the impeller (Fig. 1) has a direct influence on the filamentous growth form of the organism: Paddle impellers and rushton turbines showed less damage to freely dispersed *Penicillium chrysogenum* hyphae compared to pitched blade impellers (Jüsten et al. 1996). In bioreactor cultures of the basidiomycete *P. ostreatus*, a rushton turbine was compared with two other impeller types: a helical ribbon and an intermig impeller (see http://www.ekato.eu/en/products/ekato-rmt/products/ekato-impellers/ for the different impeller types) (Márquez-Rocha et al. 1999). At similar agitation speeds of the tested impellers, the size of fungal pellets varied in diameter with 2.3, 3.5 and 5.1 mm for cultures mixed with helical ribbon impeller, rushton turbine and intermig impeller, respectively. On the other side, the specific growth rate of *P. ostreatus* increased with decreasing pellet diameter (Márquez-Rocha et al. 1999). So far, this is the

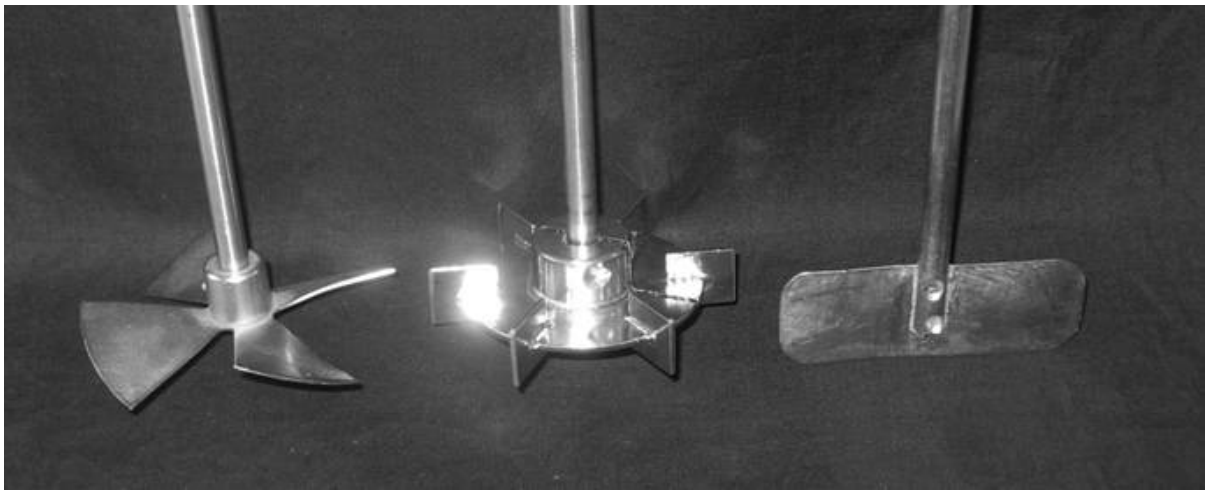


Fig. 1 Impeller types: pitched blade impeller (left), rushton turbine or disk stirrer (middle), paddle impeller or blade stirrer (right).

only study with different impeller types in basidiomycete culture, but a more general comparison between impeller and other type of agitation exists. In a stirred vessel of the type BioFloIIC (New Brunswick Scientific) with rushton turbines, the white-rot fungus *T. versicolor* grows in a pellet form with variable diameters of 0.5 to 3 mm, whereas a self-assembled magnetic stir bar reactor (MSBR) gave smaller pellets of more unique size (~1 mm) (Thiruchelvam and Ramsay 2007). This shows that impeller types have an direct impact on the fungal morphology, beside their main aim of mixing the culture broth.

Almost no studies on pellet morphology are available on large scale for basidiomycetes, but lab scale studies can give an overview (Table 2). Generally, it seems that with higher agitation rate of flask cultures the pellet diameter decreases, with diameters of 6.6 mm at 100 rpm, 1.0 to 3.5 mm at 100-200 rpm and below 1.0 mm with agitation rates higher than 200 rpm (Table 2). In a study with the white rot fungus *Lentinus tigrinus*, pellet morphology was not discussed, but the mode of agitation (stirred vessel versus airlift-reactor) had an influence on the yield of enzyme. In the stirred vessel, an agitation rate of 250 and 500 rpm gave best results for laccase yields of 4.6 and 4.0 U/ml, respectively. The used aeration rate of 1.0 vvm gave highest laccase yields, whereas higher aeration increased fungal biomass, but decreased laccase activity (Fenice et al. 2003). In contrast, *L. tigrinus* showed higher MnP yields in bubble columns compared to stirred vessels (Quaratino et al. 2006). Lower shear forces and better oxygen supply in the bubble columns might be the reason for higher enzyme yields, but it shows also that probably for each fungus a new approach is needed for defining best fermentation conditions.

When looking more closely at the pellet morphology, it is seen that the inner structure of the pellets might be influenced by agitation rate or stirrer speed. Evidence of this comes from

shaken cultures of *P. chrysosporium* (Michel et al. 1990, 1992), in which pellet morphology depends on the agitation rate of the shaking flask (Table 2). Cross sections of pellets obtained at lower speed (100 rpm, pellet Ø 6.6 mm) showed a hollow centre with an outer shell of about 1 mm. Higher agitation rates of 150 and 200 rpm resulted in smaller pellets between 1-2 mm in diameter and, concomitantly, there was an increased enzymatic yield with an optimal extra-cellular LiP activity of 341 and 376 U/l, respectively (Michel et al. 1990).

Also the working volume, which is defined as the volume of medium per vessel volume and given in percentage, has an impact on fungal morphology in flask cultures, whereas larger culture volumes seems favorable over small ones as stated by Leisola et al. (1985), who used a culture broth to flask factor of 0.3.

4. Mode of cultivation

The process of submerged cultivation can be classified into discontinuous (batch and fed-batch) and continuous cultures. In batch and fed-batch cultures the product (metabolite or biomass) is harvested at the end of the process, whereas in continuous mode the culture is kept at a steady state and harvest takes place continuously (see section 2.I for further explanations). Most of the cultivations are done in batch and sometimes in fed-batch mode, but very rarely continuously. The complexity of the problems for fermentation of filamentous organisms is even higher in continuous cultures than within normal batch operations (Papagianni 2004). An example for a continuous cultivation system is given by Yao et al. (1995), who cultivated the basidiomycete *Arthromyces ramosus* for peroxidase production. The authors used a stainless steel mesh with a specific size to retain the mycelium of *A. ramosus* inside the stirred vessel bioreactor just above its elutriation point, where the mycelial biomass would be washed out from the fermenter. A comparison between two mesh sizes (1 mm and 2 mm) and a normal continuous culture, where the culture broth was abstracted from the bioreactor without a mesh, revealed that the mesh with a size of ~ 2 mm was most adequate for *A. ramosus* cultures. In the continuous culture with a mesh size of 2 mm, the production rate of the peroxidase increased up to 3.2 times compared to the fed-batch culture and this rate was kept for over 100 h (Yao et al. 1995). The fine mesh (1 mm) retained almost all mycelia and, therefore, leads to high biomass yields but low productivity. In continuous cultures without application of a mesh, the mycelium was washed out from the fermenter. By altering the mesh size, this technique could be applied to other

filamentous organisms, having a different morphological pattern in submerged agitated cultures.

B. Conclusion

The overview from literature data given here showed that several factors in liquid fermentations have an influence on fungal morphology and, therefore, on the productivity of enzymes of interest. Some factors, such as vessel and impeller type, are defined by the construction of the bioreactor. Some cultivation parameters, such as the inoculums size, are defined before the submerged fermentation start. Others, such as oxygen concentration, pH and temperature, change during cultivation. Generally, these latter parameters can be automatically controlled in bioreactors in contrast to shake flask cultures, where controllable parameters are normally restricted to temperature and agitation. Control of fermentation parameters requires standard methods for observation of the filamentous growth, which can help to clarify the relation between fungal morphology and growth conditions (section 3.II). As already stated in section 3.I, most of the available studies focus on the morphology of filamentous ascomycetes and only some studies reveal an insight into the filamentous growth of basidiomycetes in submerged cultures. More studies on the fermentation of basidiomycetes are needed to understand their morphological behaviour in shake flask cultures, stirred bioreactors or other fermentation vessels (Fazenda et al. 2008).

II. Tools for analysis of biomass and morphology of organisms

Much is known about the submerged cultivation of unicellular bacteria and yeasts. Many textbooks, dealing with submerged fermentation (SmF) and techniques used for process control of liquid cultures, restrict the knowledge on single cell cultures. A more complex growth has to be considered when talking about the fermentation of filamentous microorganisms and fungi in particular (Paul and Thomas 1998). The determination or control of the morphology of the filamentous organisms is very important, as it has an impact on many different parameters during the cultivation: rheology of the culture broth, oxygen transfer, heat transfer and also productivity (Paul and Thomas 1998). The observation of the fungal morphology during cultivation is one important factor for the understanding of fungal behaviour and for later applications, where production of valuable metabolites is of interest.

A. General biomass determination

Online biomass monitoring of single cell cultures is a standard process using the measurement of the optical density (turbidimetry) with photometers either in a cuvette outside of the bioreactor or directly inside the vessel (Chmiel 2006). For a direct measurement of the biomass of filamentous organisms, determination of an OD does not make much sense, since the measuring of the medium with variable amounts of hyphal fragments can thus result in an under- or overestimation of biomass, e.g. when evaluating the free medium part of samples with very large pellets. In contrast, the large pellets themselves would completely darken the small light beam of the photometer.

Special developed sensors for biomass monitoring offer a more easier and simpler approach than OD_{600} measurements. Such new sensors are based on the possibility to polarise intact cell membranes which then act as small electrical capacitors. The electrical signal is then correlated to the cell surface, respectively cell amount (AberProbe™ + BiomassMonitor by www.aberinstruments.com, BIOMASS system by FOGALEnanotech www.fogalebiotech.com). Although this kind of sensors were initially developed for single cell cultures, the system was already tested also for the filamentous organism *Absidia corymbifera* grown in pellet-like form (see FOGALEnanotech www.fogalebiotech.com). Another method for direct online monitoring of cell growth in bioreactors was presented by Bittner et al. (1998). The sensor used in this study consists of a stainless steel tube with a sampling chamber at its end of 2.2×10^{-8} ml. The trapped cells can be observed by a charge-coupled device (CCD) camera

via the in situ microscope attached to the tube. Besides the biomass of the cultivated ascomycetous yeast *Saccharomyces cerevisiae*, the authors could also define cell amount and size (Bittner et al. 1998).

Another optical method is the laser scanning microscopy (a laser beam can be scattered by particles and the scattered light is collected by a photo detector), which can be used as a sensor for monitoring of particle sizes. This technique was used for on-line determination of size and concentration of aggregated spores of *Aspergillus niger* cultivated in a stirred vessel (Grimm et al. 2004). The same group analysed also later stages of growth in *A. niger* cultures by light microscopy combined with an image analysis system (Kelly et al. 2006).

Anyhow, except for the first stages of fungal growth in liquid cultures (spore germination and aggregation), sensors can not yet observe and describe the morphology of the fungal growth either in pellet like or free filamentous form. Thus, techniques to observe and analyse fungal morphology are needed. An introduction on different methods is given below.

B. Approaches to determine morphological characteristics of free hyphae and mycelial pellets

First of all, the observation of filamentous growth depends on the form of mycelia – the fungal morphology. Generally, filamentous morphology changes during cultivation and different forms of growth and development may be observed such as spore germination, first aggregation of hyphae, hyphal aggregation into small clumps and pellet formation (Fig. 1).

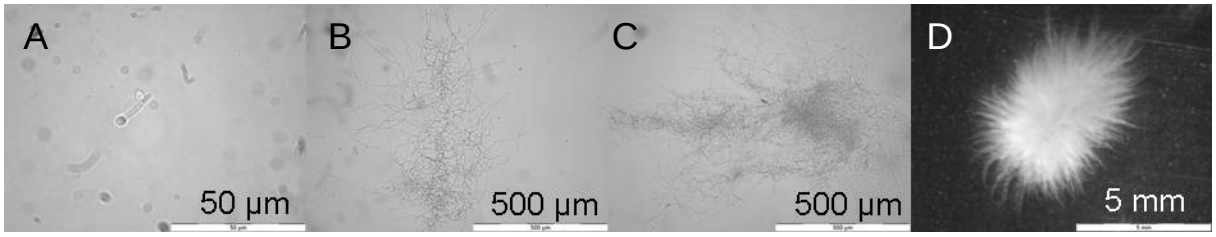


Fig. 1 Filamentous growth forms of a recombinant laccase producing *Coprinopsis cinerea* FA222 transformant (Kilaru et al. 2006, section 3.IV) in a stirred vessel. (**A**) Spore germination, (**B**) Hyphal aggregation, (**C**) Hyphal clump formation and (**D**) fungal pellet. The fungus was grown in a self-assembled stirred vessel (2 l) with a rushton turbine in glucose based medium (section 3.IV) at pH 7, 120 rpm stirrer speed and 37 °C. Images **A**, **B** and **C** were taken with the help of a microscope (Axioplan 2, Carl Zeiss MicroImaging GmbH, Göttingen) and image **D** was taken with the help of a binocular (Stemi 2000-C, Carl Zeiss MicroImaging GmbH, Göttingen). This experiment was a pre-study done for the master thesis of Max Richter (Richter 2008).

Upon spore germination (section 3.I.A), the filamentous organism can either stay in its freely dispersed hyphal form (filamentous growth) or may aggregate into clumps and pellets (pellet growth). For both morphological forms different parameters can be observed, such as tip elongation, septation and branching of hyphae, as well as pellet size, shape and hairiness (Fig. 2).

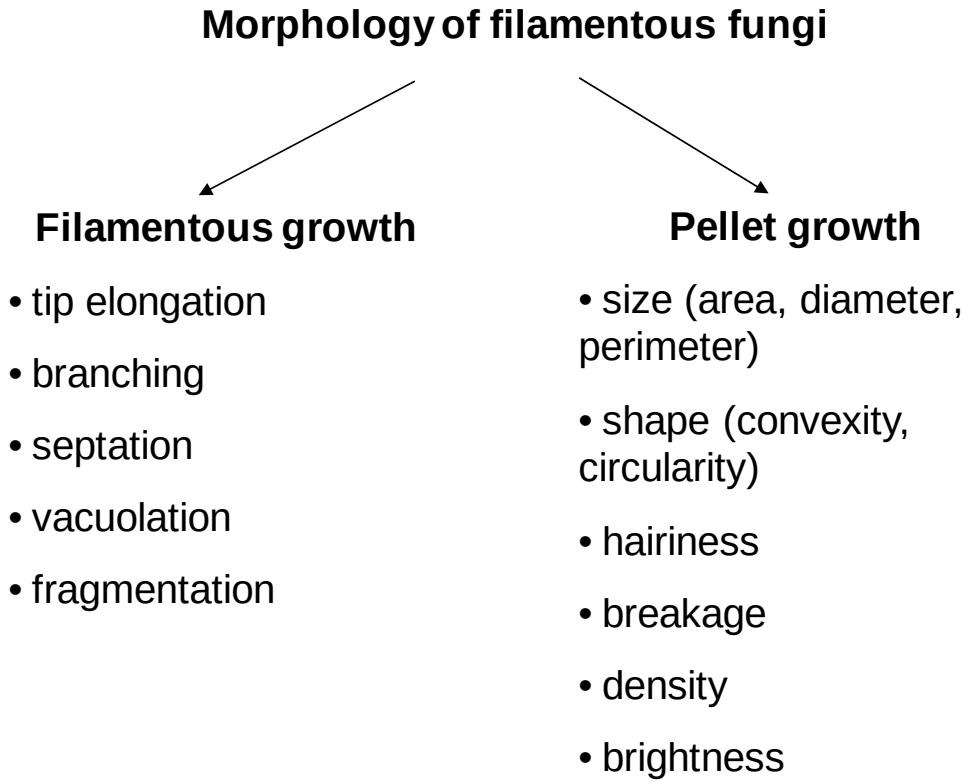


Fig. 2 Typical parameters that are observed during cultivation of fungi for their definition of the filamentous growth forms as adapted from Paul et al. (1993) and Lejeune and Baron (1998).

For pellet description, the most commonly used parameter is the pellet size which can be determined mechanically by sieving fungal cultures with different mesh sizes (Michel et al. 1990, Fang and Zhong 2002). Nevertheless, this technique restricts the user to only one parameter and, thus, other methods are more adequate than sieving to observe and analyse filamentous growth in liquid cultures. Some possible techniques are described in the following.

1. General image analysis

The most common approach to observe parameters indicated in Fig. 2 is image analysis. The image analysis consists of the sample preparation, image acquisition and image evaluation. Sample preparation includes sampling of aliquots from the fungal culture, dilution of the sample and slide preparation for image acquisition. To record the image, a microscope with appropriate magnification and a camera, normally a charge-coupled device (CCD) camera, is needed. With the following computer based analysis of the recorded image the different morphological parameters listed in Fig. 3 may be described and interpreted. Such factors which can be obtained by image analysis were listed by Paul and Thomas (1998). In their study, the authors presented four different image approaches which represent all possible forms (Fig. 1): i. dispersed morphology ii. pellet morphology iii. spore germination and iv. hyphal differentiation. The authors divided the parameters needed for all forms of filamentous growth into object size (area or projected area, perimeter, convex perimeter, …), object count (number of pellets, other objects or hyphal tips), object shape (circularity, roughness, …), object position (coordinates of an object) and object brightness (grey intensity of an object in a monochromatic image). Generally, since size and type of spore germination, hyphal differentiation and pelleted morphology vary to a high degree between fungal cultures, it may be necessary for each growth form to apply different observation techniques (Paul et al. 1993, Paul and Thomas 1998). In many studies in the literature, only pellets were observed and analysed. Parameters were for example the shape, such as hairy or smooth pellets, distribution of pellets within the culture liquid, sedimentation of pellets, size and viscosity of the culture fluid (Cox and Thomas 1992, Reichl et al. 1992b, Gehrig et al. 1998, Znidarsic et al. 2000, Žmak et al. 2006). Nevertheless, analysis can be more complex as found for *Aspergillus oryzae* in the study of Carlsen et al. (1996), who determined hyphal tip elongation, pellet size and pellet fragmentation.

2. Automated image analysis

Manual image analysis, as used in most of the above mentioned studies, is very time consuming and less adequate than computer based image analysis software. Due to lower prices and improvements of the hard- and software for image processing, automation of image analysis is nowadays a standard method. One main advantage is the tremendous gain of time, as shown for an automated image analysis via a microscope with subsequent data processing which reduced the time of pellet analysis from 67 s to 4.6 s per pellet (Cox and Thomas 1992). Nevertheless, on-line measurement together with automated image analysis

would enable to control the fermentation of filamentous organisms in liquid cultures (Reichl et al. 1992b). Up to now, different solutions for combinations of on-line observation and automated image analysis were presented. An on-line monitoring for filamentous organism was implemented using a growth chamber which was placed under the microscope (Reichl et al. 1992a, Spohr et al. 1998). The authors fixed spores of the filamentous bacterium *Streptomyces tendae* and of the fungus *A. oryzae* with poly-d-lysine on the bottom of the growth chamber and observed hyphal growth and branching after germination. Because of the small chamber volume (80 µl in Spohr et al. 1998 and 1.2 ml in Reichl et al. 1992a), such a system is restricted to basic studies for spore germination and hyphal growth. A further development to the simple growth chamber is a flow-through cell, which is connected with a bioreactor (Treskatis et al. 1997). In the study by Treskatis et al. (1997), samples with *S. tendae* cells were automatically taken and diluted and pumped into a flow-through chamber which was placed under an inverted microscope. Sample images were taken with a CCD camera. The imaged sample was then discarded and the picture was analysed by a personal computer (Treskatis et al. 1997). Although the chamber of the flow-through cell used by Treskatis et al. (1997) had a height of 2 mm, pelleted growth would be hard to observe without damaging the pellets because pellets tend to be larger than 2 mm in diameter (Table 2 in section 3.I).

A main problem which is a consequence of these ex-situ (out of the bioreactor vessel) measurement systems is the loss of culture volume, due to disposing of the sample. Especially in small fermentation system this is a bottleneck (Chmiel 2006). Anyhow, on-line measurement of the fungal morphology as described above is restricted due to the high complexity of automatic sampling and appropriate dilution of the sample. Therefore, commercial products, i.e. sensors for measuring the size and distribution of particles, are needed.

3. Methods for a more precise image analysis

For observation of cells of microorganism, different staining methods can be applied to compare between viable and dead cells (Bittner et al. 1998) in single cell cultures and in filamentous growth stages (Agger et al. 1998). For filamentous fungi, several fluorescence staining compounds were applied.

The fluorescence dye Mag fura (tetra potassium salt), which detects divalent ions (i.e. Mg^{2+}), was used to stain metabolic active hyphae of the ascomycetes *Aspergillus nidulans,*

A. oryzae and *Penicillium chrysogenum* in order to analyse fragmentation of hyphae (Cox and Thomas 1999, McIntyre et al. 2001). In another study, differentiation of *A. oryzae* hyphae were visualised by the fluorescence dye $DiOC_6$ (3,3'-dihexyloxacarbocyanine) which enrich in the mitochondria of active fungal cells (Agger et al. 1998). To have a comparison for the complete biomass, Calcofluor white was used in all three above mentioned studies since this dye stains ß1-4 glycosidic linkages present in chitin in the cell wall independently whether the cells are dead or alive. Another possibility to determine active fungal biomass is the usage of fluorescein diacetate (FDA) (Ingham and Klein 1982). FDA is proposed to be taken up into living cells by active transport. Within the cells it is hydrolysed by esterases. One product of this hydrolysis is fluorescein leading to intracellular green light emission [λ_{max} = 514nm]. In the study of Ingham and Klein (1982), the active biomass was compared with total bio dry mass, as well as glucose and oxygen consumption.

One single substance which can be used to differentiate between both, active parts and total biomass, can ease the analysis. In *A. niger* liquid cultures, the fluorescence dye acridine orange was used to differentiate between active and non-active parts in dispersed hyphae and pellets (El-Enshasy et al. 2006). This dye emits red light [λ_{max} = 650 nm] when bound to single stranded RNA which indicate active parts of the mycelium and green light [λ_{max} = 525 nm] when bound to DNA. The authors observed with this technique the influence of different agitation rates on fungal morphology. Increased agitation rates (200, 500 and 800 rpm) resulted in a more filamentous growth of *A. niger*, but also in higher shear stress and, therefore, lower biomass. Intermediate agitation speed resulted in small pellets which had an active outer zone and seems to be best for production of glucose oxidase (El-Enshasy et al. 2006).

A different approach to visualise active parts of growing hyphae is the usage of reporter genes, such as the green fluorescence protein (GFP) of the jellyfish *Aequorea victoria*. GFP emits green fluorescence when activated by UV-light and, thus, can indicate parts of active hyphae. An overexpression of GFP in a filamentous organism may help to clarify questions regarding hyphal activity, heterologous protein production and filamentous growth. For this purpose, a transformation protocol is needed for each fungus, which makes this approach currently inadequate for most of the basidiomycetes. Nevertheless, several studies with basidiomycetes exist where GFP was overexpressed under control of a promoter, whose regulation was observed with this technique. In the symbiotic fungus *Hebeloma cylindrosporum*, it was shown that the *C. cinerea cgl1* promoter fused to the *gfp* gene (*cgl1*-promoter::GFP) was repressed at higher glucose concentrations (Rekangalt et al. 2007). In a

recent study, the serine proteinase 1 (SPR1) expression was analysed via GFP production under the control of the *spr1* promoter (*spr1*-promoter::GFP) in *Agaricus bisporus* and *C. cinerea*. GFP was found differently expressed in various types of fruiting body tissue (Heneghan et al. 2009). Another development is the usage of a gene fusion construct, where a gene of interest is fused in frame to the GFP gene and, therefore, the resulting fused protein can be observed in the organism. For example, this was done for the plant pathogenic basidiomycete *Ustilago maydis* where the motor protein Kin2 was fused to GFP (Kin2::GFP) and, thus, could be localised in hyphal cells (Lehmler et al. 1997).

In the ascomycete *A. niger*, such fusion gene constructs were already applied in studies of fungal morphology in liquid culture (Xu et al. 2000, Wang et al. 2003, Talabardon and Yang 2005). In these studies, a glucoamylase-GFP construct was used to monitor secreted protein concentration and compared with protease activity. Xu et al. (2000) varied the spore inoculum level in liquid shaken cultures to optimise pellet size with regard to highest GFP yields and low protease activity. The optimal pellet had an average diameter of 1.6 mm when a spore inoculum of 4 x 10^6 spores/ml was used. Further to this, in bioreactor cultures of *A. niger*, the influence of the type of fermentation system (SmF and SSF) on heterologous protein production and secretion as well as fungal morphology was observed with the glucoamylase-GFP construct (Talabardon and Yang 2005).

An obstacle important to observe is the altered growth of positive *gfp*-transformants compared to the wild type strains (Bae and Knudsen 2000) and also between different clones (own observation documented in Fig. 3). Thus, for a definite conclusion more transformants have to be studied for their growth behaviour in liquid cultures.

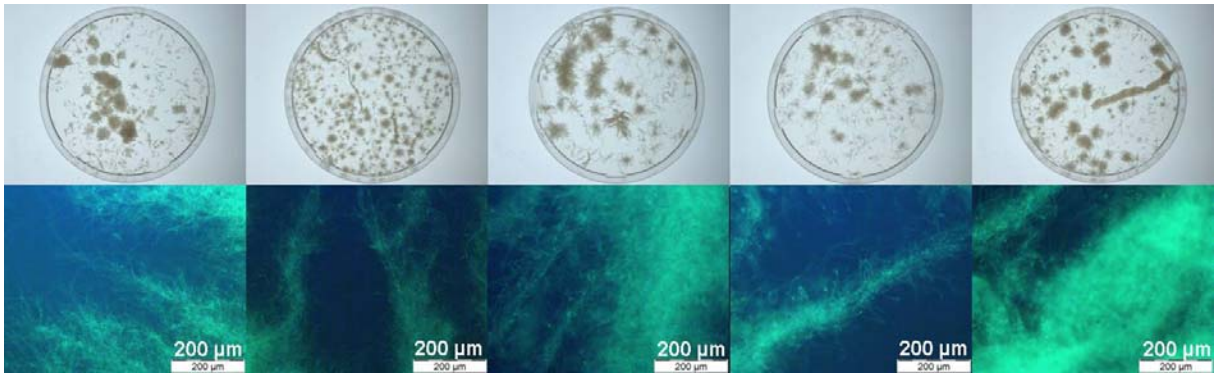


Fig. 3 Images of five different *C. cinerea* GFP expressing clones of strain FA2222 (transformation of e*gfp* under the control of the *A. bisporus gpdII*-promoter in the construct pYPH8 provided by P.J. Hoegger) grown in modified Kjalke medium in shake flasks at 120 rpm and 37 °C after inoculation with a 4 day old pre-culture. Sample of the culture broth of the different clones with different sized pellets at day 4 of cultivation are shown in Petri dishes (upper row) and fluorescence microscopic images (lower row).

Besides appointing the GFP reporter system for detection of active cells, detection of a housekeeping or otherwise interesting protein via histochemical staining or specific visible substrates in case of an enzymatic activity can bring respective information (Moukha et al. 1993, Goodell et al. 1998, Bittner et al. 1998, Jiménez-Tobon et al. 2003, Mander et al. 2006, van der Klei and Veenhuis 2007).

4. Conclusion

In summary, image analysis for description of pellet morphology and diversity in filamentous growth of fungi is a helpful and important tool in control of bioprocesses where filamentous organisms are used (Znidarsic and Pavko 2001) due to the fact that productivity of fungal metabolites and enzymes can depend on the morphology in liquid cultures (Gehrig et al. 1998).

A combination of image analysis (observation of filamentous growth) with either overexpression of GFP or other specific staining methods (detection of intracellular activities) may result in a more comprehensive overview on fungal morphology and productivity in liquid culture.

III. Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete Coprinopsis cinerea

A. Abstract

In this study, an image analysis system for fungal pellets was developed using the commercial software system analySIS® and the protocol was evaluated in morphological studies of pellet formation in submerged cultures of the basidiomycete *Coprinopsis cinerea*. Pellets were analysed on large scale (60 to 130 pellets per image, 225 to 400 pellets per culture). Morphologies of pellets were characterised by the parameters grey value, pellet area, convexity, shape factor, sphericity and pellet diameter. Threshold values were defined for all parameters for object filtering. By application of the parameter filter, aggregated hyphal fragments present in larger amounts particularly in cultures grown at higher temperature (37 °C) could be clearly distinguished in image analysis from compact pellets. At a lower growth temperature (25 °C), there was little background of loose hyphal material, fungal pellets were regularly shaped and the pellets remained constant in shape and size over a longer cultivation period.

B. Introduction

Various filamentous fungi are used in biotechnology for production of biomass, secondary metabolites, polysaccharides and/or enzymes and other proteins. Usually, filamentous fungi are cultivated in liquid medium in suitable fermenters. Typically, the fungi will grow in a pelleted form (Braun and Vecht-Lifshitz 1991, Fazenda et al. 2008). Determination of pelleted growth of filamentous organisms in liquid cultures is mostly done by image analysis, which can be conducted manually (Michel et al. 1990, Nielsen et al. 1995) or automatically (Màrquez-Rocha et al. 1999, Kelly et al. 2006). Automatic image analysis implies that pictures of the pellet culture are taken and analysed by a computer based programme regarding specific parameters, which can be e.g. pellet concentration and pellet diameter. Studies exist where such an automatic image analysis was performed on liquid cultures of basidiomycetes (Gehrig et al. 1998, Hwang et al. 2004, Kim et al. 2006).

In an early study, Michel et al. (1990) analysed manually just 15 pellets obtained from *Phanerochaete chrysosporium* cultures with respect to pellet diameter. Later on, Márquez-Rocha et al. (1990) determined by automated image analysis the diameter of

Pleurotus ostreatus pellets but only 20 pellets per culture were surveyed. However, also in more recent studies of three *Phellinus* species and of *Ganoderma resinaceum* (Hwang et al. 2004, Kim et al. 2006) only the diameter of 50 pellets were analysed per fungal sample. In contrast, Gehrig et al. (1998) gave a more complete picture of pellets of *Cyathus striatus* during a fermentation process. The authors analysed in total about 2000 pellets and a broad spectrum of parameters, such as pellet concentration, pellet diameters, total pellet volume (by using average diameter values) and pellet density (from the total biomass dry weight and the total pellet volume).

The studies have in common that all used a CCD camera to obtain photos either on the microscopic scale or on a larger scale with images of areas of up to a few cm^2 [e.g. 2.25 cm^2 in the study by Gehrig et al. (1998)]. The photos were required for determination of pellet parameters. For evaluation of the images, different image analysis software was applied, some of which were commercially (Kelly et al. 2006, Kim et al. 2006) and some of which were especially written for the analysis of pellet morphology [programme by Defren (1993) appointed in Gehrig et al (1998)].

In the following, using shake flask cultures of the basidiomycete *Coprinopsis cinerea*, a protocol is defined to be applied for morphological studies of fungal pellets and bioreactor cultures. For the analysis of the fungal morphology in liquid cultures, complete shaken flask cultures or samples taken from the bioreactor cultures are poured onto a bordered glass plate. Detailed images are taken with a CCD camera and the software analySIS® (Soft Imaging System GmbH, Münster, Germany) is used to analyse the fungal morphology from obtained images. The so obtained raw data are filtered by defined parameters that unequivocally distinguish pellets from other objects (small hyphal fragments and loosely aggregated hyphal filaments). Data processing obtains afterwards the final data describing the actual fungal morphology (Fig. 1).

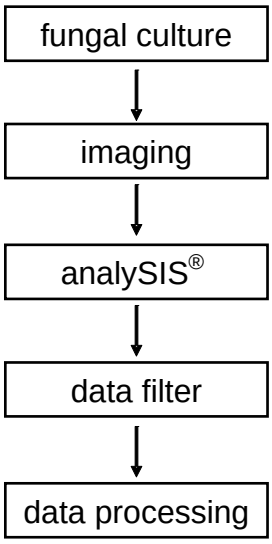


Fig. 1 Flow chart of the steps during the process of pellet morphology determination.

C. Material and Methods

1. Fungal cultures

A monokaryotic *C. cinerea* strain (FA2222) transformed with the pYSK7 plasmid expressing the laccase gene *lcc1* from *C. cinerea* under control of the *gpdII*-promoter of *Agaricus bisporus* was used (Kilaru et al. 2006). The pYSK7-transformant (clone 26) was grown on YMG-agar (per litre: 4 g yeast; 10 g malt extract; 4 g glucose; 10 g agar) plates at 37 °C until the mycelium reached the edge of the petri dish. Sterile water (ddH_2O) was poured onto the plates and the mycelium with the asexual spores was scraped with a sterile spatula from the agar. Spore solutions were filtered using a sterile funnel filled with glass wool in order to hold back the fungal hyphae. A Thoma counting chamber was used to determine spore concentrations in the solutions.

For shake flask cultures, pre-cultures inoculated with 10^6 spores/ml medium were prepared in 500 ml flasks filled with 50 ml of modified Kjalke medium [Kjalke et al. 1992; per litre: 10 g yeast, 20 g glucose, 0.5 g $CaCl_2$ x 2 H_2O, 2 g KH_2PO_4, 50 mg $MgSO_4$ x 7 H_2O]. Inoculated flasks were incubated for 4 days at 37 °C as stationary (standing) cultures. Pre-cultures were homogenised by an Ultra-Turrax® (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 sec at 8000 rpm and 30 sec at 9500 rpm (rotations per minute). For main-cultures, 500 ml flasks with 100 ml of sterile modified Kjalke medium supplemented with 0.1 mM $CuSO_4$ were inoculated with each 5 ml of the homogenised pre-culture. Cultivation took place at 25 °C and 37 °C on a rotary shaker at 120 rpm for 4 and 10 days, respectively. Per culture day and cultivation temperature, two or three parallel cultures were analysed.

Fungal shaken cultures were poured onto a glass plate (28.5 cm x 38.5 cm, Fig. 2) whose edges were sealed by a silicone border in order to keep the liquid samples on the plate. Water (200 to 300 ml) was added to equally distribute the mycelium as a monolayer of pellets on the glass plate. When necessary, pellets lumped together were manually dispersed with the help of a spatula or forceps.

Total fungal biomass was determined after taking images by filtering complete cultures through a Büchner funnel containing a cellulose filter of known dry weight. The filters together with the wet biomass were dried at 80 °C and the dry weights of the biomass were determined.

2. Imaging

To record pellet growth, the glass plate with a fungal sample was placed onto the illuminated translucent plate of a camera stand (Kaiser Copylizer eVision ini.tial HF, Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany) with a digital CCD camera (Color View II, Soft Imaging System GmbH, Münster, Germany) installed at a distance of 28 cm to the glass plate. Images were recorded with the CCD camera to which a Lametar 2.8/25 objective (Jenoptik GmbH, Jena, Germany) was fixed. Three non-overlapping photographs (dashed lines in Fig. 2) were taken of each culture.

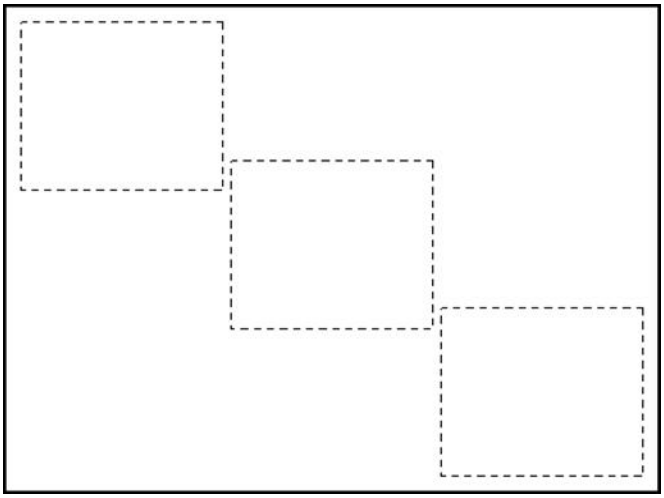


Fig. 2 Glass plate (solid line representing the silicon border; size 28.5 x 38.5 cm) onto which liquid fungal cultures are poured and positions of fields (dashed lines; each field size 6.4 x 8.5 cm) of which photographs are taken.

3. Analysis of the images

Pictures were evaluated using analySIS® (Soft Imaging System GmbH, Münster, Germany), a software tool for the analysis of microscopic images of biological materials. The magnification, defined by the specific objective at the CCD camera used at a distance of 28 cm to the specimen, was set to be 0.132 (equivalent to 0.0418 mm/pixel).

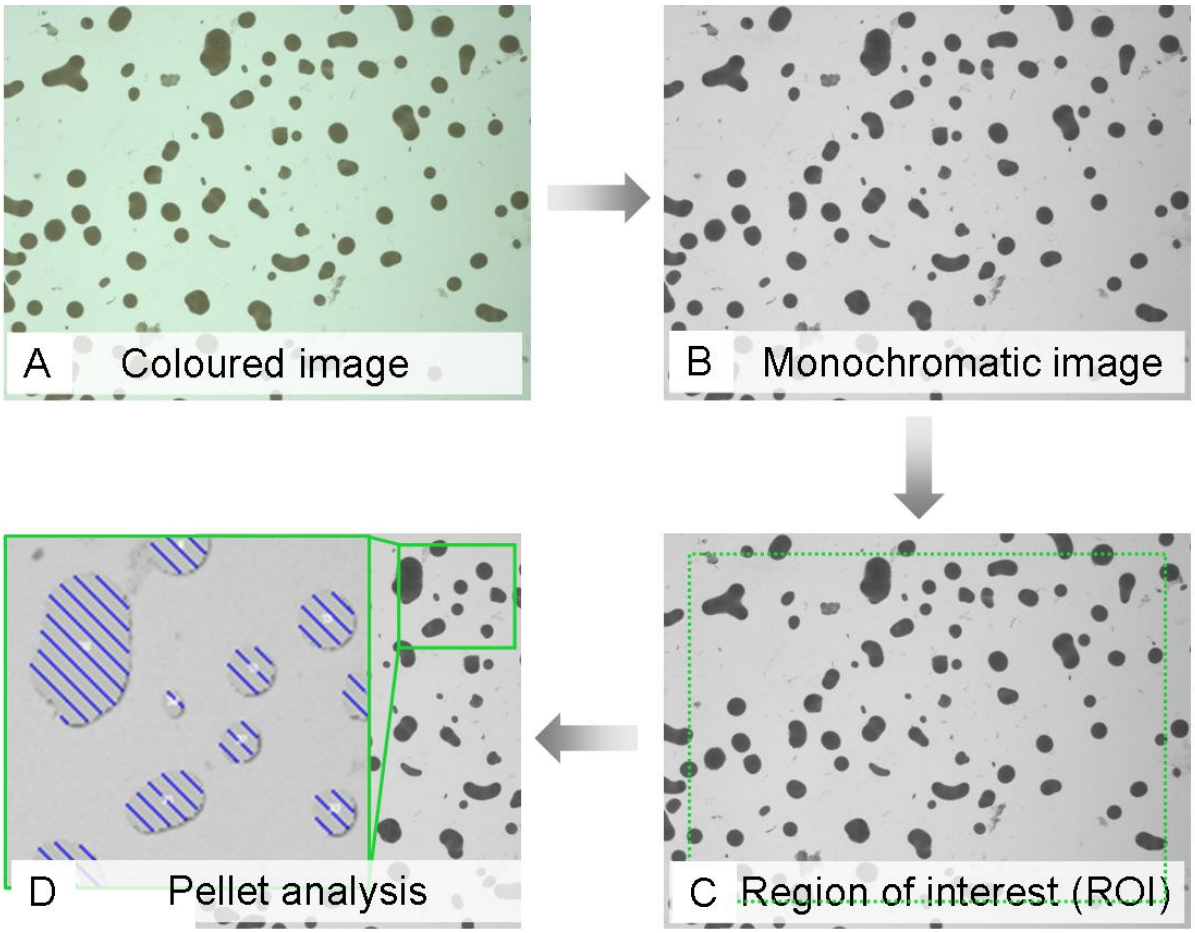


Fig. 3 Processing of photographs of fungal cultures with analySIS®. A picture of the fungal culture (**A**) is reduced to a monochromatic image (**B**). A region of interest (ROI) for pellet determination is defined (**C**) and the pellets are detected as documented in Fig. 5 by analySIS® (**D**) after setting a specific threshold (see Fig. 4) for best distinction of pellets and background.

The subsequent steps for the process of automated image analysis using predefined functions of the software analySIS® are explained in the following:

- Function RGB reduction: The coloured photograph (Fig. 3A) were reduced to their RGB (red, green and blue) colours. Of the three pictures obtained, the green monochromatic images (Fig. 3B) gave the best contrast between pellets and background. The green monochromatic images were therefore used for further analysis in form of a grey value scale.
- Function Define ROI: A region of interest (ROI, Fig. 3C) had to be defined and loaded into a picture. This ROI defines the area in the image, where objects were analysed. With the magnification as defined above, a ROI of 40.66 cm^2 was used (Fig. 3C).
- Function Define measurement: In analySIS®, a pre-defined set of object parameters can be chosen, of which the following were selected for pellet detection and description (Fig. 3D): grey value mean, area, convexity, shape factor and sphericity.

Moreover, the average diameters of the individual pellets were determined in analySIS® from 180 measurements of diameters per object, i.e. one at every degree.

- Function Set threshold: To distinguish the compact mycelial pellets from loose hyphal fragments as background, the settings of the analySIS® software had to be manually adjusted in the programme to a specific threshold determined by the background in a given monochromatic image. Usually, the threshold was set to 180 in order to reject the peak of smaller objects (hyphal fragments) and other background within the bimodal distribution of greyness level as seen in Fig. 4.

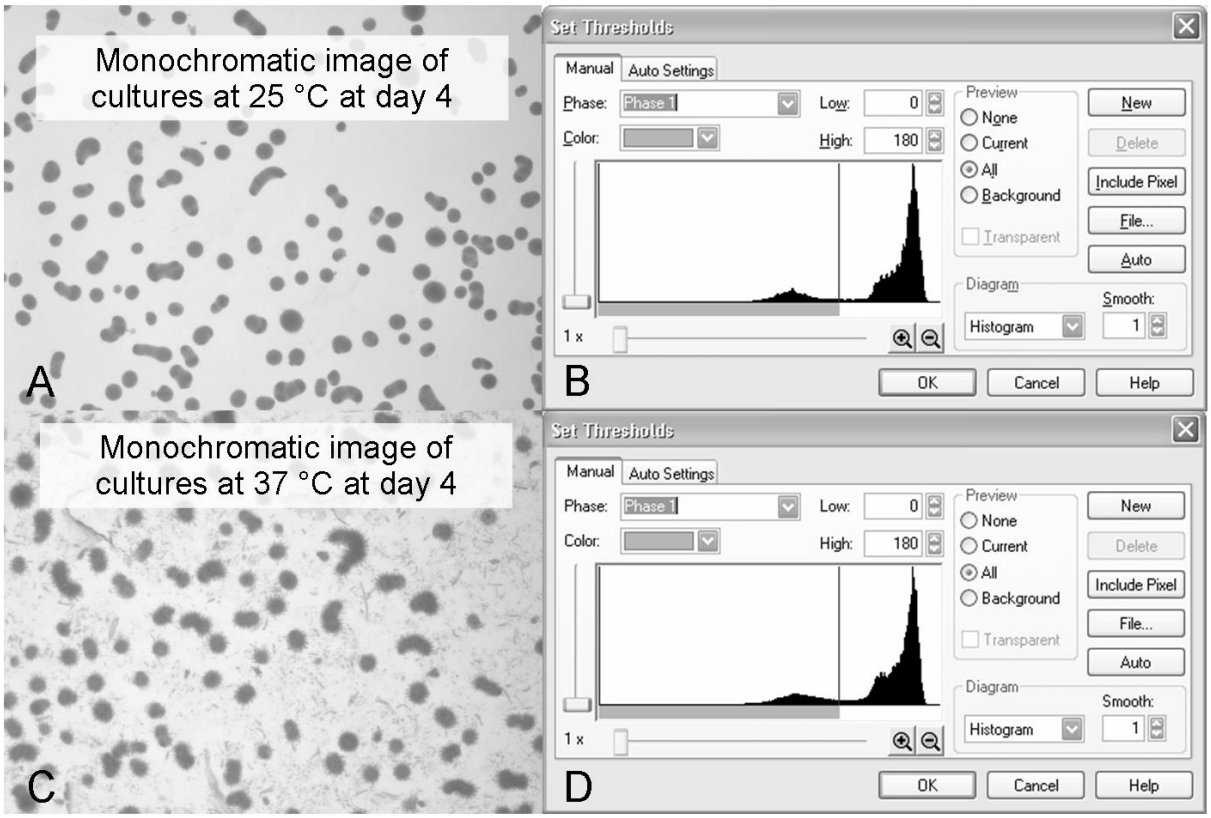


Fig. 4 Differences in the background noise of culture images taken at day 4 of cultivation of *C. cinerea* in modified Kjalke medium at 25 °C and 37 °C, respectively (**A** and **C**). The bimodal distribution of greyness levels (on a scale from 0 = black to 255 = white; black is at the left and white is at the right side of the histograms **B** and **D**) deduced from these images and the corresponding settings for defining the background threshold for the monochromatic images in **A** and **C** are presented in **B** and **D**, respectively.

- Function Define Detection: A projected area per object is determined by counting the pixels per object being connected within the area due to a same grey value contrast. A minimum pixel amount of 10 per object was set to exclude any remaining smaller loosely aggregated filamentous fragments from further analysis. Detection of the fungal pellets covered by the ROI was done twice: Include and Exclude. In case of the Include function, all pellets inside the ROI plus those that cross the ROI borders

were measured. In case of the Exclude function, only pellets localised fully within the ROI were detected (Fig. 5).

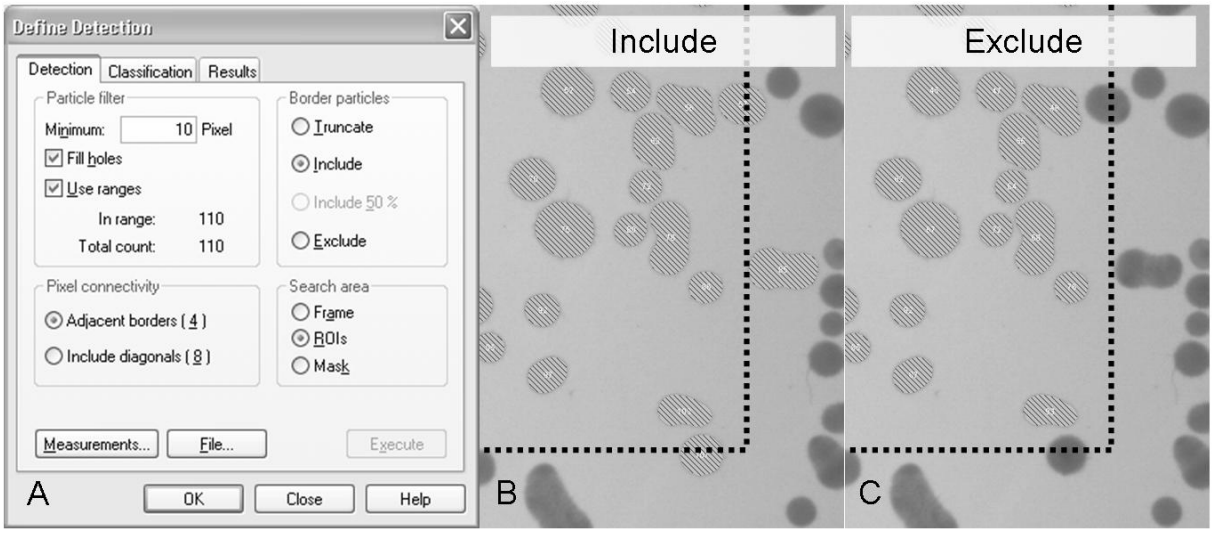


Fig. 5 Each image was analysed twice by analySIS®. The settings for the determination (**A**) were used for an Include-recording (**B**) of the ROI (dashed line). For an Exclude-recording (**C**), the border particles were respectively set onto function Exclude.

- Function Particle Results: Parameters for all pellets were calculated for the Include and for the Exclude setting, respectively, and obtained data were saved in separate spreadsheet files, where each row presents the information for one specific object and each column specific object parameters as defined above.

4. Data processing

All calculations were done for the raw data set and for filtered data sets. Object data from the three analysed images per culture were combined (Include and Exclude measurements separately) and the amount of objects, the average value of the projected object areas and the average object diameter were calculated with the help of a self-implemented programme constructed with pre-defined analysis objects (average with standard deviation and general histogram used to automatically cluster objects into 19 distinct groups of 0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1.0, 1-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 21-25, 25-30, 30-50 and 50-500 mm^2 pellet area) of the programming language LabView™ (www.ni.com/labview). The object data were saved automatically in individual spreadsheet files for further analysis of Include and Exclude data in Excel (Excel 2002, Microsoft). The average Include data and, respectively, the average Exclude data for the projected area and the object diameter from two or three parallel cultures per growth condition were combined

and averaged. Next, values derived from Include and Exclude measurements were averaged in order to include a proportionate fraction of objects into the analysis that either touch or cross the ROI border lines so that the complete ROIs are best covered. For calculating the average amounts of objects per culture, the total amounts of objects in the three ROIs analysed per culture were determined and averaged between the cultures of a same growth condition separately for the Include and Exclude data set, respectively. Subsequently, the values derived from Include and Exclude measurements were averaged and this average value was multiplied by a factor of 9.0 resulting in the average number of pellets per 100 ml culture liquid.

D. Results

Upon inoculation of fresh *C. cinerea* cultures in modified Kjalke medium, compact round to oval pellets dominated at day 4 of cultivation in the shaken cultures, both at 25 °C and 37 °C. In the monochromatic images of the cultures, pellets had a strong grey-shade. However, at the higher cultivation temperature the background including fine hyphal material was generally higher and, in addition, lighter shaded loosely aggregated hyphal fragments were also present (Fig. 6). In older cultures at day 10 of cultivation at 25 °C, there were no larger differences of pellet structure in the views of the cultures as compared to day 4 of cultivation. In contrast, in addition to dark-shaded compact pellets and loose aggregates of filamentous fragments, pellets of less dense structure and lighter grey shading accumulated in the aging cultures grown for 10 days at 37 °C (Fig. 6).

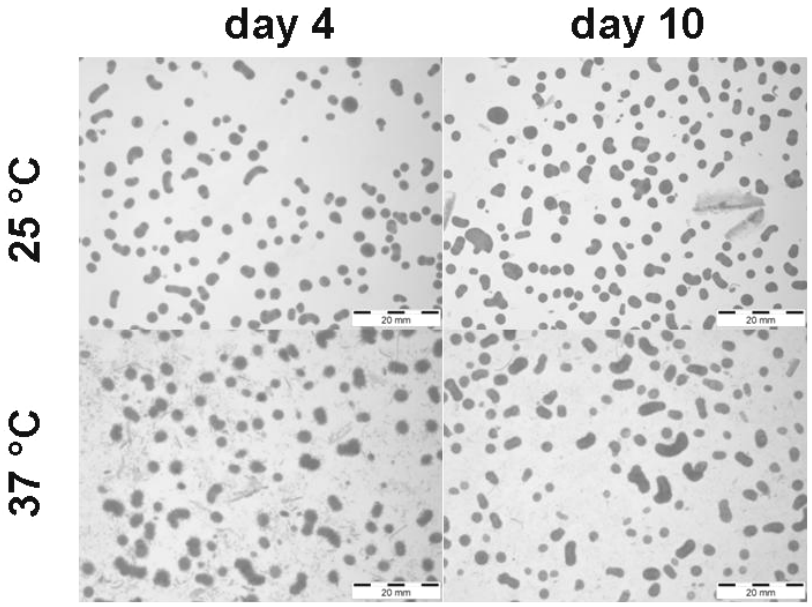
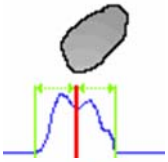
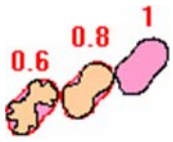
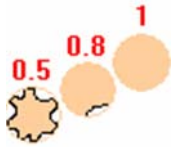
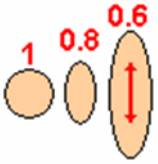


Fig. 6 Monochromatic images of *C. cinerea* cultures grown for different times in modified Kjalke medium at 25 °C and 37 °C, respectively. The size bars in the images represent 20 mm.

Particularly in the 37 °C cultures, the projected areas of some of the loosely aggregated filamentous fragments were larger than the minimum size of 10 pixel (projected area = 0.017 mm^2) per object as fixed for pellet recognition in the analysis software (see below and Fig. 7 for further details). Such filamentous fragments will thus interfere with pellet analysis. In addition to the mean grey value and the projected area of an object, convexity, shape factor and sphericity (Table 1) were therefore chosen as other parameters in order to better describe pellet morphology and clearly distinguish between fungal pellets and loose filamentous aggregates. Threshold values for filtering as given in Table 1 were empirically defined in order to confidently exclude any loose filamentous aggregates from further pellet analysis.

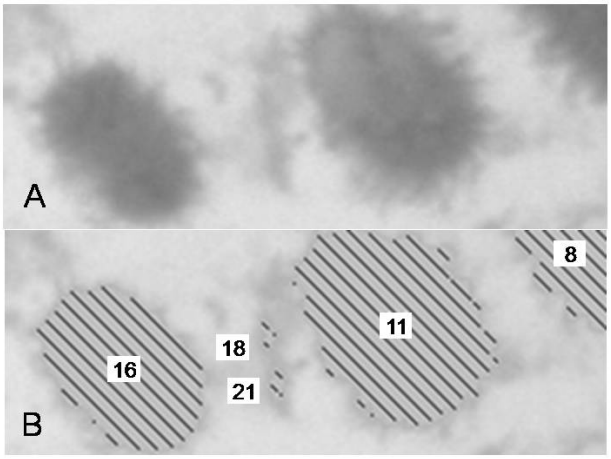
For processing the raw data sets in the spreadsheet files obtained from the function "Particle Results", at least 3 of the measured values per row (each row defining one detected object) have to pass the defined threshold values for positive filtering of an object as a pellet. If not achieved, a row will automatically be deleted from the raw data set. Fig. 7 demonstrates

Table 1 Parameters for definition of pellet morphology

Parameter provided by the analysis® software		Description as given by the analysis® software	Threshold value for positive filtering as set in this study
	Grey value mean	The arithmetic mean of all grey values of the particle	≤ maximum object grey value in a given image minus 10 % of the maximum grey value *
	Area	The area of a particle is (number of pixels of the particle) times (calibration factors in X and Y direction).	≥ 0.1 mm^2
	Convexity	The fraction of the particle's area and the area of its convex hull	≥ 0.7
	Shape factor	The shape factor provides information about the "roundness" of the particle. For a spherical particle the shape factor is 1, for all other particles it is smaller than 1	≥ 0.5
	Sphericity	Describes the sphericity or 'roundness' of the particle by using central moments	≥ 0.3

* Note that for the grey value mean, the absolute values may vary between different images because of the different background values; e.g. for a maximum grey value of 180 in an image, the maximum value for positive filtering is 162 (= 180 - 10% of 180).

examples of such positive and negative filtering. Each recognised object in Fig. 7A was identified by a number and overlaid with a shading representing the projected area of an object (Fig. 7B). When comparing the objects in the original monochromatic image (Fig. 7A) with the shades overlaid by the analySIS® programme (Fig. 7B), it becomes clear that the objects with the number 18 and 21 having a projected area above 10 pixel (0.06 and 0.09 mm^2, respectively) are not pellets but loosely aggregated filaments. The filter implemented for the pellet data however remove these objects from the raw data set, because the threshold values for parameters grey value, area and shape factor were not reached (see Fig. 7 data sheet). In other cases of loosely aggregated filaments, the threshold values e.g. for the combination grey value, shape factor and sphericity or for the combination grey value, convexity and shape factor were not reached (data not shown). In extensive empirical data analysis, a minimum of three parameters were found to be necessary for save pellet filtering from other objects in order not to eliminate very small pellets of low grey shading and/or low convexity from the raw data sets (not further shown).



Object No	Grey value mean	Area [mm²]	Convexity	Shape factor	Sphericity	Pellets [yes/no]
8	140	12.12	1.05	0.48	0.34	yes
11	149	9.57	0.89	0.51	0.51	yes
16	144	6.47	0.94	0.75	0.43	yes
18	179	0.06	0.75	0.48	0.87	no
21	178	0.09	0.83	0.44	0.43	no
Threshold value	≤162	≥0.1	≥0.7	≥0.5	≥0.3	

Fig. 7 Monochromatic pellet images taken from a *C. cinerea* culture at day 4 of cultivation at 37 °C (**A**), shades overlaid by analysis® on present objects defining object areas for calculation (**B**) and a data sheet for these objects. The data for each object obtained by the software were used for distinguishing pellets and loosely aggregated hyphal fragments, respectively. Values shaded in grey did not reach the defined limits.

The defined filter were applied to the raw data sets (Include and Exclude separately), the Include and Exclude data were combined and averaged as described in the methods in order to obtain the average number of pellets per culture. After applying the filter, the number of objects from the raw data sets reduced by 2-24%, respectively, depending on the type of cultures analysed (Table 2). In total, about 225 to 400 pellets (30 to 160 per individual image) were analysed per individual culture. The calculated total pellet numbers per culture varied from about 2300 to 3400 (Table 2).

Table 2 Pellet distribution in *C. cinerea* cultures at day 4 and day 10 of cultivation in modified Kjalke medium

Culture age	Cultivation temperature	Raw object data				Filtered object data				Reduction in object number per flask [%]
		No of total analysed objects*	Object area [mm²]	Object diameter [mm]	No of objects per flask	No of total analysed pellets*	Pellet area [mm²]	Pellet diameter [mm]	No of pellets per flask	
Day 4	25 °C	804	4.85 ± 4.24	2.57 ± 1.32	2411 ± 63	786	4.81 ± 3.20	2.57 ± 1.06	2355 ± 58	2
	37 °C	860	4.13 ± 5.27	2.06 ± 1.88	3866 ± 283	677	5.10 ± 5.35	2.40 ± 1.80	3043 ± 86	21
Day 10	25 °C	1247	4.58 ± 3.89	2.36 ± 1.39	3739 ± 240	1139	4.95 ± 3.67	2.51 ± 1.24	3415 ± 267	9
	37 °C	839	5.24 ± 5.51	2.66 ± 1.81	2516 ± 199	763	5.49 ± 4.72	2.70 ± 1.38	2286 ± 223	9

* For each culture condition, three flasks were analysed except for day 4 of cultivation at 37 °C where only two flasks were used. Absolute numbers of objects analysed per culture condition were added from the two or three cultures, respectively.

With the time in 25 °C cultures, there was a strong increase in absolute numbers of detected pellets per culture (about 2400 pellets at day 4 to about 3400 pellets at day 10) and a little increase in average pellet area [about 4.8 mm2 at day 4 to about 5.0 mm2 at day 10; both values corresponded well with the peak value in histograms of the individual pellet areas (not shown)], whereas the average pellet diameter decreased slightly from about 2.57 mm at day 4 to about 2.51 mm at day 10. The percentage of non-pellet objects (loose hyphal aggregates) increased from 2% at day 4 of cultivation to 9% at day 10 of cultivation. These data from automated image analysis confirm the impression of the former rough overview that pellets in cultures at 25 °C change little in shape and size within the 6 following days of incubation.

The situation in the 37 °C cultures was different. Absolute pellet numbers decreased from about 3000 pellets per flask at day 4 of cultivation to 2300 per flask at day 10 of cultivation, but the average pellet area [corresponded again well with the peak value in histograms of the individual pellet areas (not shown)] increased from 5.1 mm^2 at day 4 of cultivation to about 5.5 mm^2 at day 10 of cultivation along with the average pellet diameter (from 2.40 mm at day 4 to 2.70 mm at day 10). As documented in Fig. 8, the 4 day-old cultures at 37 °C had generally a higher background of small hyphal fragments and a larger fraction of total detected objects (21%; Table 2) were identified in the raw data set as loose filamentous aggregates. At day 10 of cultivation, the amount of hyphal fragments in the background was lower (Fig. 8) and, in parallel, also the amount of loose aggregated filaments detected by the automated image analysis (9%; Table 2). In summary, the data

imply that major morphological changes occur at 37 °C during the period of cultivation from day 4 to day 10.

At both growth temperatures, the differences in average pellet numbers and average pellet areas (Table 2) corresponded well with changes in biomass. The 25 °C cultures had a mycelial biomass of 5.6 ± 0.2 g/l and 8.3 ± 0.1 g/l at day 4 and day 10 of cultivation, respectively. The 37 °C cultures had a biomass of 8.3 ± 0.2 g/l and 5.5 g/l ± 0.3 g/l at day 4 and day 10 of cultivation, respectively. At 25 °C as the suboptimal temperature for growth of *C. cinerea* (Kües 2000), 10 days are obviously required to achieve highest biomass yields whereas the reduction in total biomass at 37 °C implies that the fungus is at day 10 of cultivation already in a major phase of biomass degeneration.

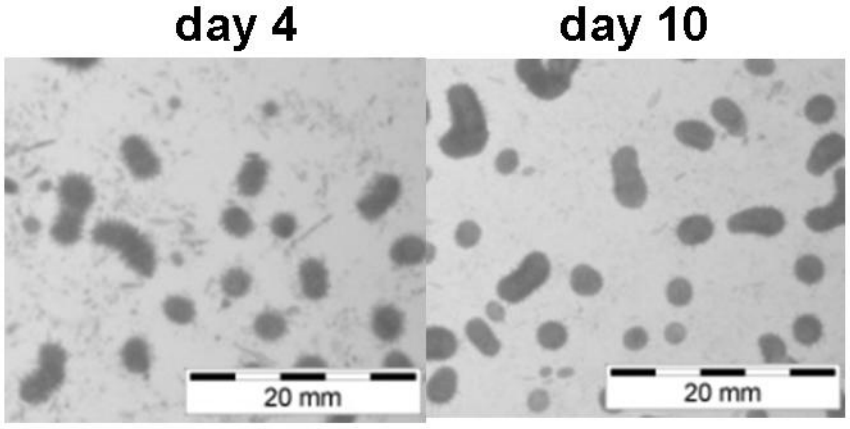


Fig. 8 Enlargement of sectors of photographs from Fig. 6 showing fungal pellets, small hyphal fragments and loose filamentous aggregates in the culture broth of *C. cinerea* cultivated in modified Kjalke medium at 37 °C for 4 and 10 days, respectively.

E. Discussion

Pellet morphology is known to be influenced by culture conditions and, in turn, to affect biomass formation and production yields in submerged fungal fermentations (Papagianni 2004, Fazenda et al. 2008). Effects of temperature and age on submerged growth of *C. cinerea* used as an example in this study are obvious from the photographs shown in Fig. 6 and Fig. 8 and from the data presented in Table 2. This fungus has its growth optimum at 37 °C (Kües 2000). Accordingly, we observe that cultures at this temperature have a faster growth rate, reach the highest possible biomass levels earlier and undergo faster biomass degeneration than cultures kept at the lower temperature of 25 °C (Table 2 and Rühl et al. unpublished results). With the lower growth rate and the delay in biomass degeneration, the overall pellet structures of the cultures grown at 25 °C were much more compact and uniformly than the structures of pellets grown at 37 °C. Moreover in the 25 °C

cultures, there was much less background of small hyphal fragments and of loose aggregated hyphal filaments (Fig. 6 and Fig. 8). *C. cinerea* is a filamentous basidiomycete that can easily be manipulated by genetic transformation (Binninger et al. 1987, Granado et al. 1997). The fungus has thus found interest for production of glycosylated enzymes from higher basidiomycetes that will not or only poorly be expressed and most possible wrongly be glycosylated in established ascomycete systems for recombinant enzyme production (Ogawa et al. 1998, Kikuchi et al. 1999, Kilaru et al. 2006). Laccases are for examples enzymes in focus for recombinant production with *C. cinerea* in efficient fermentation processes (Kilaru 2006, Kilaru et al. 2006, Rühl et al. unpublished results). Studying fungal pellet morphology will be beneficial for optimising the fermentation processes and enzyme production yields.

An efficient and easy to apply routine technique for observation and large scale pellet analysis for *C. cinerea* but also other filamentous fungi is presented in this work. By taking three images per culture, 225 to 400 pellets per culture are simultaneously and easily characterised by applying specific functions of the commercial analySIS® software. If higher pellet numbers of the same culture are required, further non-overlapping images might easily be taken of other areas of the glass plates onto which the pellets were poured (compare Fig. 2). Pellets for taking photographs are easily spread on the bordered glass plate from cultures with a pellet number of about 2000-2500 per 100 ml. With increasing pellet numbers, in some cases pellets were found packed in densely clusters on the glass plate and had to be separated manually with a spatula or forceps. When wanting most accurate numbers of pellets per culture, simply diluting the cultures is not as advisable since the fungal pellets sink quickly and are difficult to be kept evenly dispersed in solution. Evenly distributing of pellets from dense cultures on the glass plate was therefore in our hands the most time consuming step in the procedure leading in the worst case to 20 min preparation time per culture until images could be taken. Subsequent usage of the analySIS® software helped in a fast and, especially, representative determination of pellet morphology, as up to 400 pellets (or more) can be analysed per culture in a few minutes. Considering the parameters grey value, pellet area, convexity, shape factor and sphericity with the implemented filter, fungal pellets and filamentous aggregates could clearly be recognised at the same time and distinguished as such both when relative low and when relative high amounts of these two growth forms were obtained in *C. cinerea* cultures. Such data will allow to quantify the nature of mycelial aggregates, fragmentation of pellets as well as pellet regrowth (Papagianni 2006).

Most literature studies on fungal morphology during submerged cultivation consider only small samples of pellets (see examples for basidiomycetes in the introduction). Gehrig et al. (1998), in contrast, observed approximately 2000 pellets by image analysis in a *C. striatus* culture by taking pictures of pellets (1-6 mm in diameter) present in a reference area of 15 mm x 15 mm. Total pellet volume was defined by the authors from determination of an average pellet diameter measured from the images of fungal pellets. Moreover, Kelly (2006) studied the ascomycete *Aspergillus niger* and similarly determined the pellet diameter of 500 to 1000 pellets per *A. niger* culture. The description of the method used by Kelly (2006) is not presented in much detail in the publication but it appears that also this author used a relatively small reference area with maximum 30 to 35 pellets when these were comparably small and laying quite crowded on the area, forcing the researcher to take many different images for obtaining high numbers of different pellets for large scale analysis. In other cases, pellets of *A. niger* were observed on 1 mm-deep cavity slides in case of very small sizes under a microscope or in case of larger pellets on an adjustable camera stand equipped with a CCD camera fitted with a macroscopic zoom lens. Since the size of the images are restricted by the width of the cavity slide, also in these studies numerous pictures have to be taken for analysing high pellet numbers (Cox and Thomas 1992, Paul and Thomas 1998, Papagianni 2006).

The protocol presented here allows analysis of much higher numbers of pellets (60-130 pellets) per single image. Moreover, additional parameters of morphology were considered in this study. For simplification of analysis, Gehrig et al. (1998) and Kelly (2006) assumed a spherical shape of the fungal pellets. The photographs and images in Fig. 3 to 8 show that fungal pellets may be stretched in one dimension and that pellets can be of fringy shape. Therefore, a definition of the projected area calculated from all connected pixels of an object by the software analySIS® will give much more accurate pellet area values than is possible by only considering the pellet diameters. Although not analysed in more detail in this study, the independently collected individual values for the extra parameters convexity, shape factor and sphericity of the pellets are available from the same analysis if a more deep insight into such extra growth parameters for a more detailed description of pellet morphology in *C. cinerea* will be required. Furthermore, analysing the individual grey shades of pellets can serve to define a relative distribution of more compact and less dense pellets in a culture. The usefulness of such data for morphological classification of fungal pellets by automated image analysis has been demonstrated before for *A. niger* (Cox and Thomas 1992, Paul and Thomas 1998).

IV. Fermentation of *C. cinerea* in shake flasks and bioreactors

A. Abstract

In this study, laccase transformants of the basidiomycete *Coprinopsis cinerea* strain FA2222 (carrying the *C. cinerea* laccase gene *lcc1* subcloned in an expression vector under the control of the *gpdII*-promoter from *Agaricus bisporus*) was used and compared to a transformant carrying a vector without a laccase gene. In standard *Coprinopsis* medium at 37 °C, maximal laccase yields of 3.0 U/ml were achieved with the best *lcc1* transformant in shake flask cultures. A reduction in temperature from 37 °C to 25 °C increased laccase yields up to 9 U/ml and had severe effects on the morphology of the mycelial pellets formed during cultivation. Therefore, automated image analysis was used to characterise morphological differences in shaken flask and stirred bioreactor cultures of the *lcc1* transformant during growth at different cultivation conditions. The most important parameter determined by automated image analysis is the projected area of the pellet, which derives from the 2-dimensional image of the pellet. In addition, the outer pellet structures were analysed under the microscope by cross sections of selected pellets.

Compared to shake flask cultures, in stirred bioreactors (4.5 l) the *lcc1 transformant* was cultivated at 37 °C and the pH of the medium was controlled. Laccase activity was very low at pH 8 with values of 0.5 after 6 days of cultivation, whereas at pH 7 and pH 6 activities of 1.1 and 1.3 U/ml were reached, respectively.

In shake flask cultures (100 ml), the *lcc1 transformant* and the control transformant, both differed in pellet morphology when growing at 25 °C and 37 °C, respectively. At 25 °C, pellets had a smooth surface, whereas at 37 °C pellets had a hairy appearance. At the lower temperature, pellets were more compact than pellets grown at the cultivation temperature of 37 °C. The outer layer of pellets formed at 25 °C consists of small densely aggregated cells. The inner medulla is formed by loosely aggregated hyphae that during aging appear to degenerate. The inner medulla of pellets grown at 37 °C was similar to pellets derived at 25 °C, whereas the cells of the outer layer are less dense aggregated. Generally, the outer layers of pellets from 37 °C are wider than the one of pellets grown at 25 °C.

In automated image analysis of both transformants, the projected pellet area varied between 1 to 10 mm^2, whereas also smaller fragments and pellets were present. Most pellets of the *lcc1* transformant were in the range of 3 to 5 mm^2 and most pellets of the control

transformant in the range of 2 to 4 mm^2, respectively. In all cultures, the pellet diameter changed over the time. The diameter of pellets levelled at 1.5 to 2.5 mm for the control transformant from day 8 of the cultivation onwards. For the *lcc1* transformant, the diameter balanced after 6 days in a range of 2.0 to 3.0 mm.

In bioreactor cultures, pellet morphology was much more variable in sizes ranging from smaller pellets and fragments (< 0.4 mm^2) to very large pellets (> 20 mm^2). Pellet size was influenced by the pH, at pH 6 and pH 7 most pellets showed an average projected area of around 7 to 12 mm^2 and pellets grown at pH 8 of around 3 to 5 mm^2. The outer appearance of the pellets in the stirred bioreactor resembled those obtained in shake flask cultures at 37 °C.

B. Introduction

In nature, laccases occur in fungi, plants and insects (Mayer and Staples 2002), and also in bacteria (Claus 2003). Laccases are multi-copper oxidases that oxidise phenolic and a number of other organic compounds. Because of their wide substrate range, laccases are interesting enzymes for various biotechnological applications in the food, the textile and the pulp and paper industries, in soil bioremediation and in wood composite production (chapter 1 and Hüttermann et al. 2001, Couto and Toca-Herrera 2006, Riva 2006, Widsten and Kandelbauer 2008).

For native laccase production, fungal strains are selected that are most efficient in secretion of enzymes. Generally, native laccases are won from basidiomycetes in liquid cultures which are treated with suitable inducers for improving enzyme production (e.g. copper, ethanol, 2,5-xylidine and other phenolic compounds) (Lomascolo et al. 2003, Tavares et al. 2005, Jang et al. 2006, Myasoedova et al. 2008). Also other cultivation parameters can increase natural laccase production, as it was shown for *Pleurotus ostreatus* and *Phanerochaete chrysosporium* in shake flask cultures where increased agitation speed resulted in higher laccase yield (Michel et al. 1990, Hublik and Schinner 2000). Nevertheless, shake flask cultures may be suboptimal for production of enzymes from aerobic organisms, since aeration is limited due to a restricted liquid/air interphase. In contrast, in stirred bioreactors, highly dispersed bubbles of compressed air or 100% oxygen lead to a better aeration. Thus and because of the limiting cultivation volume in shake flask cultures, fermentation in stirred bioreactors is preferred in industrial production processes. An industrial scale production of native laccase in a stirred bioreactor was first described for the white-rot fungus

Trametes versicolor (*Polyporus versicolor*) more than 25 years ago (Fåhraeus and Reinhammar 1967, Kharazipour 1983). Since then, several studies on industrial scale production of laccases either in submerged (SmF) or in solid state fermentation (SSF) were published (reviewed by Couto and Toca-Herrera 2007). Although highest laccase activities in bioreactor scale exceed 700 U/ml (Table 1), yields are still not high enough for best economic industrial production. Besides, the used basidiomycetes grow slow and are restricted to the laccases secreted natively by the fungus. Overexpression of these laccases in fungi upon gene transformation is an alternative way for high-level enzyme production (Kilaru 2006).

In this study, expression of the *C. cinerea* laccase Lcc1 under control of a foreign promoter (the *gpdII*-promoter of *Agaricus bisporus*) in shake flask and bioreactor cultures was observed. Beside standard parameters, such as enzyme activities, biomass dry weights and glucose concentrations, I analysed morphological alterations of fungal pellets including the inner structure of fungal pellets as obtained in shake flask cultures of a *lcc1 transformant* and a control transformant not expressing laccase. On a more technical scale, the *lcc1* transformant was grown in a 5 l bioreactor, where different constant pH values were tested for their influence on laccase production and fungal morphology. To my knowledge, this is the first study observing the morphology of *C. cinerea* in liquid shake flasks or stirred bioreactor cultures.

C. Material and Methods

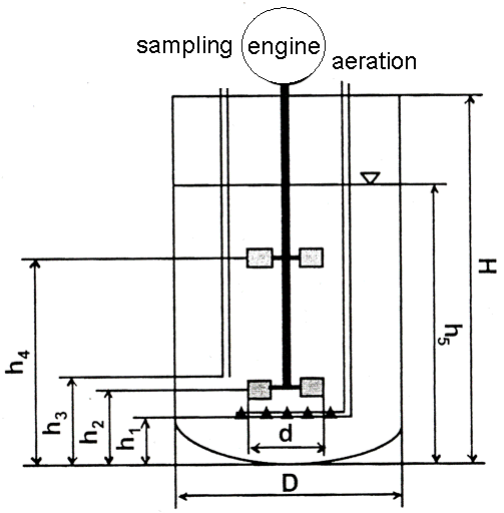
1. Fungal cultures

A monokaryotic *C. cinerea* strain (FA2222) transformed with the pYSK7 plasmid expressing the laccase gene *lcc1* from *C. cinerea* under control of the *gpdII*-promoter of *A. bisporus* and a control transformant (pCc1001) were used (Kilaru et al. 2006). The clone with the highest laccase activity in liquid culture (known as clone 26 in Kilaru 2006) was used throughout the study.

Fungi were grown on YMG-agar (per litre: 4 g yeast; 10 g malt extract; 4 g glucose; 10 g agar) plates at 37 °C until the mycelium reached the border of the petri dish. Sterile water (ddH_2O) was poured onto the plate and the mycelium with the asexual spores was scraped with a sterile spatula from the agar. The spore solution was filtered using a sterile funnel filled with glass wool in order to hold back the fungal hyphae. A Thoma counting chamber was used to determine the spore concentration in the solution.

For shake flask cultures, a pre-culture with 10^6 spores/ml medium were prepared in 500 ml flasks, filled with 50 ml of modified Kjalke medium (Kjalke et al. 1992) per litre: 10 g yeast, 20 g glucose, 0.5 g $CaCl_2$ x $2H_2O$, 2 g KH_2PO_4, 50 mg $MgSO_4$ x $7H_2O$. The inoculated flasks were incubated for 4 days at 37 °C as stationary (standing) cultures. The pre-culture was homogenised by an Ultra-Turrax® (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 sec at 8000 rpm and for 30 sec at 9500 rpm (rotations per minute). For the main-culture, 500 ml flasks with 100 ml of sterile Kjalke medium supplemented with 0.1 mM $CuSO_4$ were inoculated with each 5 ml of the homogenised pre-culture. Cultivation took place at 25 °C and 37 °C on a rotary shaker at 120 rpm.

Bioreactor cultures were performed in a New Brunswick Scientific 5 l stirred vessel bioreactor (New Brunswick, Edison, NJ, USA; Fig. 1). The fermenter was filled with 4.5 litre of modified Kjalke medium (per litre: 10 g yeast, 20 g glucose, 0.1 g $CaCl_2$ x $2H_2O$, 1.5 g KH_2PO_4, 50 mg $MgSO_4$ x $7H_2O$) supplemented with 0.1 mM $CuSO_4$ and inoculated with the spores to a final amount of 10^6 spores/ml. Cultivation took place at 37 °C and 120 rpm at pH 6, pH 7 and pH 8.



Specification	Description	Dimension 5 l
Height of the vessel	H	31 cm
Installation height:		
aeration pipe	h1	2.3 cm
lower rushton turbine	h2	4.7 cm
sampling tube	h3	5.5 cm
upper rushton turbine	h4	16 cm
Filling height	h5	20.8 cm
Diameter reactor vessel	D	17 cm
Diameter rushton turbine	d	8.2 cm
Diameter agitator shaft		9.5 mm
Blades/ruhston turbine		6
Working volume		4.5 l
Temperature		37 °C
Agitation speed		120 min^{-1}
Aeration rate		180 l/h

Fig. 1 Scheme of the used fermenter (after Grimm 2006). Triangles indicate the air outlet of the aeration pipe.

2. Determination of the dry weight (DW) in gram per litre [g/l]

Following growth, volumes of the fungal cultures were measured and the liquid was poured into a Büchner funnel containing a cellulose filter of known dry weight. The filters together with the wet biomass were dried at 80 °C and the dry weights were determined.

3. Laccase assay

Laccase activity was determined in 100 mM sodium acetate buffer (pH 5.0) at room temperature with ABTS (2,2‘-azino-bis [3-ethylbenzthiazoline-6-sulfonate], AppliChem GmbH, Darmstadt, Germany) as a substrate. The conversion of the substrate was observed with the help of a spectrophotometer (DU 800, Beckman Coulter, Fullerton, CA, USA) at 420 nm (ε = 36000 M^{-1} cm^{-1}) (Matsumura et al. 1986). One unit of enzyme activity was defined as the amount of substrate in µmol transformed per min and enzymatic activities are given in U per volume.

4. Glucose determination

The glucose concentration was determined indirectly with the Glucose (HK) Assay Kit from Sigma (Sigma-Aldrich Chemie GmbH, Munich, Germany). Glucose was enzymatically phosphorylated and afterwards oxidised with NAD (nicotinamide adenine dinucleotide) to 6-phosphogluconate. During the oxidation, NAD is reduced to NADH, which correlates directly with the glucose concentration and which was measured at 340 nm with a spectrophotometer. Glucose standards (Sigma, G 3285) were measured with the same method in concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625 g/l, respectively. The supernatant samples of the fungal cultures were diluted to fit in the NADH absorption curve for the glucose standards.

5. C/N determination of culture supernatant

Tin capsules (HEKAtech GmbH, Wegberg, Germany) were weighted and filled with an adequate amount of supernatant (40-120 µl to reach ~800 ng dry weight/capsule). The tin capsules with the supernatant were lyophilised, weight again and analysed with a CHNS-O elemental analyser (Carlo Erba Instruments, EA 1108-Elemental Analyzer, Milan, Italy) according to the instructions of the supplier.

6. Morphological observation

Pelleted growth was observed and analysed using an automated image analysis system described earlier in this study (section 3.IV, Rühl and Kües 2009). For shake flask cultures, pellets were clustered in distinct groups according to their pellet area (0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1.0, 1-3, 3-5, 5-7, 7-9, 9-11, 11-13, 13-15, 15-17, 17-19, 19-21, 21-23, 23-25 mm^2) and, additionally, according to their convexity (0.50-0.525, 0.525-0.55,

0.55-0.575, …, 0.975-1.00) and diameter (0-0.5, 0.5-1.0, 1.0-1.5, …, 9.5-10.0) by using the automated image analysis system. The pellet concentration (pellets per culture) was determined as well.

In bioreactor cultures, the pellets were clustered according to their projected pellet area into broader groups, due to inhomogeneous growth among the fermentation runs at constant pH 7 (0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1.0, 1-3, 3-5, 5-9, 9-13, 13-17, 17-21, 21-25, 25-50 mm^2).

7. Embedding of mycelium pellets into Roti®-Plast (Fa. Roth, Karlsruhe, Germany)

Around 10 mycelial pellets were collected per fungal liquid culture per day of incubation and fixed in FAE solution (100 ml contain 90 ml of 70% ethanol (EtOH), 5 ml of acetic acid and 5 ml of 37% formaldehyde) followed by several embedding steps. The pellets rested at least for 4 h at room temperature (RT) in the following chemicals: 70% EtOH, 80% EtOH, 90% EtOH, 96% EtOH, 96% EtOH/isopropanol 1:1, isopropanol, isopropanol/Roti®-Histol 3:1, isopropanol/Roti®-Histol 1:1, isopropanol/Roti®-Histol 1:3, 3 times pure Roti®-Histol, saturated Roti®-Plast in Roti®-Histol for at least 8 h, saturated Roti®-Plast in Roti®-Histol at 40 °C for at least 12 h, 3 times pure melted Roti®-Plast at 60 °C for at least 12 h. The embedded pellets were fixed onto a wooden block, trimmed to fit in the microtome and 10 µm cuttings were performed with a rotary microtome (R. Jung, Heidelberg, Germany). The cuttings were put onto a gelatinised microscope slide and washed three times with pure Roti®-Histol, once with isopropanol/Roti®-Histol 1:1 and twice with pure isopropanol for at least 5 min each. At the end, the slides were stored at RT for evaporation of the isopropanol and pictures were taken with a binocular (Stemi 2000-C, Carl Zeiss MicroImaging GmbH, Göttingen) for analysis of the cross section.

D. Results

1. Cultural growth and laccase production at 25 °C and 37 °C

The laccase producing *lcc1* transformant and the control transformant were inoculated into modified Kjalke medium and cultivated in parallel at 25 °C and 37 °C, respectively.

Biomass (DW) production, glucose consumption and nitrogen consumption (C/N ratio) followed the same general pattern for the *lcc1* transformant and the control transformant at 25 °C, respectively at 37 °C (Fig. 2 A, B and C). Growth at 25 °C was delayed compared to

growth at 37 °C, in accordance to the optimal growth temperature of the fungus (Kües 2000). DW values of ~ 1 g/l were reached at day 2 of cultivation for the *lcc1* transformant in 25 °C and 37 °C cultures and of ~ 3 g/l for the control transformant in 25 °C and 37 °C cultures at day 4 of cultivation, respectively (Fig. 2A). Until day 6, the controls had slightly higher dry weights compared to the *lcc1* transformant. All cultures reached their maximum DW of around 8-10 g/l at day 6 of cultivation. Afterwards, the biomass declined in the cultures at 37 °C with DW values of 7 and 5 g/l at days 8 and 10 of cultivation, respectively. At 25 °C, from day 6 to day 8 the DW of both strains remained high with a slight increase for the *lcc1* transformant culture that reached at day 8 a maximum biomass DW of 9 g/l. Afterwards, the biomass slowly declined (Fig. 2A).

Along with biomass production, glucose was consumed in the first 6 days of cultivation in all control cultures, irrespectively of temperature. Glucose in *lcc1* transformant cultures was also fully depleted at day 6 in cultures grown at 37 °C, whereas at 25 °C the glucose was fully depleted at day 8 of cultivation (Fig. 2B). The C/N ratios in the cultures followed exactly the glucose concentration curves. At day 6, respectively day 8 of cultivation, the C/N-ratio levelled down to about 4 which was subsequently kept for the rest of the cultivation period. Generally, the 37 °C cultures showed a more drastic decrease in the glucose concentration compared to the 25 °C cultures. This again is reflected in the C/N-ratio which decreased faster at 37 °C (Fig. 2C).

Along with biomass production, as well as C and N consumption, laccase activity in the supernatant increased in comparable manner at the temperatures of 25 °C and 37 °C until day 6, in both cases to a level of about 3 U/ml. In the next 4 days, the activity at 37 °C decreased continuously to final levels below 0.3 U/ml. In contrast, the 25 °C cultures reached their maximum activities of around 9 U/ml at day 8 of cultivation. For comparison, the control transformant showed no measurable activity at 37 °C, but reached activities of about 3 U/ml at day 6 in 25 °C comparable to the *lcc1* transformant activities at 37 °C (Fig. 2D).

The initial pH of the Kjalke medium (pH 6.0) decreased in all cultures until day 4, when values of pH 5.0 to 5.5 where reached. Subsequently, when highest biomass values were obtained (Fig. 2A), a dramatic increase of the pH was observed at day 6 (pH 7.8) in the 37 °C cultures and final values of almost pH 9 occurred at day 10. The 25 °C cultures showed a slightly delayed increase in the pH of the supernatant from day 6 onwards. Values of around pH 7 were observed at day 8 and a final pH of around 8 was reached at day 10 of cultivation (Fig. 2C). A direct correlation to enzymatic activities is not seen.

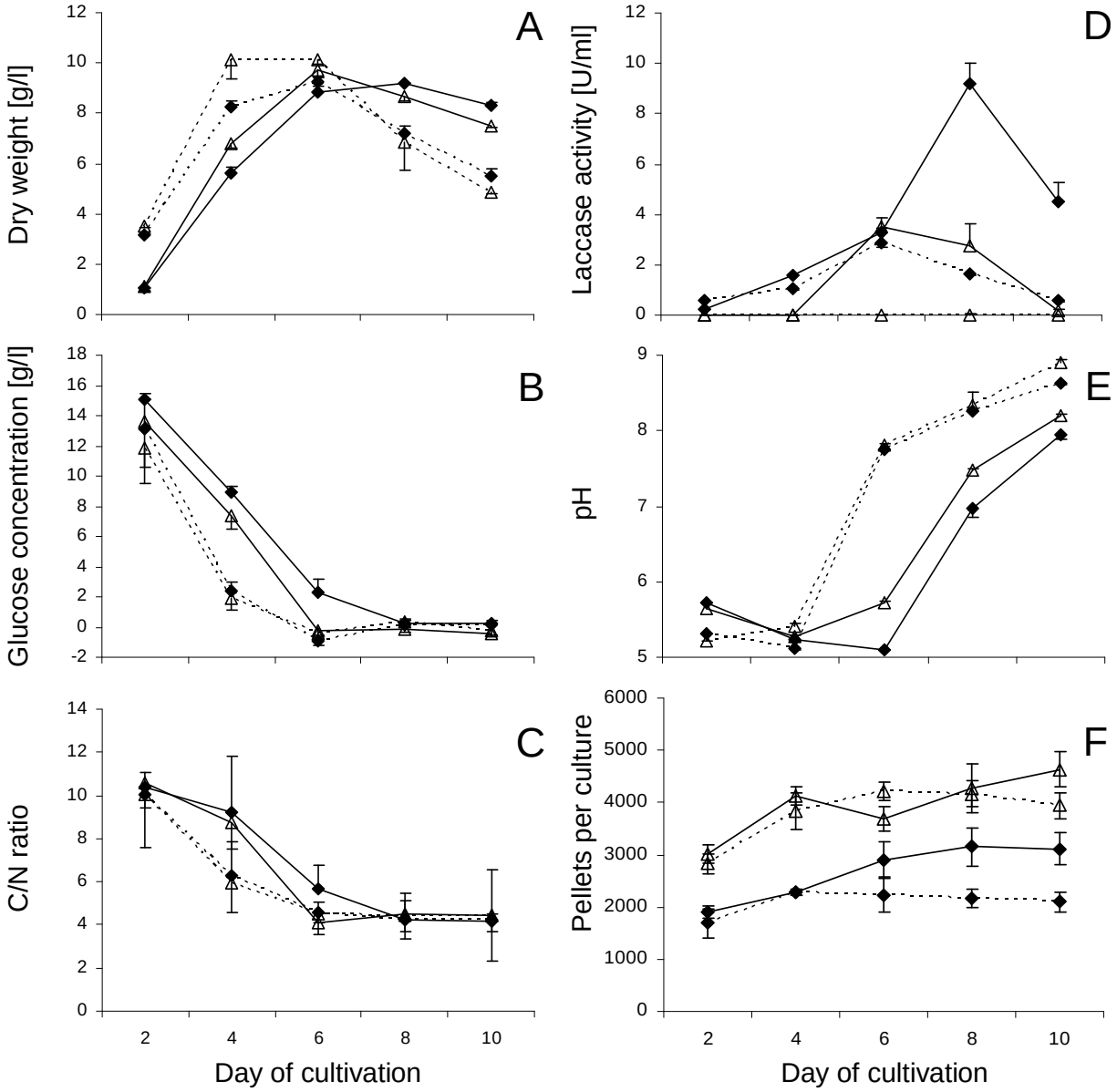


Fig. 2 Shaken cultures of the *lcc1* transformant (closed diamond) and control transformant (open triangle) at 25 °C (solid line) and 37 °C (dashed line) in modified Kjalke medium: dry weight of the fungal biomass (**A**), glucose concentration (**B**), C/N ratio (**C**), laccase activity (**D**) and pH of the supernatant (**E**), as well as number of pellets per culture with a projected area ≥ 0.2 mm^2 (**F**). Values are averages ± standard deviation calculated from three parallel cultures.

Pellet formation was also followed up in the cultures. Here, unlike total biomass production, pellet concentration differed drastically between the transformants, but not as much as between the two different temperatures a strain was cultured at. Pellet concentration reached amounts of 3000 and 2200 pellets per culture from day 6 onwards for the *lcc1* transformant at 25 °C and 37 °C, respectively. More than 4000 pellets per culture were obtained for the control transformant at both cultivation temperatures (Fig. 2F).

In 25 °C cultures irrespectively of the strain used, the pellet amount increased slightly over the time until day 10, whereas pellet concentration of cultures of both transformants at 37 °C had their maximum levels at day 6 of cultivation. Pellet amounts slightly decreased from day 6 onwards until the end of the cultivation. Highest pellet numbers in 25 °C cultures of the *lcc1* transformant correlated with highest biomass and highest enzyme production, whereas such a correlation at 37 °C with the generally lower enzymatic yield was not obvious, in terms of enzymatic activity.

2. Pellet morphology in shake flask cultures

Fungal growth and pellet formation in cultures of the *lcc1* transformant and the control transformant was followed up over the time by automated image analysis as described earlier (section 3.III, Rühl and Kües 2009). Selected photographs of the pellets in the culture supernatants are shown in Fig. 3. For both transformants, the growth at 25 °C was generally more homogenous than the growth at 37 °C.

At 25 °C at the initial phase of growth at day 2 of cultivation, for both transformants pellets were small of irregular fringy shape and had a loosely aggregated, thin mycelium. With prolonged cultivation time at day 4 of cultivation, pellets reached round and sometimes oval shapes. At day 6 of cultivation, the pellets of both transformants showed the highest density throughout the cultivation as can be deduced from their intransparent look in the photographs. Until the end of the cultivation, the pellets retained their round and oval shapes, but reduced in their overall density as is documented by the lighter grey shading of pellets in the photographs (Fig. 3, 25 °C).

At 37 °C, both transformants produced already large and dense pellets at day 2 at the beginning of the cultivation. However from day 4 onwards, high amounts of free mycelium (hyphal fragments) were present in the supernatant cultures. In cultures of the control transformant, the amount of hyphal fragments seemed to be higher than in cultures of the *lcc1* transformant (Fig. 3, 37 °C). Along with producing masses of mycelium fragments, overall, the density of the pellets in both transformants reduced much more drastically at 37 °C at the end of the cultivation than in the 25 °C cultures as indicated by the more light pellet colours in the photographs taken on day 8 and day 10 of cultivation, respectively (Fig. 3).

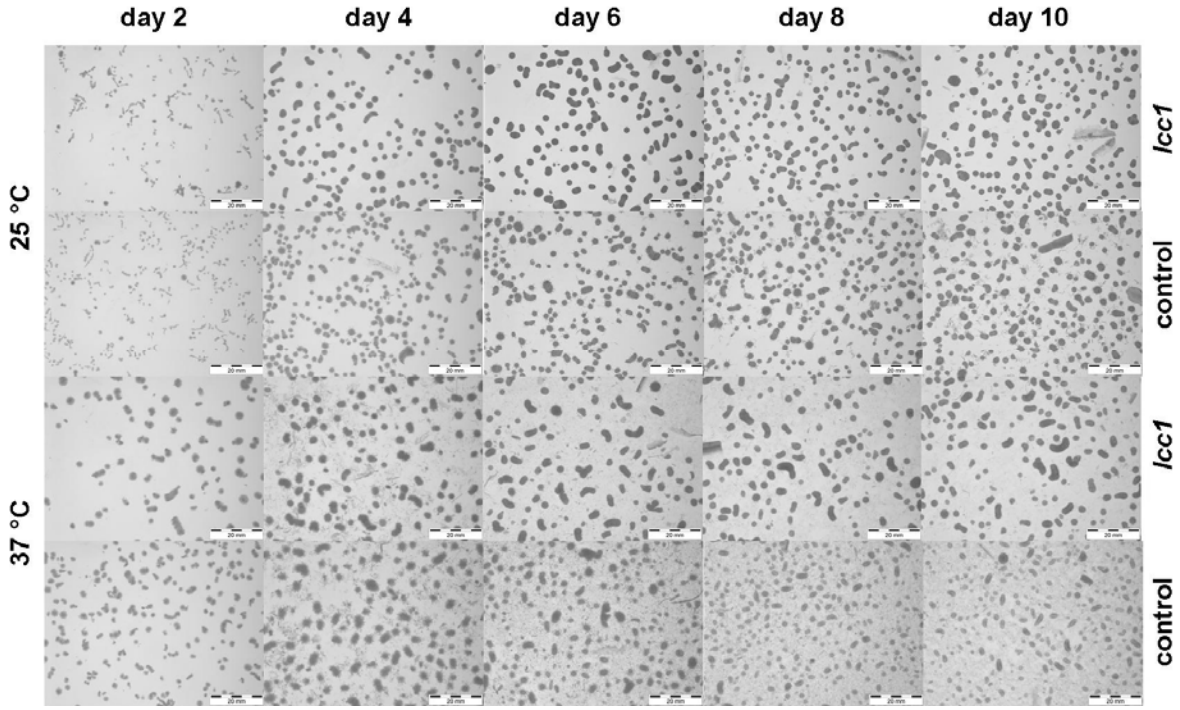


Fig. 3 Photographs of pellets in the supernatant from the *lcc1* transformant and the control transformant cultivated at 25 °C and 37 °C, respectively, in modified Kjalke medium. Scale bars in the lower right corner of the images indicate 20 mm in size.

In order to characterise pellet morphologies in more detail, in total about 800-1200 pellets per transformant, culture condition and day of cultivation were characterised by automated image analysis. The study revealed that the distribution of the projected pellet area of the pellets showed differences in size. At 25 °C, after 2 days of cultivation most of the pellets derived from *lcc1* transformant cultures covered a projected area of 1-3 mm^2, whereas pellets of the control transformant were smaller with most of them having a projected area below 0.4 mm^2 (Fig. 4). At 37 °C after 2 days of cultivation, the pellets reached already a size of 1-5 mm^2 and 3-7 mm^2 in control and in *lcc1* transformant cultures, respectively (Fig. 4).

Over the whole cultivation time, small pellets and loosely aggregated hyphal fragments covering a projected area lower than 0.2 mm^2, which were not excluded from pellet raw data by the automated image analysis system (section 3.III, Rühl and Kües 2009), were present in all cultures. At 37 °C, highest amounts of these small pellets and loosely aggregated hyphal fragments were reached from day 4 onwards. Their fraction on the detected pellets was almost 25% at day 4 and 50% at day 6 for the *lcc1* transformant and control transformant, respectively. With prolonged cultivation time, the amount of small pellets and loosely aggregated hyphal fragments increased slowly up to 10% and 25% also in the 25 °C cultures at day 10 for the *lcc1* transformant and control transformant, respectively (Fig. 4,

left row). However at both temperatures and for both strains, most pellets were larger than 0.2 mm^2 with the projected area mostly in the range of 1-5 mm^2. Generally, the *lcc1* transformant cultures had slightly larger pellets than the control transformant with the maximum amount of pellets having a projected area between 3-7 mm^2 with average values between 4.8 and 6.0 mm^2 from day 4 of cultivation onwards. Pellets from control transformant cultures had smaller average areas with values of 2.2 to 4.7 mm^2 (Table 1, Fig. 4). Overall, the *lcc1* transformant at 37 °C showed a more diverse distribution of the projected pellet area in a range of 1-10 mm^2 with a high number of smaller pellets or loosely aggregated hyphal fragments (< 0.2 mm^2) until day 6 (Fig. 4). Generally, the results on the projected pellet areas are reflected in the data on pellet diameters.

When analysing the pellet diameter, pellets at 25 °C of both transformants at day 2 showed a sharp peak of the distribution curve. Most pellets had a diameter of 1.0 mm to 1.5 mm both for the control and for the *lcc1* transformant. For both transformants, the diameter increased to stable values of 2.0-2.5 mm at day 4 and day 6 onwards, whereas at day 10 also smaller fragments with diameters of 0.5-1.0 mm occurred (Fig. 5 left row).
In 37 °C cultures at day 2, larger diameters were reached with peaks at sizes of 0.5-1.0 mm and 2.0-2.5 mm for the control transformant, and 3.0-3.5 mm for the *lcc1* transformant. This distribution of pellets split into diameters of < 1.0 mm and 2.5-3.5 mm after day 2. The larger pellets (> 1.0 mm) approach over the time with diameters of 1.5-2.5 mm for the control transformant and 2.0-3.0 mm for the *lcc1* transformant. In contrast similarly to the amount of pellets with a smaller projected area, the amount of pellets with smaller diameters increased over the time for the control transformant and the *lcc1* transformant to values of 20% and 40% at day 6, respectively (Fig. 5 left row).
Analysing the regularity of the shape of pellets, the convexity of the pellets was very variable at the beginning of cultivation, but sharp peaks of convexity values of 0.95-0.975 were obtained at the end of cultivation. The *lcc1* transformant cultures at 25 °C showed a somewhat different picture as sharp peaks for values of the convexity were reached already after day 4 of cultivation and kept on during further incubation (Fig. 5 right row).

Table 1 Average pellet area in mm^2 of pellets of the *lcc1* transformant and control transformant cultivated in shake flasks in Kjalke medium at 25 °C and 37 °C, respectively

		day 2	day 4	day 6	day 8	day 10
25 °C	*lcc1*	1.39 (± 1.51)	4.81 (± 3.20)	5.96 (± 4.68)	5.61 (± 4.41)	4.95 (± 3.67)
	control	0.63 (± 0.80)	3.11 (± 2.06)	4.71 (± 4.06)	4.13 (± 3.87)	3.32 (± 3.72)
37 °C	*lcc1*	7.06 (± 5.11)	5.10 (± 5.35)	4.94 (± 5.28)	5.88 (± 5.53)	5.49 (± 4.72)
	control	3.21 (± 2.68)	3.63 (± 3.73)	2.57 (± 3.33)	2.47 (± 2.69)	2.16 (± 2.94)

Values are averaged of three replicates and standard deviations are given in brackets.

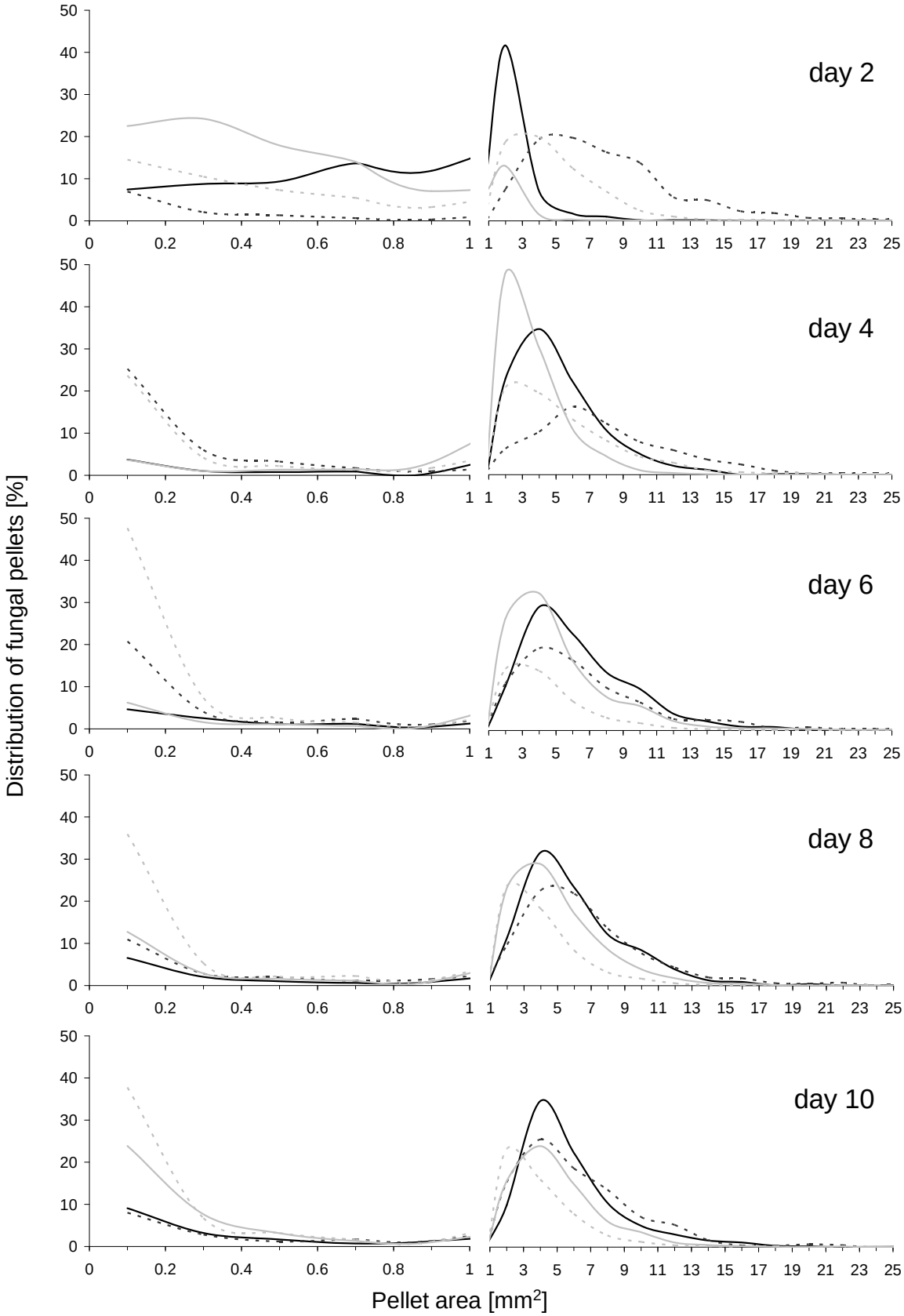


Fig. 4 Distribution of pellets from *C. cinerea* shake flask cultures over the fermentation time [h] according to their projected pellet area [mm^2]. The *lcc1* transformant (black line) and control transformant (grey line) were grown at 25 °C (solid line) and 37 °C (dashed line) in modified Kjalke medium, respectively. The distribution is given in % as a mean value of 3 replicates. Curves were drawn by extrapolation of calculated values in % for size ranges (see Material and Methods), whereas calculated values are fixed within curves in position of the median value of a range. To indicate the change of scale, curves were interrupted at the value 1 mm^2 for the pellet areas.

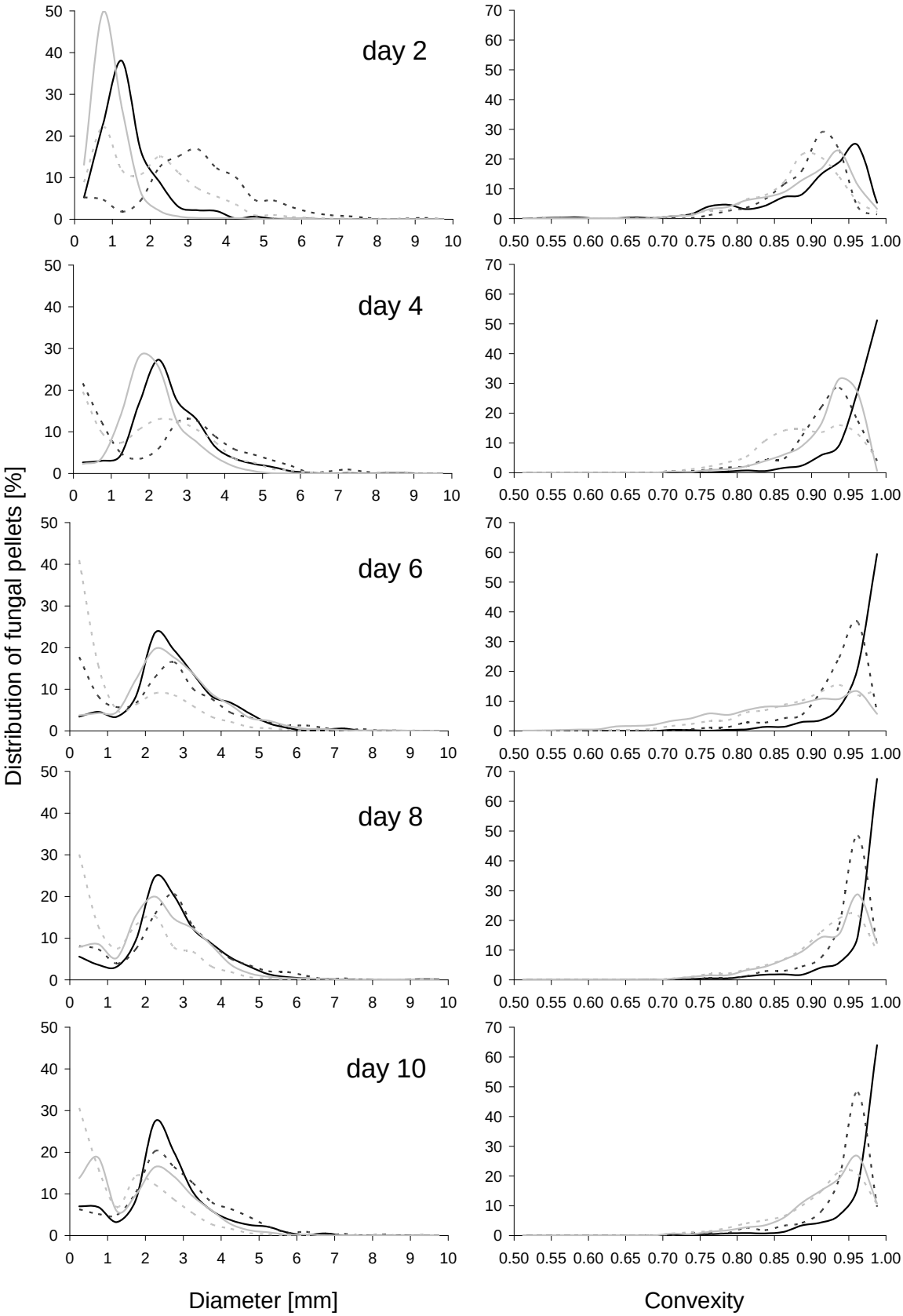


Fig. 5 Distribution in % of pellets of the shaken flask cultures of the *Icc1* transformant (black line) and the control transformant (grey line) grown at 25 °C (solid line) and 37 °C (dashed line) in modified Kjalke medium and analysed by pellet diameter (left series of graphs) and pellet convexity (right series of graphs). Curves were drawn by extrapolation of calculated values in % for size ranges (see Material and Methods), whereas calculated values are fixed within curves in position of the median value of a range. The distribution is given in % as a mean value of 3 replicates.

3. Micro-morphological analysis of *C. cinerea* pellets

Photographs of pellets taken by a CCD camera attached to a binocular revealed that pellet surfaces varied tremendously between 25 °C and 37 °C cultures (Fig. 6). At 25 °C, the pellets of both transformants showed a very smooth surface area. In contrast, pellets derived from cultures at 37 °C had a more hairy look.

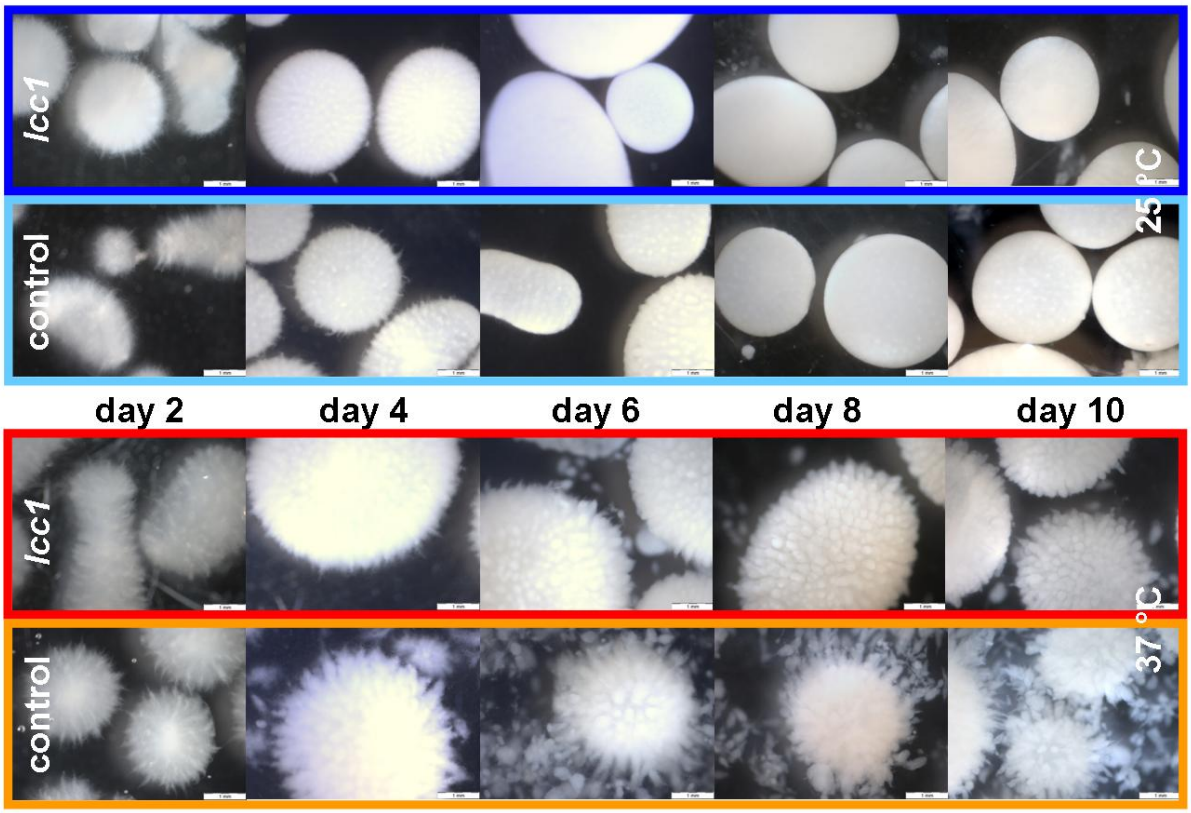


Fig. 6 Photographs of pellets obtained from the *lcc1* transformant and control transformant cultures grown at 25 °C and 37 °C, respectively. Scale bars in the lower right corner indicate 1 mm.

To study the outer surface in more detail, we analysed microtome cuttings of pellets. Each second day, average sized pellets from cultures were taken, embedded in paraffin wax and cut into 12 µm slices with a rotary microtome. Slices representing the middle of the pellet (the largest diameter) were photographed using a CCD camera fixed onto a binocular (Fig. 7). The differences between pellets grown at 25 °C and 37 °C are very obvious when comparing the microtome cuttings. The pellets of 37 °C cultures already reached their final size after 2 days, whereas at 25 °C the final size was reached within 4 days (compare Fig. 4 and Fig. 7). At the first day of analysis (day 2 of cultivation), pellets were uniformly structured without an outer zone. While at day 4 of cultivation such a differentiation became visible. At 25 °C, the smooth pellets of both transformants have a very dense and small mycelial outer zone (rind) and a less dense inner region (medulla). However, the outer

mycelial ring in pellets from the control transformant seems to be less even (Fig. 7). In pellets of 37 °C cultures, the outer mycelial zone is irregular hairy and seems to be less compact than the rind of pellets formed at 25 °C although, generally, the outer zone of pellets formed at 37 °C is much broader than those formed at 25 °C (Fig. 7). No obvious differences can be seen between pellets of the *lcc1* transformant and the control transformant of 37 °C cultures. We noticed that pellets from 10 day-old cultures were more instable. Thus microtome cuttings were harder to obtain.

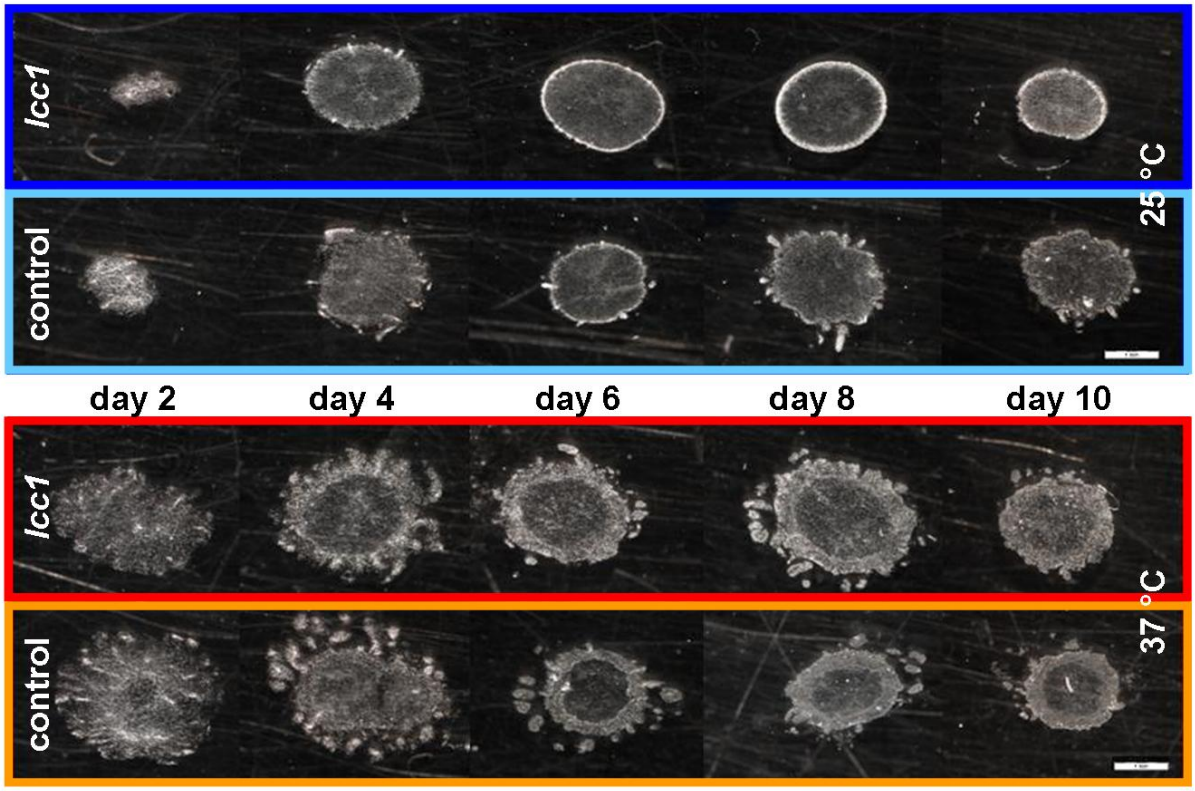


Fig. 7 Microtome cuttings of paraffin embedded pellets from *lcc1* transformant and control transformant cultures grown at 25 °C (upper rows of photos) and 37 °C (lower rows of photos) in modified Kjalke medium, respectively. The bar in the lower right part indicates 1 mm. Per day of cultivation and transformant, at least 3 pellets were analysed.

To better compare the general structure of pellets from different cultures with each other, a ratio of the width of the outer zone to the complete diameter was calculated. For this purpose, each three photos of microtome cuttings were analysed with the software analySIS® (Soft Imaging System GmbH, Münster, Germany). Obtained data are presented in Fig. 8. Because differentiation into an outer and an inner zone were only observed from day 4 onwards, pellets derived from day 2 of cultivation were excluded from the analysis. The ratio width outer zone to diameter did not differ over the time for a strain within a culture or between strains at the same cultivation condition. The ratios for both strains were around 5

for 37 °C over the whole period of cultivation, with minor differences being within the range of the standard deviation (4.9 ± 1.1). At 25 °C, the ratios were more than twofold higher than at 37 °C with values ranging from 12 to 17 (14.1 ± 3.6).

Although the outer zones in pellets from 25 °C cultures of *lcc1* transformants appeared to be more compact than those of pellets of the control transformant, differences in the ratio (diameter/outer zone) between pellets of the *lcc1* transformant and pellets of the control transformant were marginal for both cultivation temperatures.

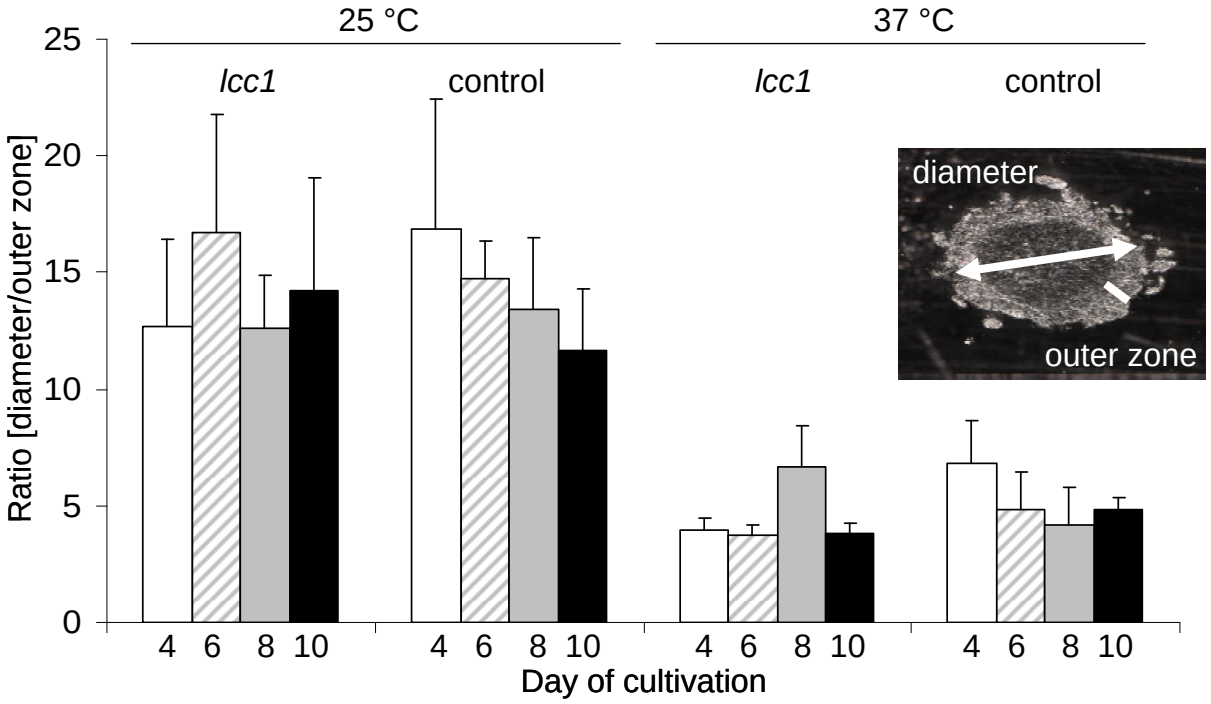


Fig. 8 Comparison of the ratio (pellet diameter/width outer zone) measured from cross sections of pellets of the *lcc1* transformant and control transformant cultivated at 25 °C and 37 °C, respectively, at day 4 (white), day 6 (shaded grey), day 8 (grey) and day 10 (black) of cultivation. The error bars indicate the standard deviation of three different pellet cross sections. The image shows an example for a microtome cutting of a pellet with arrows indicating the diameter of the pellet (large arrow) and the width of the outer zone (small bar) as used for calculation of the ratio: pellet diameter/width outer zone.

4. Laccase production in a stirred bioreactor

Fermentation of the *lcc1* transformant was conducted in a 5 l bioreactor (New Brunswick Scientific) at 37 °C and an agitation rate of 120 rpm that was mediated by two six-bladed Rushton turbines (Fig. 1). Runs were performed at three different constant pH values: pH 6, 7 and 8. Until day 3 after inoculation, culture samples were taken via the sampling tube twice a day. Afterwards, only one sample was taken every 24 h.

Up to day 6, the fungus grew in all cultures in form of pellets and mycelial fragments allowing mixing of the cultures by the Rushton turbines of the bioreactor. Afterwards, by the increase in biomass and viscosity, growth was non-homogenous due to increased adhesion of mycelial hyphae at peripherals of the bioreactor, such as sensors, impellers and aeration pipe (see Fig. 1 for details in bioreactor construction). Therefore, fermentation were stopped at day 6 of cultivation.

Generally, an increase in biomass indicated by the dry weight (DW) and laccase yields in the supernatant were achieved with decreasing pH values in the fermentation runs. Thus, highest values in biomass were obtained at pH 6, respectively lowest values at pH 8. Nevertheless, the biomass curves showed a similar constant increase at all pH values. Highest biomass values of 4.8 ± 0.8, 4.3 ± 0.4 and 3.3 ± 0.8 g/l were reached for pH 6, pH 7 and pH 8, respectively, at day 6 of cultivation (Fig. 9A). The laccase activity within the supernatant showed a similar increase until day 3 after inoculation. At this point, the laccase activity curve of pH 8 flattens in increase unlike the laccase activity curves at pH 6 and pH 7. At pH 6 and pH 7, laccase activity further increased with prolonged cultivation time, reaching highest activities of 1.3 ± 0.3 and 1.1 ± 0.2 U/ml at day 6 of cultivation. The laccase activity obtained at pH 8 (0.5 ± 0.1 U/ml) was only half the amount than that at pH 6 and pH 7 (Fig. 9B).

Since a constitutive promoter (*gpdII* from *A. bisporus*) was used for the recombinant laccase production, a comparison between the laccase activity and the biomass might show effects other than by the biomass itself. Thus, laccase activities per biomass DW values were calculated (Fig. 9C). At the beginning of cultivation, the laccase per biomass specific activity curves [U/gDW] were alike for all pH values. However, after 3 days of cultivation upon reaching a value of 183 ± 72 U/gDW, the curve for the cultivation at pH 8 stopped abruptly in increase in order to slowly decrease to a final value of 163 ± 26 U/gDW at day 6 of cultivation. After day 3 of cultivation, biomass specific activities of cultivation at pH 6 and at pH 7, in contrast, constantly increased further over the next days and reached their highest values of 296 ± 57 U/gDW and 246 ± 28 U/gDW after 5 and 6 days of cultivation, respectively.

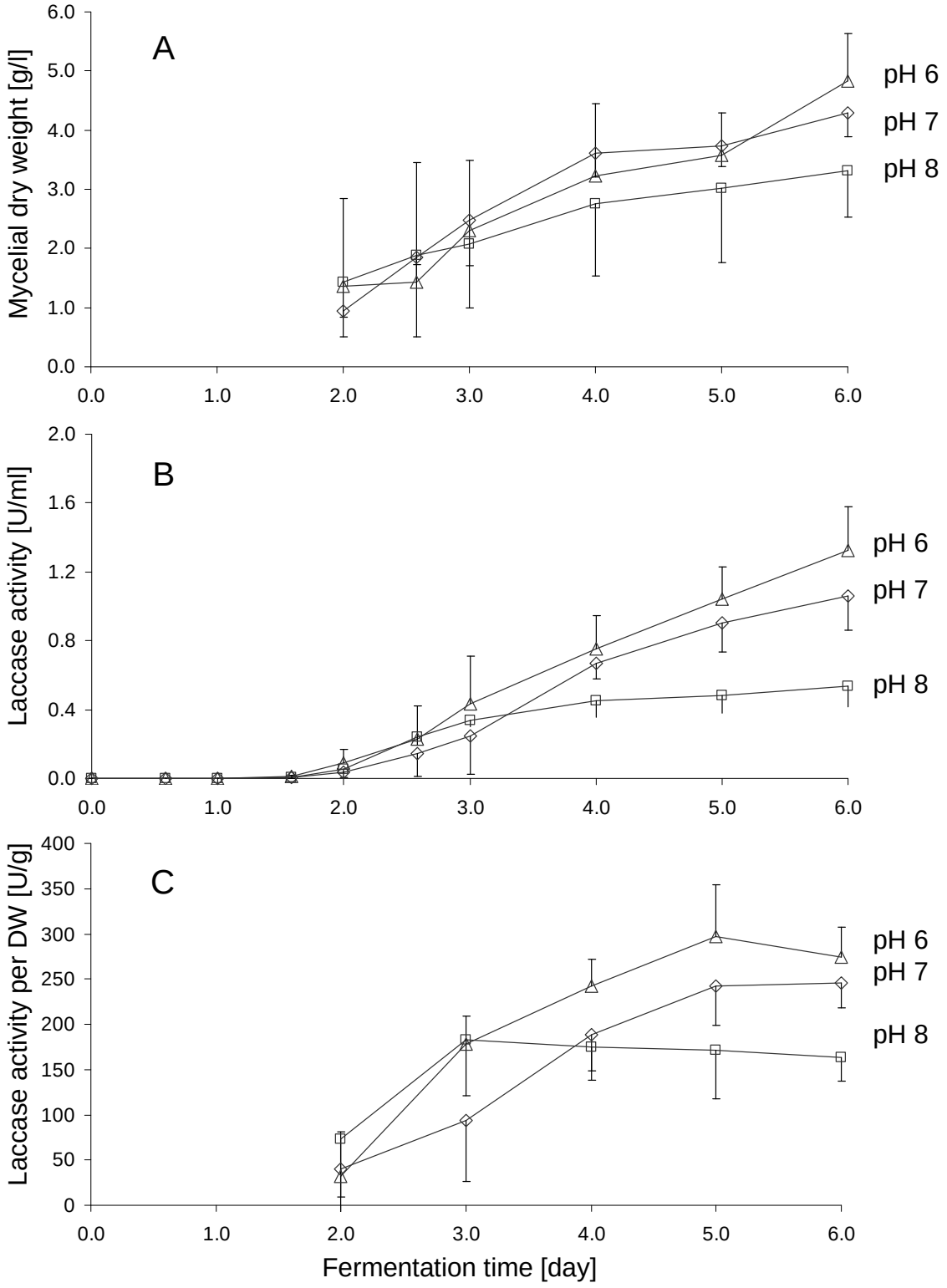


Fig. 9 Biomass curve (**A**) stated by the fungal dry weight (DW) and laccase activities of the supernatant per volume (**B**) and g DW (**C**) of fermentations of the *lcc1* transformant cultivated in a 5 l stirred tank reactor in modified Kjalke medium at pH 6 (triangle), pH 7 (diamond) and pH 8 (square). Values were averaged from three fermentation runs per pH and bars above or below the curves indicate the standard deviation.

5. Pellet morphology in stirred bioreactor cultures

Also for cultivation in the 5 l bioreactor, pellet morphology was observed. Per day and culture about 15-20 ml solution were harvested with about 60 to 250 pellets that were evaluated per image by automated image analysis. In all fermentations in the 5 l bioreactor, pellets of measurable size were first detected after 2 days of cultivation. In the cultures kept at constant pH 6, at the beginning almost 40% of the pellets had a projected area of 1-3 mm^2 with an average pellet size of 1.1 mm^2 (± 0.9 mm^2) (Fig. 10, Table 2). After day 2 of cultivation, the pellets showed a broader distribution in size of 1-7 mm^2 and a smaller amount of pellets being in the range of up to 10 mm^2. The average pellet area increased until day 4 (7.6 ± 4.8 mm^2) and changed only marginally until the end of the cultivation (7.9 ± 5.7 mm^2) (Table 2). From day 4 onwards until the end of the cultivation, also pellets with a projected area above 21 mm^2 were detected accounting for about 10% of all pellets per culture (Fig. 10).

At pH 7, already at the start of the fermentation there was a different distribution pattern of pellet size as compared to pH 6. At day 2 of cultivation, average pellet area was 3.7 ± 3.5 mm^2 (Table 2). Afterwards, the sizes of the pellet areas varied dramatically with most pellets having a projected area in the range of 5-9 mm^2 and 13-17 mm^2, respectively (Fig. 10). Additionally, also very small (< 0.2 mm^2) and large (> 21 mm^2) pellets were detected from day 3 onwards over the whole cultivation period with up to 21% of the pellets at day 5 being larger than 21 mm^2. At the end of the fermentation runs, values of the average pellet area at pH 7 were similar to values derived at pH 6 with an average projected area of 7.3 ± 11.0 mm^2 at day 6 of cultivation, although values at pH 7 had a much a higher standard deviation than at pH 6, because of the overall broad pattern of size distribution.

In fermentation experiments at pH 8, the pellets were smaller than those produced at pH 6 at the beginning of cultivation with values between 1-3 mm^2 that however increased from day 3 onwards to a more widespread average projected area of 1-9 mm^2 after 4 days, with the highest amount of pellets being in the range of 1-3 mm^2. Small pellets or loosely aggregated hyphal fragments of sizes between 0.2-0.4 mm^2 were observed over the whole cultivation time, whereas in later stages the amount increased to around 20% at day 5 and day 6 of cultivation, respectively (Fig. 10). Generally, more smaller pellets and loosely aggregated hyphal fragments occurred at pH 7 and 8, compared to pH 6 (Fig. 10).

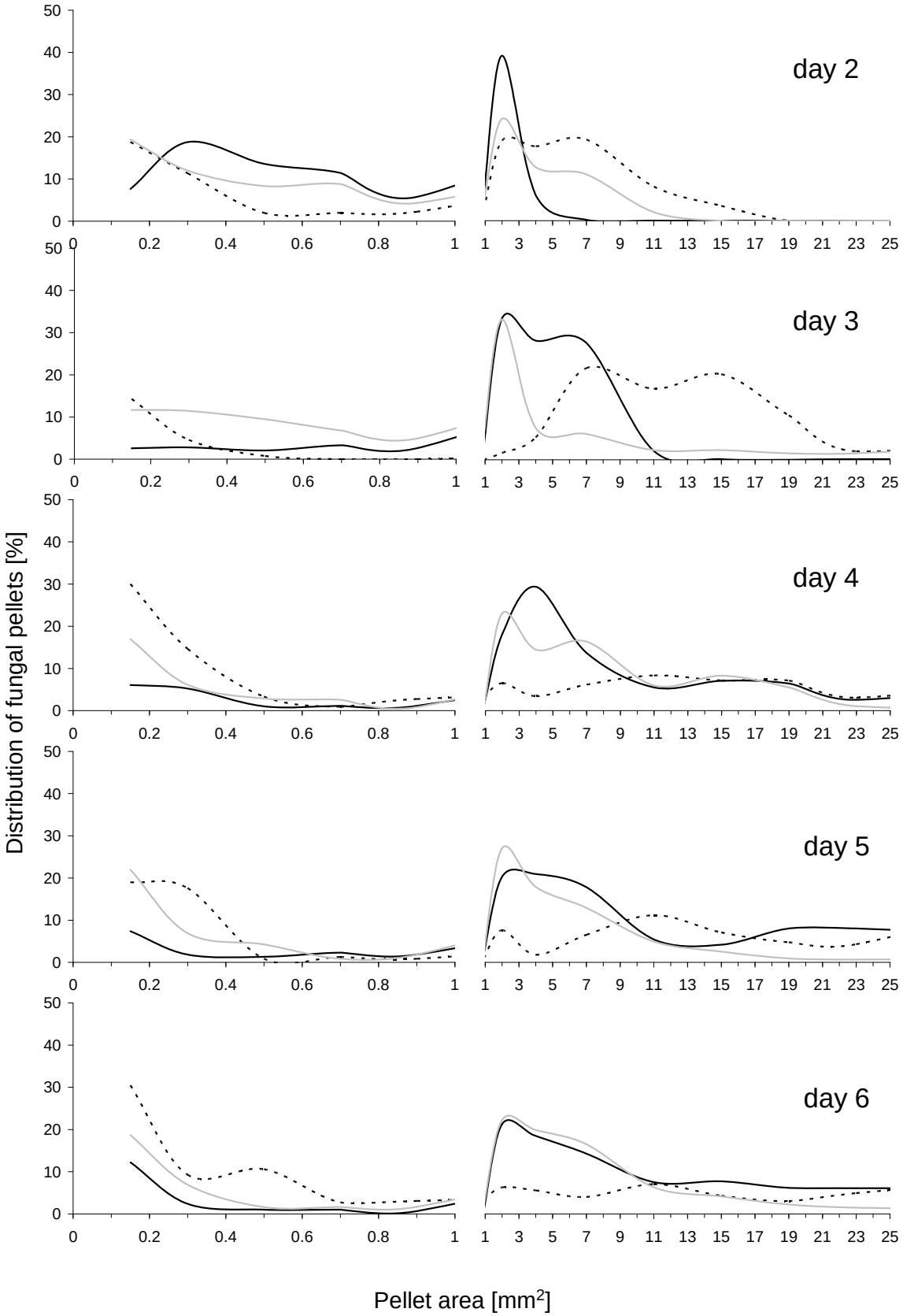


Fig. 10 Distribution in % of pellets with an area equal or larger than 0.1 mm² in fermentation experiments at pH 6 (black solid line), pH 7 (black dotted line) and pH 8 (grey solid line), respectively. The percentages are average values of 3 fermentation runs per pH value. Curves were drawn by extrapolation of calculated values in % for size ranges (see Material and Methods), whereas calculated values are fixed within curves in position of the median value of a range. To indicate the change of scale, curves were interrupted at the value 1 mm² for the pellet areas.

Table 2 Average pellet area in mm^2 of pellets equal or larger than 0.1 mm^2 of the *lcc1* transformant cultivated in Kjalke medium in a 5 l bioreactor at 37 °C at three different pH values

	day 2	day 3	day 4	day 5	day 6
pH 6	1.12 (± 0.89)	3.73 (± 2.57)	7.64 (± 4.82)	8.32 (± 4.79)	7.92 (± 5.68)
pH 7	3.66 (± 3.45)	11.09 (± 8.86)	7.45 (± 9.89)	12.02 (± 14.28)	7.28 (± 11.01)
pH 8	2.05 (± 2.29	4.22 (± 6.81)	5.13 (± 5.64)	3.34 (± 4.26)	4.44 (± 5.30)

Values are averaged of three fermentation runs per pH and standard deviations are given in brackets. Note that the average values did not correspond to the peak values shown in Fig. 10.

E. Discussion

In this study, the changing morphology of *C. cinerea* in modified Kjalke medium in liquid shaken and in stirred bioreactor cultures were observed over the time together with enzyme production by a recombinant laccase producing strain (*lcc1* transformant) and a control transformant without extra laccase gene (pCc1001). Results show that morphology of fungal pellets is influenced by the cultivation temperature and the pH of the medium. A correlation between fungal morphology and laccase secretion was not obvious.

1. Shake flask cultures: laccase and biomass production

In shake flask cultures, laccase yields of the *lcc1* transformant were highest at 25 °C, although part of the enzymatic activities seemed to derive from natural production of laccase as deduced from the low enzymatic activities in the supernatant of the control transformant. In contrast, the control did not produce laccases at the higher temperature of 37 °C unlike the *lcc1* transformant (Fig. 2). It is thus assumed that at 37 °C all laccase activity is due to recombinant enzyme production. Generally, enzymatic activities were found to be higher at the lower temperature. Best enzymatic yields at both temperatures correlated with high biomass amounts, depletion of glucose in the medium and a shift in pH from slightly acidic to alkaline. The two transformants had comparable growth behaviours with a faster biomass production at 37 °C as compared to 25 °C.

After 2 days of cultivation, both transformant cultures grown at 37 °C already had 3times higher dry weights compared to the 25 °C cultures. This faster growth at the beginning of the culture might have been due to the required adaptation of the fungus to the lower growth temperature of 25 °C, because the pre-culture was solely cultivated at 37 °C. During the continuous cultivation in the following 2 days, *lcc1* transformant and control transformant cultures showed an analogous increase in the growth curves (Fig. 2A) at 25 °C and 37 °C, respectively. Although the maximum biomass dry weight was reached 2 days earlier in

cultures at 37 °C, the maximum dry weight for all temperatures was in the range of 8-10 g/l. These findings indicate a longer lag-phase and a lower biomass duplication time at 25 °C compared to 37 °C.

Accordingly, also depletion of glucose in the cultures showed a 2-days shift between 25 °C and 37 °C cultures, respectively. The depletion of glucose and highest biomass yields more or less coincided with an increase in the pH. Most obvious is the 2-days difference between 25 °C and 37 °C in change of the pH of the supernatant (Fig. 2E). The shift of ~2 days in alkalisation of the medium may derive from a better growth at 37 °C resulting in an active alkalisation of the medium by the higher fungal biomass and in earlier autolysis of the fungus.

Analysing laccase production by the control transformant at 25 °C suggests that native laccase is already produced by *C. cinerea* at lower pH values as highest activity occurred at pH 5.7 (Fig. 2D und 2E). Contradictory to this, Hublik and Schinner (2000) reported for *P. ostreatus* a correlation of laccase production with the pH in the culture supernatant reaching maximum laccase activities at pH 8.5. In another study, the basidiomycete *Trametes pubescence* was cultivated in a stirred vessel without controlling the pH. Similar to our observation in shake flask cultures of *C. cinerea*, the pH decreased at the beginning of the culture and increased sharply after the glucose got depleted. Herein, the maximum laccase activity was reached at pH 7 for batch and at pH 5 for fed-batch cultures (Galhaup et al. 2002). Thus, laccase activities in *T. pubescence* seem to depend rather on the glucose concentration than on the pH of the cultivation medium. How compare such natural laccase yields to our results with the recombinant laccase producer? There was comparable laccase activity by the *lcc1* transformant prior to day 6 of cultivation at 25 °C and 37 °C, but at 25 °C extra laccase activity occurred at day 8 of cultivation (Fig. 2D). Production of the recombinant laccase was under the control of the *gpdII*-promoter from *A. bisporus* believed to be a constitutive promoter (Harmsen et al. 1992, Kilaru and Kües 2005). However in the *lcc1* transformant at 25 °C, the *gpdII*-promoter appeared not to behave constitutively. Currently, it is unclear whether this is due to the ectopic place of the vector integration into the genome of *C. cinerea* or due to a failure of appropriate regulation of the *A. bisporus* promoter in the foreign host at 25 °C.

2. Shake flask cultures: pellet morphology

Enzymes in filamentous fungi are believed to be secreted by physiologically active hyphal tips (Moukha et al. 1993, Conesa et al 2001). Therefore, it is very reasonable to study fungal growth and morphology during cultivation and fermentation. Here, the pellet production and

morphology was compared between two transformants, one of which recombinantly produced laccase.
Clear differences in pellet morphology were visible between different cultivation temperatures and to a less degree also between the two transformants when grown at the same temperature.

For both transformants, the projected area of their pellets showed similar sizes at both cultivation temperatures. Independently of the temperature, most pellets of the control transformant showed a projected area of approximately 3 mm^2 and most pellets of the *lcc1* transformant a projected area of 5 mm^2, respectively. One reason for this difference between the recombinant laccase producer and the control is probably the slightly faster growth of the control transformant resulting in a faster increase in total biomass (Fig. 2A) and in a higher increase in the amount of pellets per culture (Fig. 2F). As reported earlier for other fungi (Lejeune and Baron 1998, Fang and Zhong 2002), an increase in pellet amount may be due to pellet breakage. Already at day 2 of cultivation, independently of the temperature of cultivation, there were about 1200 pellets more in the cultures of the control transformant than in cultures of the *lcc1* transformant. Although pre-cultures of the transformants were handled exactly the same, it could also have been that a higher number of mycelial debris were produced for the control transformant during maceration of the pre-culture. In addition, germination tests on YMG agar plates indicated higher amounts of active spores for the control transformant (9.8 ± 0.8 % germination rate) compared to the *lcc1* transformant (5.1 ± 1.5 % germination rate). Also pre-cultures in modified Kjalke medium showed higher biomass yields for the control transformant (4.2 ± 0.4 g/l DW) compared to the *lcc1* transformant (3.1 ± 0.8 g/l) (experiments not further shown).

Maximum pellet size in liquid modified Kjalke medium was reached for both transformants at 25 °C at day 4 of cultivation and at 37 °C at day 2 of cultivation, respectively (Fig. 4). After the increase of pellet diameter and projected area at 25 °C until day 4, both parameters were more or less stable over the whole cultivation period of both transformants. A similar behaviour was reported for the triterpene producer *Ganoderma lucidum* when the fungus was cultivated in shake flask cultures in a complex medium based on sucrose as C-source. Also in this study, the distribution of pellet diameter increased during the first 4 days and was subsequently quite stable over the rest of the culture (Fang and Zhong 2002).

At 37 °C, most pellets reached their final size after 2 days, whereas the biomass and the pellet concentration still inclined until day 4 and day 6 for the control and the *lcc1*

transformant, respectively. In addition to the higher number of pellets which may account for the elevated biomass yield, the increase in dry weight is probably also due to the more dense and packed nature of the pellets at day 4 compared to day 2 of cultivation. These morphological differences can be seen in the microtome sections of pellets grown at 37 °C (Fig. 7). Pellets showed a much thicker outer zone after 4 days of cultivation, compared to pellets derived from 2 day-old cultures. This outer zone, present from day 4 onwards, were found in 25 °C and 37 °C cultures, although the outer zone at 25 °C was generally smaller and seemed to be denser than those formed in pellets at 37 °C.

Another difference is the surface of the pellets, which can be divided into smooth and hairy pellets at 25 °C and 37 °C, respectively. A similar effect of temperature on the morphology was also reported for *Rhizopus nigricans*, showing a smooth growth at 19 °C and a fluffier one at 23 °C (Žnidarsic et al. 2000). The reason for this might be a reduced growth rate at the lower temperature and, thus, the actual surface is relatively more influenced over the time by the shear forces. Nevertheless, a sufficient explanation is not known.

To be sure that the morphological phenotypes of the outer zone were not specific to the used *C. cinerea* transformant, in ongoing research we tested so far 4 additional recombinant *lcc1* expressing clones (clone 3, 23, 32 and 35). The clones were cultivated at 37 °C and every second day from day 4 onwards pellets were embedded and cross sections with the microtome were produced (Fig. 11). Superficically in the cuttings, there were no differences between the strains but between the age of the cultures in producing the more compact outer rind only later in cultivation (from day 4 of cultivation, Fig. 11). The ratios of outer zones to pellet diameters were calculated as described above at day 4, day 6 and day 8 (Fig. 12). Once an outer rind was formed the ratios (pellet diameter/width outer zone) varied marginally with values between 2.8 and 4.6 giving an average over all clones of 3.6 ± 0.7. Also in this experiment of comparing more *C. cinerea* clones, complete microtome cuttings for observation of the ratio between outer and inner zone could not be obtained for pellets derived after 10 days, due to instability of the pellets at this stage of growth. Further work will need to evaluate the formation of the outer zone in cultures incubated at 25 °C.

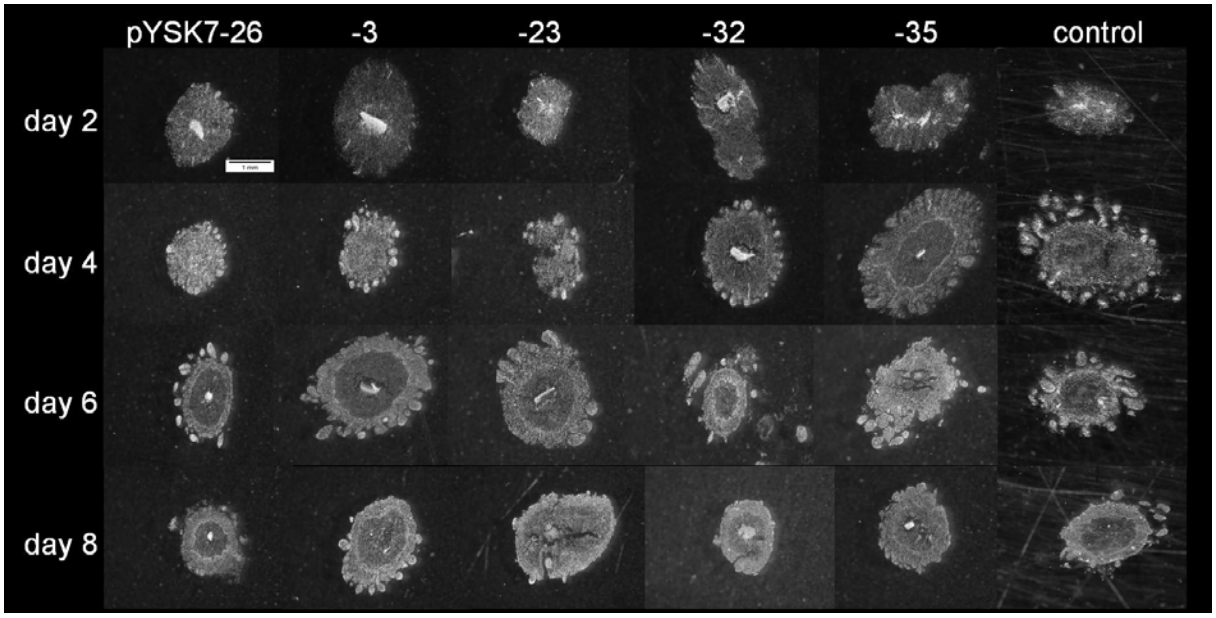


Fig. 11 Microtome cuttings of paraffin embedded pellets from different *lcc1* transformant clones (pYSK7-26, -3, -23, -32, -35) and the control transformant from cultures grown at 37 °C in modified Kjalke medium. Note that the lcc1 transformant pYSK7-26 and the control transformant are the same strains whose behaviour is described in more detail in the results section of this chapter. The bar in the upper left part indicates 1 mm. Per day of cultivation and transformant at least 3 pellets were analysed.

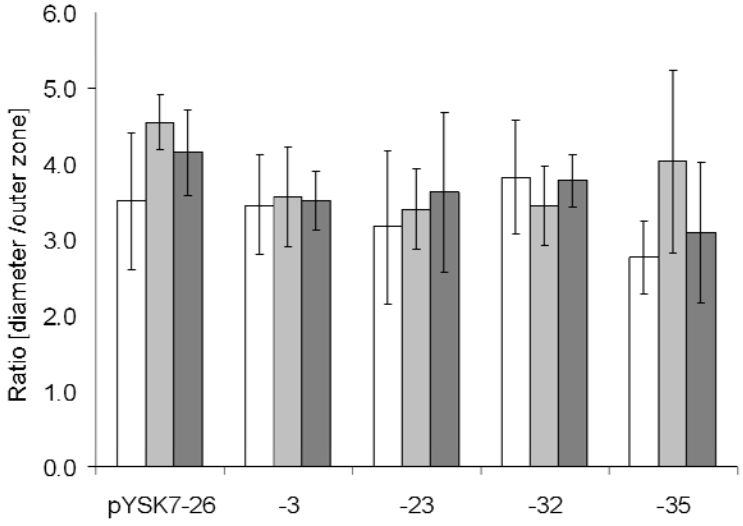


Fig. 12 Comparison of the ratios (pellet diameter/width outer zone) determined from cross sections of different *lcc1* clones (pYSK7-clone number) cultivated in modified Kjalke medium at 37 °C. Samples were compared after 4 days (white bar), 6 days (light gay) and 8 days (dark grey) of cultviation. The error bars indicate the standard deviation of three different pellet cross sections.

To describe the outer surface or form of the pellets without cross sections, definition of the convexity might help. Cox and Thomas (1992) analysed the convex area ratio with values of 0.9 for smooth pellets and 0.5 for hairy pellets in cultures of *A. niger*. When comparing the

convexity of the pellets derived from shake flask cultures (Fig. 5) with the microtome cuttings of the pellet (Fig. 7), it becomes clear that there is a connection between convexity and hariness of the pellet. Pellets of the *lcc1* transformant grown at 25 °C cultures had a smooth surface and also a high convexity value, whereas pellets from 37 °C cultures showed a lower convexity throughout the cultivation. Although, in our study the convexity decreased with prolonged cultivation time, differentiates from the *Aspergillus niger* pellets described in the study of Cox and Thomas (1992). The *A. niger* pellets showed a more hairy look at the end of the cultivation and, thus, a lower convexity. This might be due to shear forces fractioning the outer hairy parts of the pellets, as can be deduced from the microtome cuttings (Fig. 7 and Fig. 11).

3. Fermentation in a stirred bioreactor

When cultivating basidiomycete fungi in liquid medium, an expanded lag phase can result in a whole batch process of several weeks (Fazenda et al. 2008). In this study with *C. cinerea*, we used a very fast growing basidiomycete, which completely colonised the bioreactor in less than one week from spore inoculum. Upon inoculation of a fermenter with spores, one can distinguish as phases of cultivation: i) phases of spore agglomeration, ii) phases of spore germination and iii) phases of growth (Grimm et al. 2004, Kelly et al. 2006). In the experiment with *C. cinerea* cultivated in the Kjalke medium described here, the biomass dry weight was more or less similar at the beginning of the measurement (day 2) for all three different pH values tested. Thus, agglomeration of *C. cinerea* spores, which were used for inoculation, might have been similar at all tested pHs. In contrast, for *Aspergillus oryzae* it was reported that spore agglomeration is more intense at higher pH values resulting in pellet like growth, whereas at a lower pH filamentous growth is favourable due to germination of freely dispersed spores (Carlsen et al. 1996). However in this study, we did not quantify the germination rate of the *C. cinerea* spores at different pH values. This might be one point to consider in future fermentation studies.

Compared to the shaken cultures, the final biomass dry weights per volume of medium of the bioreactor cultures were lower, which might be due to problems related to cultivation in the bioreactor, such as a low stirring speed leading to reduced oxygen concentration in the medium, lower shearing stress and an uneven mixing of the culture broth, and clumping of the mycelium around the bioreactor peripherals leading to oxygen undersupply of cells. 10^6 spores per ml final volume were applied in stirred bioreactor cultures as inoculum. Reducing

spore amounts might help to solve the growth problem and, further on, may result in higher laccase yields.
In shake flask cultures, homogenised pre-cultures, which were inoculated with 10^6 spores/ml final medium and grown for 4 days, were used for the main culture. In future fermentation studies in stirred bioreactors, pre-cultures similar to the ones applied in shake flask experiments will be used unlike the direct inoculation with spores used in this study for bioreactor cultures.

The laccase activity per volume and biomass varied to a high degree over the tested range of pH values. pH 6 seems to be the better than pH 7 and pH 8 among the tested values for production of recombinant laccase Lcc1 by the used *lcc1* transformant. At day 6 of cultivation in the bioreactor in medium at pH 6, the laccase activity reached 1.3 U/ml which was more than 2times lower than in shaken cultures. Fermentation was interrupted prior of reaching the stationary phase, due to blocking of the peripherals and clumping of the mycelium. Higher activities in stirred bioreactors might have been achieved at a later time point. However, to increase biomass and laccase yields, the impeller diameter can be raised or a higher stirring speed can be used. Analogously to the results with *C. cinerea* from the studies here, Hess et al. (2002) observed a decrease in the laccase activity of *Trametes multicolor* grown in a stirred bioreactor cultures compared to shake flask cultures. The authors stated that the decrease may be due to shear stress of the stirrer or morphological changes in the growth of the fungus (Hess et al. 2002). In *C. cinerea* cultures of the *lcc1* transformant cultivated at pH 8, in contrast the situation appears to be very difficult. Laccase production compared to available biomass was relatively well at the first 3 days of cultivation, but afterwards there was no further increase but a low gradual decrease. Currently it is not known whether the *gpdII*-promoter activity is negatively affected by pH 8 or the protein secretion, a high protease activity, lower laccase stability or others.

As in shake flask cultures, growth occurred predominantly in form of pellets and no or little free filamentous growth was observed in bioreactor cultures, although smaller aggregated hyphal fragments were present in the stirred vessel at all pH values. By increasing the stirrer speed a more filamentous growth form might be achieved by suppressing pellet growth and/or fragmentation of hyphal filaments, as it was shown for *A. niger*. Increased power input via stirring resulted in a lower biomass production, but higher respiration of the fungus (El-Enshasy et al. 1999). Nevertheless, pellet formation might be better for the production of specific metabolites. Pellet formation can be positively influenced by various factors such as different bioreactor constructs, pH, inoculum and detergents (Vecht-Lifshitz et al. 1990 in

Hess et al. 2002). In this study, we showed that the pH had an influence on the morphology of *C. cinerea*, where at the higher pH value a more fragmented growth was observed compared to the lower pH value. In the pH 8 cultures, laccase yields were lower, but currently we do not know whether this was due to suppression of pellet in favour to the filamentous growth form. Whatsoever, pellets are formed in cultures in bioreactors at pH 6 and 7 and the higher production rate of the recombinant laccase Lcc1 makes cultivation at lower pH values (pH 6 and pH 7) not to be more adequate than at pH 8. Pellets derived from pH 6 and pH 7 cultures had similar average projected areas of 7.9 and 7.3 mm^2, respectively, at the end of the fermentation, whereas the average pellet area at pH 8 was only 4.4 mm^2. In contrast to growth at pH 6, pellet distribution at pH 7 in the different runs differed from each other with a high standard deviation of the average pellet areas showing that the pattern of pellet formation at pH 7 differs from the one at pH 6.

More input is needed to understand the process of bioreactor cultures of *C. cinerea* regarding its growth and morphological characteristics.

V. References

Agger T, Spohr AB, Carlsen M and Nielsen J (1998) Growth and product formation of *Aspergillus oryzae* during submerged cultivations: verification of a morphologically structured model using fluorescent probes. Biotechnology and Bioengineering 57:321-329

Bae YS and Knudsen GR (2000) Cotransformation of *Trichoderma harzianum* with ß-glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. Applied and Environmental Microbiology 66:810-815

Binninger DM, Skrzynia C, Pukkila PJ and Casselton LA (1987). DNA-mediated tranformation of the basidiomycete *Coprinus cinereus*. EMBO Journal 6:835-840

Bittner C, Wehnert G and Scheper T (1998) In situ microscopy for on-line determination of biomass. Biotechnology and Bioengineering 60:24-35

Boh B and Berovic M (2007) *Grifola frondosa* (Dicks.: Fr.) SF Gray (Maitake mushroom): Medicinal properties, active compounds, and biotechnological cultivation. International Journal of Medicinal Mushrooms 9:89-108

Böker R (1990) Untersuchungen zu Wirkstoffsynthesen und Fermentationen an *Atropurpurascens* und *Cyathus striatus* (Basidomycetes). Dissertation, Universität Kaiserslautern, Kaiserslautern, Germany

Braun S and Vecht-Lifshitz SE (1991) Mycelial morphology and metabolite production. Trends in Biotechnology 9:63-68

Carlsen M, Spohr AB, Nielsen J and Villadsen J (1996) Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. Biotechnology and Bioengineering 49:266-276

Carmichael RD and Pickard MA (1989) Continuous and batch-production of chloroperoxidase by mycelial pellets of *Caldariomyces fumago* in an airlift fermenter. Applied and Environmental Microbiology 55:17-20

Chmiel H (2006) Bioreaktoren. In: Chmiel H (Ed.) Bioprozesstechnik. Spektrum Akademischer Verlag, München, Germany

Cho EJ, Oh JY, Chang HY and Yun JW (2006) Production of exopolysaccharides by submerged mycelial culture of a mushroom *Tremella fuciformis*. Journal of Biotechnology 127:129-140

Claus H (2003) Laccases and their occurrence in prokaryotes. Archives of Microbiology 179:145-150

Conesa A, Punt PJ, van Luijk N, van den Hondel CAMJ (2001) The secretion pathway in filamentous fungi: A biotechnological view. Fungal Genetics and Biology 33:155-171

Couto SR and Toca-Herrera JL (2006) Industrial and biotechnological applications of laccases: a review. Biotechnology Advances 24:500-513

Couto SR and Toca-Herrera JL (2007) Laccase production at reactor scale by filamentous fungi. Biotechnology Advances 25:558-569

Cox PW and Thomas CR (1992) Classification and measurement of fungal pellets by automated image-analysis. Biotechnology and Bioengineering 39:945-952

Cox PW and Thomas CR (1999) Assessment of the activity of filamentous fungi using Mag fura. Mycological Research 103:757-763

Cui YQ, Okkerse WJ, van der Lans RGJM and Luyben KCAM (1998) Modelling and measurements of fungal growth and morphology in submerged fermentations. Biotechnology and Bioengineering 60:216-229

Cui YQ, van der Lans RGJM and Luyben KCAM (1997) Effect of agitation intensities on fungal morphology of submerged fermentation. Biotechnology and Bioengineering 55:715-726

Defren, K (1993) Untersuchung der Auswirkungen von Rührertyp und Rührergröße auf das Fermentationsverhalten des Pilzes *Cyathus striatus*. VDI-Fortschrittsbericht Nr. 91, Reihe 17, VDI-Verlag, Düsseldorf, Germany

Dominguez A, Couto SR and Sanroman MA (2005) Dye decolorization by *Trametes hirsuta* immobilized into alginate beads. World Journal of Microbiology & Biotechnology 21:405-409

Dominguez A, Gomez J, Lorenzo M and Sanroman A (2007) Enhanced production of laccase activity by *Trametes versicolor* immobilized into alginate beads by the addition of different inducers. World Journal of Microbiology & Biotechnology 23:367-373

Dosoretz CG, Chen AHC and Grethlein HE (1990) Effect of oxygenation conditions on submerged cultures of *Phanerochaete chrysosporium*. Applied Microbiology and Biotechnology 34:131-137

El-Enshasy H, Hellmuth K and Rinas U (1999) Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger*. Applied Biochemistry and Biotechnology 81:1-11

El-Enshasy H, Kleine J and Rinas U (2006) Agitation effects on morphology and protein productive fractions of filamentous and pelleted growth forms of recombinant *Aspergillus niger*. Process Biochemistry 41:2103-2112

Fåhraeus G and Reinhammar B (1967) Large scale production and purification of laccase from cultures of fungus *Polyporus versicolor* and some properties of laccase A. Acta Chemica Scandinavica 21:2367-2378

Fang QH and Zhong JJ (2002) Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. Biotechnology Progress 18:51-54

Fazenda ML, Seviour R, McNeil B and Harvey LM (2008) Submerged culture fermentation of "higher fungi": the macrofungi. Advances in Applied Microbiology 63:33-103

Fenice M, Giovannozzi SG, Federici F and D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. Journal of Biotechnology 100:77-85

Galhaup C, Wagner H, Hinterstoisser B and Haltrich D (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme and Microbial Technology 30:529-536

Gehrig I, Bart HJ, Anke T and Germerdonk R (1998) Influence of morphology and rheology on the production characteristics of the basidiomycete *Cyathus striatus*. Biotechnology and Bioengineering 59:525-533

Goodell B, Yamamoto K, Jellison J, Nakamura M, Fujii T, Takabe K and Hayashi N (1998) Laccase immunolabelling and microanalytical analysis of wood degraded by *Lentinus edodes*. Holzforschung 52:345-350

Granado JD, Kertesz-Chaloupková K, Aebi M and Kües U (1997) Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Molecular and General Genetics 256:28-36

Grimm LH (2006) Sporenaggregationsmodel für die submerse Kultivierung koagulativer Myzelbildner. Dissertation, FIT-Verlag für Innovation und Technologietransfer, Paderborn, Germany

Grimm LH, Kelly S, Hengstler J, Göbel A, Krull R and Hempel DC (2004) Kinetic studies on the aggregation of *Aspergillus niger* conidia. Biotechnology and Bioengineering 87:213-218

Grimm LH, Kelly S, Krull R and Hempel DC (2005) Morphology and productivity of filamentous fungi. Applied Microbiology and Biotechnology 69:375-384

Ha HC, Honda Y, Watanabe T and Kuwahara M (2001) Production of manganese peroxidase by pellet culture of the lignin-degrading basidiomycete, *Pleurotus ostreatus*. Applied Microbiology and Biotechnology 55:704-711

Harmsen MC, Schuren FHJ, Moukha SM, Vanzuilen CM, Punt PJ and Wessels JGH (1992) Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. Current Genetics 22:447-454

Heneghan MN, Porta C, Zhang C, Burton KS, Challen MP, Bailey AM and Foster GD (2009) Characterization of serine proteinase expression in *Agaricus bisporus* and *Coprinopsis cinerea* by using green fluorescent protein and the *A. bisporus* SPR1 promoter. Applied and Environmental Microbiology 75:792-801

Hess J, Leitner C, Galhaup C, Kulbe KD, Hinterstoisser B, Steinwender M and Haltrich D (2002) Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. Applied Biochemistry and Biotechnology 98-100:229-241

Hublik G and Schinner F (2000) Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. Enzyme and Microbial Technology 27:330-336

Hüttermann A, Mai C and Kharazipour A (2001) Modification of lignin for the production of new compounded materials. Applied Microbiology and Biotechnology 55:387-394

Hwang HJ, Kim SW, Xu CP, Choi JW and Yun JW (2004) Morphological and rheological properties of the three different species of basidiomycetes *Phellinus* in submerged cultures. Journal of Applied Microbiology 96:1296-1305

Ingham ER and Klein DA (1982) Relationship between fluorescein diacetate stained hyphae and oxygen utilization, glucose-utilization, and biomass of submerged fungal batch cultures. Applied and Environmental Microbiology 44:363-370

Jang MY, Ryu WY and Cho MH (2006) Enhanced production of laccase from *Trametes* sp. by combination of various inducers. Biotechnology and Bioprocess Engineering 11:96-99

Jiménez-Tobon G, Kurzatkowski W, Rozbicka B, Solecka J, Pocsi I and Penninckx MJ (2003) In situ localization of manganese peroxidase production in mycelial pellets of *Phanerochaete chrysosporium*. Microbiology-SGM 149:3121-3127

Jüsten P, Paul GC, Nienow AW and Thomas CR (1996) Dependence of mycelial morphology on impeller type and agitation intensity. Biotechnology and Bioengineering 52:672-684

Kelly S (2006) Fluiddynamischer Einfluss auf die Morphogenese von Biopellets filamentöser Pilze. Dissertation, FIT-Verlag, Paderborn, Germany

Kelly S, Grimm LH, Jonas R, Hempel DC and Krull R (2006) Investigations of the morphogenesis of filamentous microorganisms. Engineering in Life Sciences 6:475-480

Kharazipour A (1983) Optimierung eines Verfahrens zur Herstellung von Bindemitteln für Holzwerkstoffe auf der Basis von Ligninsulfonat und Laccase. Dissertation, Georg-August-Universität Göttingen, Göttingen, Germany

Kikuchi M, Ogawa K, Yamazaki T, Kajiwara S, Sugio A, Nakamura S and Shishido K (1999) Secretional expression of a *Bacillus subtilis* xylanase gene in the basidiomycete *Coprinus cinereus*. FEMS Microbiology Letters 178:277-282

Kilaru S (2006) Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology. Dissertation, Cuvillier Verlag, Göttingen, Germany

Kilaru S and Kües U (2005) Comparison of *gpd* genes and their protein products in basidiomycetes. Fungal Genetics Newsletters 52:18-24

Kilaru S, Hoegger PJ, Majcherczyk A, Burns C, Shishido K, Bailey A, Foster GD and Kües U (2006) Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Applied Microbiology and Biotechnology 71:200-210

Kim HM, Kim SW, Hwang HJ, Park MK, Mahmoud YAG, Choi JW and Yun JW (2006) Influence of agitation intensity and aeration rate on production of antioxidative exopolysaccharides from submerged mycelial culture of G*anoderma resinaceum*. Journal of Microbiology and Biotechnology 16:1240-1247

Kirk TK, Schultz E, Connors WJ, Lorenz LF and Zeikus JG (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Archives of Microbiology 117:277-285

Kjalke M, Andersen MB, Schneider P, Christensen B, Schulein M and Welinder KG (1992) Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. Biochimica et Biophysica Acta 1120:248-256

Koroleva OV, Stepanova EV, Gavrilova VP, Yakovleva NS, Landesman EO, Yavmetdinov IS and Yaropolov AI (2002) Laccase and Mn-peroxidase production by *Coriolus hirsutus* strain 075 in a jar fermentor. Journal of Bioscience and Bioengineering 93:449-455

Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiology and Molecular Biology Reviews 64: 316-353

Lecault V, Patel N and Thibault J (2007) Morphological characterization and viability assessment of *Trichoderma reesei* by image analysis. Biotechnology Progress 23:734-740

Lehmler C, Steinberg G, Snetselaar K, Schliwa M, Kahmann R and Bölker M (1997) Identification of a motor protein required for filamentous growth in *Ustilago maydis*. EMBO Journal 16:3464-3473

Leisola MSA, Thaneiwyss U and Fiechter A (1985) Strategies for production of high ligninase activities by *Phanerochaete chrysosporium*. Journal of Biotechnology 3:97-107

Lejeune R and Baron GV (1998) Modeling the exponential growth of filamentous fungi during batch cultivation. Biotechnology and Bioengineering 60:169-179

Lomascolo A, Record E, Herpoel-Gimbert I, Delattre M, Robert JL, Georis J, Dauvrin T, Sigoillot JC and Asther M (2003) Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. Journal of Applied Microbiology 94:618-624

Mander GJ, Wang HM, Bodie E, Wagner J, Vienken K, Vinuesa C, Foster C, Leeder AC, Allen G, Hamill V, Janssen GG, Dunn-Coleman N, Karos M, Lemaire HG, Subkowski T, Bollschweiler C, Turner G, Nüsslein B and Fischer R (2006) Use of laccase as a novel, versatile reporter system in filamentous fungi. Applied and Environmental Microbiology 72:5020-5026

Manzoni M and Rollini M (2001) Isolation and characterization of the exopolysaccharide produced by *Daedalea quercina*. Biotechnology Letters 23:1491-1497

Márquez-Rocha FJ, Guillén GK, Sánchez JE and Vázquez-Duhalt R (1999) Growth characteristics of *Pleurotus ostreatus* in bioreactors. Biotechnology Techniques 13:29-32

Martin AM and Bailey VI (1985) Growth of *Agaricus campestris* Nrrl-2334 in the form of pellets. Applied and Environmental Microbiology 49:1502-1506

Matsumura E, Yamamoto E, Numata A, Kawano T, Shin T and Murao S (1986) Structures of the laccase-catalyzed oxidation products of hydroxy benzoic acids in the presence of ABTS (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)). Agricultural and Biological Chemistry 50:1355-1357

Mayer AM and Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551-565

Mazumder S, Bose S, Bandopadhyay A, Alam S and Mukherjee M (2008) Study of laccase production by *Pleurotus ostreatus* in a 5 l bioreactor and application of the enzyme to determine the antioxidant concentration of human plasma. Letters in Applied Microbiology 47:355-360

McIntyre M, Dynesen J and Nielsen J (2001) Morphological characterization of *Aspergillus nidulans*: growth, septation and fragmentation. Microbiology-UK 147:239-246

Michel FC, Grulke EA and Reddy CA (1990) Development of a stirred tank reactor system for the production of lignin peroxidases (ligninases) by *Phanerochaete chrysosporium* Bkm-F-1767. Journal of Industrial Microbiology 5:103-112

Michel FC, Grulke EA and Reddy CA (1992) Determination of the respiration kinetics for mycelial pellets of *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 58:1740-1745

Moukha SM, Wosten HAB, Asther M and Wessels JGH (1993) In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. Journal of General Microbiology 139:969-978

Myasoedova NM, Chernykh AM, Psurtseva NV, Belova NV and Golovleva LA (2008) New efficient producers of fungal laccases. Applied Biochemistry and Microbiology 44:73-77

Nielsen J, Johansen CL, Jacobsen M, Krabben P and Villadsen J (1995) Pellet formation and fragmentation in submerged cultures of *Penicillium chrysogenum* and its relation to penicillin production. Biotechnology Progress 11:93-98

Ogawa K, Yamazaki T, Kajiwara S, Watanabe A, Asada Y and Shishido K (1998) Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and –degradation activities using novel heterologous protein expression vectors. Applied Microbiology and Biotechnology 49: 285-289

Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances 22:189-259

Papagianni M (2006) Quantification of the fractal nature of mycelial aggregation in *Aspergillus niger* submerged cultures. Microbial Cell Factories 5:5 doi:10.1186/1475-2859-5-5

Paul GC, Kent CA and Thomas CR (1993) Viability testing and characterization of germination of fungal spores by automatic image analysis. Biotechnology and Bioengineering 42:11-23

Paul GC and Thomas CR (1998) Characterisation of mycelial morphology using image analysis. Advances in Biochemical Engineering/Biotechnology 60:1-59

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J and van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends in Biotechnology 20:200-206

Quaratino D, Fenice M, Federici F and D'Annibale A (2006) Mn-peroxidase production by *Panus tigrinus* CBS 577.79: response surface optimisation and bioreactor comparison. Journal of Chemical Technology and Biotechnology 81:832-840

Radzio R and Kück U (1997) Synthesis of biotechnologically relevant heterologous proteins in filamentous fungi. Process Biochemistry 32:529-539

Reichl U, King R and Gilles ED (1992a) Effect of temperature and medium composition on mycelial growth of *Streptomyces tendae* in submerged culture. Journal of Basic Microbiology 32:193-200

Reichl U, King R and Gilles ED (1992b) Characterization of pellet morphology during submerged growth of *Streptomyces tendae* by image analysis. Biotechnology and Bioengineering 39:164-170

Rekangalt D, Verner MC, Kües U, Walser PJ, Marmeisse R, Debaud JC and Fraissinet-Tachet L (2007) Green fluorescent protein expression in the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. FEMS Microbiology Letters 268:67-72

Richter M (2008) Optimierung der rekombinanten Laccaseproduktion in *Coprinopsis cinerea*. Master thesis, Molecular Wood Biotechnology and Technical Mycology, Büsgen-Institute, Georg-August University of Göttingen, Göttingen, Germany

Riva S (2006) Laccases: blue enzymes for green chemistry. Trends in Biotechnology 24:219-226

Rühl M and Kües (2009) Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete *Coprinopsis cinerea*. Current Trends in Biotechnology and Pharmacy 3:241-253

Ryan DR, Leukes WD and Burton SG (2005) Fungal bioremediation of phenolic wastewaters in an airlift reactor. Biotechnology Progress 21:1068-1074

Spohr A, Dam-Mikkelsen C, Carlsen M, Nielsen J and Villadsen J (1998) On-line study of fungal morphology during submerged growth in a small flow-through cell. Biotechnology and Bioengineering 58:541-553

Talabardon M and Yang ST (2005) Production of GFP and glucoamylase by recombinant *Aspergillus niger*: Effects of fermentation conditions on fungal morphology and protein secretion. Biotechnology Progress 21:1389-1400

Tavares APM, Coelho MAZ, Coutinho JAP and Xavier AMRB (2005) Laccase improvement in submerged cultivation: induced production and kinetic modelling. Journal of Chemical Technology and Biotechnology 80:669-676

Thiruchelvam AT and Ramsay JA (2007) Growth and laccase production kinetics of *Trametes versicolor* in a stirred tank reactor. Applied Microbiology and Biotechnology 74:547-554

Treskatis SK, Orgeldinger V, Wolf H and Gilles ED (1997) Morphological characterization of filamentous microorganisms in submerged cultures by on-line digital image analysis and pattern recognition. Biotechnology and Bioengineering 53:191-201

Tucker KG and Thomas CR (1992) Mycelial morphology - the effect of spore inoculum level. Biotechnology Letters 14:1071-1074

van der Klei I and Veenhuis M (2007) Protein targeting to yeast peroxisomes. Methods in Molecular Biology 390:373-391

Vanhoutte B, Pons MN, Thomas CR, Louvel L and Vivier H (1995) Characterization of *Penicillium chrysogenum* physiology in submerged cultures by color and monochrome image analysis. Biotechnology and Bioengineering 48:1-11

Vecht-Lifshitz SE, Magdassi S and Braun S (1990) Pellet formation and cellular aggregation in *Streptomyces tendae*. Biotechnology and Bioengineering 35:890-896

Wang L, Ridgway D, Gu TY and Moo-Young M (2003) Effects of process parameters on heterologous protein production in *Aspergillus niger* fermentation. Journal of Chemical Technology and Biotechnology 78:1259-1266

Widsten P and Kandelbauer A (2008) Laccase applications in the forest products industry: A review. Enzyme and Microbial Technology 42:293-307

Xu JF, Wang LP, Ridgway D, Gu TY and Moo-Young M (2000) Increased heterologous protein production in *Aspergillus niger* fermentation through extracellular proteases inhibition by pelleted growth. Biotechnology Progress 16:222-227

Yao PX, Matsumoto N, Tsujimura H, Takaya M and Toda K (1995) Enhanced production of peroxidase in continuous culture of *Arthromyces ramosus* by selective bleeding of mycelium. Journal of Fermentation and Bioengineering 79:307-312

Znidarsic P, Komel R and Pavko A (2000) Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets. World Journal of Microbiology & Biotechnology 16:589-593

Znidarsic P and Pavko A (2001) The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. Food Technology and Biotechnology 39:237-252

Žmak PM, Podgornik A, Podgornik H and Koloini T (2006) Impact of pellet size on growth and lignin peroxidase activity of *Phanerochaete chrysosporium*. World Journal of Microbiology & Biotechnology 22:1243-1249

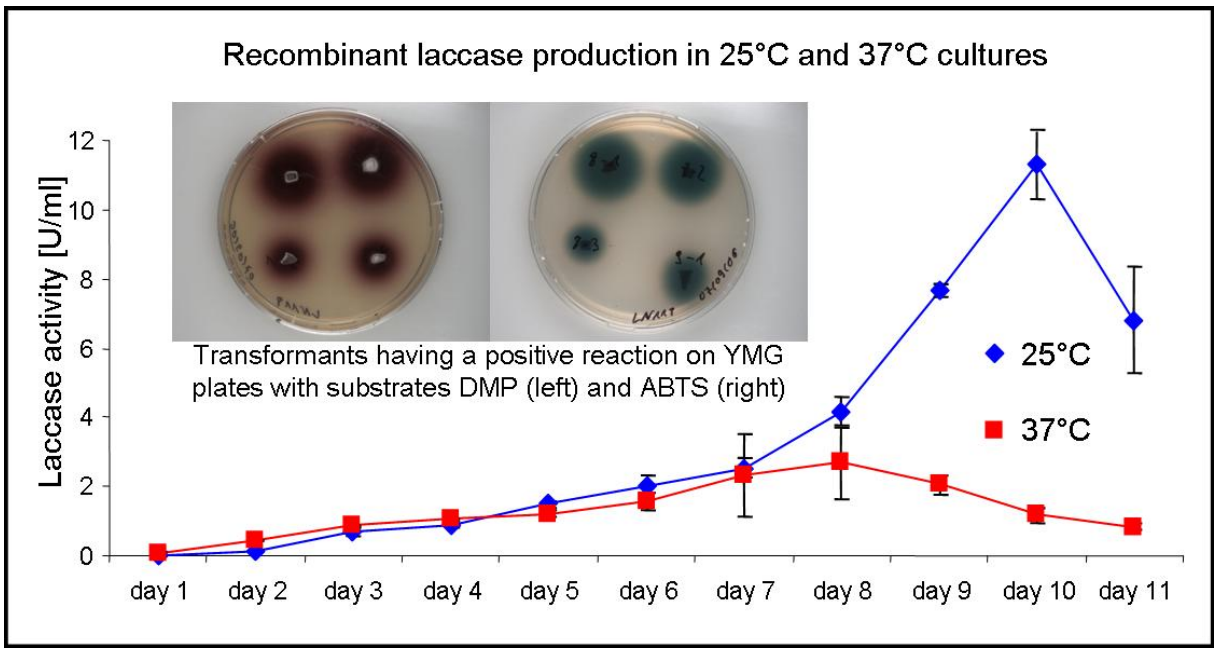
Chapter 4

Native and recombinant laccase production with *Coprinopsis cinerea*

I. Secretion of natural laccases in *Coprinopsis cinerea*

II. Optimisation of recombinant laccase production in *Coprinopsis cinerea*

III. References



I. Secretion of natural laccases in *Coprinopsis cinerea*

A. Abstract

Basidiomycetes as main producers for laccases in nature secrete various forms of this type of phenoloxidase under different environmental conditions being either laccase isoforms encoded by the same gene or isoenzymes encoded by different laccase genes. The homobasidiomycete *Coprinopsis cinerea* for example has seventeen different laccase genes.

In this work, ten monokaryotic *C. cinerea* strains were studied for their ability to produce laccases in two different media at 25 °C and 37 °C. For nine strains, laccase activity was increased at 25 °C compared to 37 °C. Yields of up to 1 U/ml and above were achieved for seven strains when cultivated at 25 °C in the glucose-based modified Kjalke medium. A zymogram of the supernatants of these strains resulted in a total of 10 different bands indicating different isoenzymes and/or different isoforms of laccases to be produced by the different strains. By analysis of the different bands via LC-MS/MS five different isoenzymes were detected: Lcc1, Lcc2, Lcc5, Lcc9 and Lcc10. Based on the zymogram, Lcc1 and Lcc5 were expressed in all of the strains, whereas Lcc2, Lcc9 and Lcc10 occurred only in three strains. These findings are discussed according to the effect of cultivation conditions and genetic background of strains in comparison to other studies, where different isoenzyme patterns of basidiomycetes were observed.

B. Introduction

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) are phenoloxidases capable of oxidising phenolic and aromatic compounds (Leonowicz et al. 2001). In nature, they are widespread in the fungal kingdom particularly in the basidiomycetes, but they also occur in plants, insects and bacteria (Claus 2004). In higher fungi, laccase genes often occur in larger families (Hoegger et al. 2004), coding for different isoenzymes (Bollag and Leonowicz 1984). Such paralogous laccase genes can be differentially regulated (Palmieri et al. 1997, Faraco 2009).

Basidiomycete laccases are probably the most studied phenoloxidases with respect to their production, biochemical characteristics and potential biotechnological use (Baldrian 2006, Kilaru 2006). However, up to now, only a few applications are marketable (section 1.II), amongst other reasons likely due to the generally too low production yields of active enzymes.

To increase the yield of laccase(s) in liquid cultures of native fungal strains, several strategies can be performed. One widely used method is the screening for high producing wildtype strains of white-rot fungi, which are known to secrete naturally detectable amounts of laccases (Herpoel et al. 2000, Lomascolo et al. 2002, Myasoedova et al. 2008). Additionally, optimisation of culture conditions (aeration, agitation, medium, pH, T) to improve laccase production is mainly studied in white-rot fungal cultures (Pointing et al. 2000, Arora and Gill 2001, Hou et al. 2004, Prasad et al. 2005, Dong et al. 2005, D'Souza et al. 2006, Tavares et al. 2006). Further strategies include the usage of detergents (Pointing et al. 2000, Lomascolo et al. 2002) as well as inducing substances, such as copper (Palmieri et al. 2000, Tavares et al. 2005) and other more complex compounds (2,5-xylidine, ferulic acid, vanilin, violuric acid) (D'Souza et al. 1999, Herpoel et al. 2000, De Souza et al. 2004, Kollmann et al. 2005). Lignocellulosic substances, such as cotton stalk, wheat bran, wood and other waste materials of the agricultural, forestral and food industry, which are general substrates for white-rot fungi, may also act as inducers (Ardon et al. 1996, Arora and Gill 2001, Stajic et al. 2006). The inducing effects of the lignocellulosic based media components and the inducing compounds on native laccase secretion result in normally more than one enzyme secreted in the culture broth: in some *Trametes* species 20 different isoenzymes and/or isoforms of the individual laccases were detected (Dong et al. 2005) and *Pleurotus* strains also produce several laccase isoenzymes (Palmieri et al. 1997).

Besides in cultures of the wood-degrading basidiomycetes, also in liquid cultures of saprotrophic litter degraders laccase activity can be found, such as described for six strains of the ink cap species *Coprinus* sp. (Ikehata et al. 2004). Recently, Anh et al. (2007) tested 20 *Coprinus* species, of which two (*Coprinus radians* and *Coprinus verticillatus*) produced laccase under non-inducing culture conditions. Another ink-cap mushroom, the homobasidiomycete *Coprinopsis cinerea*, has 17 different laccase genes divided into two gene subfamilies (Kilaru et al. 2006a). Native production of laccase during different stages of growth and development in *Coprinopsis cinerea* on solid medium was studied by Navarro-González (2008), but not in liquid shaken cultures with complex medium and inducing compounds.

In this study, the effect of medium and temperature on the laccase production by several monokaryotic strains of *C. cinerea* in liquid shaken culture is presented. Differences in the overall laccase activity in the supernatant and in the isoenzym pattern were observed. Laccases found in cultures with high enzyme yield and high expressed bands were identified by LC-MS/MS.

C. Material and methods

1. *C. cinerea* strains and culture conditions

Ten different monokaryotic strains of *Coprinopsis cinerea* were used in this work (Table 1).

Table 1 *Coprinopsis cinerea* strains used in this work (Kertesz-Chaloupková et al. 1998)

Strain	Genotype	Source (Reference)
218	*A3 B1, trp-1.1,1.6*	P. J. Pukkila (Binninger et al. 1987)
306	*A43 B43*	P. J. Pukkila (Pukkila 1996)
AT8	*A43 B43, trp-3, ade-8*	L. A. Casselton (Kües et al. 1992)
FA2222	*A5 B6, acu-1, trp-1.1,1.6*	L. A. Casselton (Mutasa et al. 1990)
H5	*A5 B6*	B. C. Lu (Lewis 1961)
JV6	*A42 B42*	L. A. Casselton (Binninger et al. 1987)
LN118	*A42 B42, ade-2, trp-1.1,1.6*	L. A. Casselton (Mutasa et al. 1990)
LT2	*A6 B6, trp-1.1,1.6*	L. A. Casselton (Mutasa et al. 1990)
Okayama7	*A43 B43*	*Coprinus* research community, FGSC #9003 (May et al. 1991)
PG78	*A6 B42, pab-1, trp-1.1,1.6*	L. A. Casselton (Granado et al. 1997)

Fungi were grown on solid YMG/T medium (per litre: 4 g yeast extract; 10 g malt extract; 10 g glucose; 0.1 g tryptophan; 10 g agar was added for solidification) at 37 °C. For a preculture, ten mycelial agar plugs of Ø 6 mm were put into a 500 ml flask filled with 50 ml of either modified Kjalke (Kjalke et al. 1992, per litre: 10 g yeast extract, 20 g glucose, 0.5 g $CaCl_2$ x 2 H_2O, 2 g KH_2PO_4, 50 mg $MgSO_4$ x 7 H_2O) or YMG/T liquid medium. Flasks were incubated for 4 days at 37 °C as standing cultures. Afterwards, the preculture was homogenised by an Ultra-Turrax® (IKA Werke GmbH & Co. KG, Staufen, Germany) for around 30 sec at 8000 rpm (rotations per minute) and 30 sec at 9500 rpm. For the main-cultures, 500 ml flasks were filled with 100 ml of modified Kjalke or YMG/T medium, both supplemented with 0.1 mM $CuSO_4$ and inoculated with 5 ml of a homogenised preculture. Cultivation took place at 25 °C or 37 °C on a rotary shaker at 120 rpm. Every day, samples of 1 ml were taken from the shaken culture and stored at -20 °C for further analysis.

2. Laccase activity assay

Laccase activity was determined in 100 mM sodium acetate buffer (pH 5.0) at room temperature with ABTS (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonate], AppliChem GmbH, Darmstadt, Germany). The conversion of ABTS was observed spectroscopically at 420 nm (ε = 36000 M^{-1} cm^{-1}) (Matsumura et al. 1986). One unit of enzyme activity was defined as the amount of substrate in µmol transformed per min and the activities are given in U per volume.

3. SDS-PAGE

For separation of proteins, polyacrylamide gel-electrophoresis (PAGE) was performed with 4% stacking and 12% resolving gels: **4%**: 2.5 ml of 0.5 M Tris pH 6.8, 1.3 ml acrylamide (37%), 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS (ammonium peroxodisulfate, Sigma-Aldrich, Steinheim, Germany), 10 µl TEMED (N,N,N',N'-tetramethylethylenediamine, Amersham Bioscience AB, Uppsala, Sweden) and filled up to 10ml with dH_2O. **12%**: 2.5 ml of 1.5 M Tris pH 8.8, 4.0 ml acrylamide (37%), 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS, 5 µl TEMED and filled up to 10ml with dH_2O.
The unfrozen supernatant samples were concentrated with a Vivaspin 2 (10.000 MWCO) from Sartorius (Sartorius GmbH, Göttingen, Germany). For the zymograms, a protein amount equal to 40 mU of laccase activity per well and for Coomassie-stained gels the undiluted concentrated supernatants were used. The samples were mixed 1:1 with the loading buffer that contained 0.06 M Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 0.025% bromphenol blue (w/v). Separation was performed at a constant current of 15 mA until the samples reached the resolving gel and continued at 25 mA for migration of proteins in the resolving gel.

4. Staining

For Coomassie staining, gels were fixed in 12% TCA (trichloracetic acid) (w/v) for at least 1 h and stained overnight in colloidal Coomassie solution (10% phosphoric acid (v/v), 10% ammonium sulfate (w/v) and 0.12% Coomassie Brilliant Blue G250 (Serva electrophoresis GmbH, Heidelberg, Germany) (w/v) in water/methanol (80/20, v/v) solution. The gels were washed with water until an optimal contrast between bands and the background level were reached.
For native laccase staining, gels were washed in 100 mM sodium acetate buffer (pH 5.0) and then incubated with 5-10 mM MBTH (3-methyl-2-benzothiazolinon-hydrazone hydrochloride) and 5-10 mM DHPPA (3,4-dihydroxyhydrocinnamic acid) in the same buffer until the protein bands showed up.

Tests showed that SDS in the gels and in the loading buffer had no influence on the detection of native laccase activity in the gels.

5. Protein identification

Protein bands of the Coomassie-stained gel were cut with a razor blade and gel pieces were digested as described by Havlis and Shevchenko (2004) with some modifications. Briefly, gel

pieces were washed twice with water for 15 min and subsequently once with 50 % ethanol for destaining. Gel pieces were dehydrated with 100 % acetonitril for 20 min and dried in a vacuum centrifuge (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) for 10 min. Subsequently the gel pieces were soaked in 2 µM trypsin in 50 mM ammoniumbicarbonate buffer (pH 8) at 4 °C for 1 h and digested for 30 min at 58 °C. Peptides were extracted from the gel pieces on a shaker first with 150 µl 5% (v/v) formic acid for one hour, followed by two extractions with 150 µl 5 % formic acid, 50% acetonitril. Extracts were combined, dried in the vacuum centrifuge to total dryness and stored at -20 °C until further proceeding. For further processing, peptides were dissolved in 15 µl of 5 % formic acid and 3 times 4 µl were injected to a 12 cm capillary column packed with 3 µm paticles of Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) for peptide separation. Analysis of the peptides was performed by ESI-LC-MS (HP 1100 Agilent; Esquire 3000, Bruker Daltonik). Each sample was analysed in a mass range of the ion trap from 200 to 1500 m/z. Analysis of the raw data was done with Daltonic DataAnalysis version 3.0 (Bruker Daltonic GmbH; Bruker Daltonic esquire 5.0). Proteins were identified by searching the data against a database of the annotated genome of *C. cinerea* using local Mascot software (www.matrixscience.com). The following settings were used for the database search: fixed modification: carbamidomethylation; variable modification: oxidation; peptide mass tolerance: 1.4 Da; peptide charge: 1+,2+,and 3+; MS/MS tolerance: 0.4 Da; missed cleavages allowed: 1. For final identification and predicted function protein BLAST against NCBI database, PFAM (http://pfam.sanger.ac.uk/), and Interpro (http://www.ebi.ac.uk/interpro/) were used.

D. Results

1. Laccase secretion depends on medium and temperature

Ten monokaryotic *C. cinerea* strains were tested for their ability to produce laccase(s) under different cultivation parameters in either the complete medium YMG/T or in the complete medium modified Kjalke, both supplemented with 0.1 M $CuSO_4$, in liquid shaken cultures at 25 °C and 37 °C, respectively. All strains showed good growth in both media and all cultures were completely grown after 12 days. With the exception of strain 218 (2.28 U/ml) and PG78 (1.02 U/ml) at 25 °C, laccase production in the rich *C. cinerea* standard growth medium YMG/T was generally very low or not existing (Table 2). Changing the medium to modified Kjalke gave reasonable laccase activity yields at 25 °C for all tested strains but monokaryon LN118 (Table 2). At the optimal growth temperature for *C. cinerea* at 37 °C, laccase

production for most strains was negligible (Table 2). The highest activities were obtained in Kjalke medium at 25 °C with monokaryons AT8 (8.07 U/ml), 306 (3.10 U/ml), FA2222 (2.94 U/ml) and 218 (2.82 U/ml). Monokaryon LN118 was the only strain that under all tested growth conditions failed to produce laccase activities above just detectable levels.

2. Different isoenzymes account for the laccase activity of different strains

Levels of laccase activities of strains 218 and PG78 at 25 °C in modified Kjalke and YMG/T medium were comparable (Table 2) as well as isoenzyme pattern for the strains in the two media – only variations in the strength of minor bands occurred (Fig. 1).

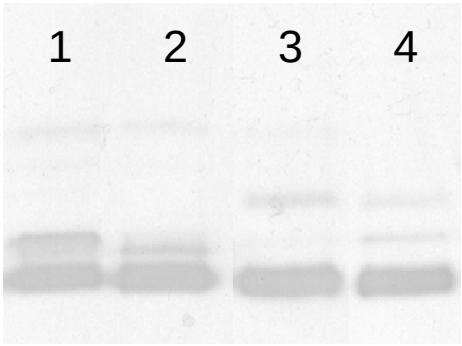


Fig. 1 Native PAGE of supernatants of PG78 (lanes 1+2) and 218 (lanes 3+4) cultures grown at 25°C in YMG/T (lanes 1+3) and modified Kjalke (lanes 2+4) stained with the laccase substrates MBTH and DHPPA.

Table 2 Maximal laccase activity of monokaryotic strains of *C. cinerea* cultivated in different media (modified Kjalke and YMG/T) at 25 °C and 37 °C

C. cinerea monokaryons	Laccase activities in U/ml of strains grown in modified Kjalke 25°C	modified Kjalke 37°C	YMG/T 25°C	YMG/T 37°C
218 *	2.82 (9)	0.02 (8)	2.28 (11)	0.01 (3)
306	3.10 (7)	0.59 (5)	0.50 (5)	0.53 (3)
AT8 * °	8.07 (8)	0.07 (4)	0.05 (12)	0.00
FA 2222 *	2.94 (9)	0.01 (6)	0.01 (7)	0.00
H 5	0.90 (7)	0.11 (4)	0.02 (4)	0.00
LN 118 * °	0.01 (11)	0.08 (5)	0.00	0.01 (9)
LT2 *	0.24 (9)	0.01 (5)	0.00	0.00
Okayama 7	0.53 (7)	0.24 (4)	0.03 (4)	0.03 (2)
JV6	0.96 (8)	0.00	0.00	0.00
PG78 * +	0.96 (8)	0.90 (5)	1.02 (7)	0.01 (5)

Both media were supplemented with 0.1 mM $CuSO_4$. Over a period of 12 days, laccase activities in the supernatant were measured at a daily basis. Numbers in brackets indicate the day of cultivation at which the highest activity was detected. Auxotrophies of strains: * tryptophan; ° adenine; + para-aminobenzoic acid.

Supernatants from cultures of *C. cinerea* monokaryons grown in modified Kjalke medium at 25 °C that showed laccase activities of about 1 U/ml and higher (Table 2) were concentrated via a Vivaspin tube (10,000 MWCO) up to 30fold and concentrated culture supernatants were loaded for native gel electrophoresis either for establishing a zymogram by detection of

enzymatic activties (40 mU of laccase activity per well) or for Coomassie staining of the complete secretome (25 µl of the concentrated culture supernatant per well).

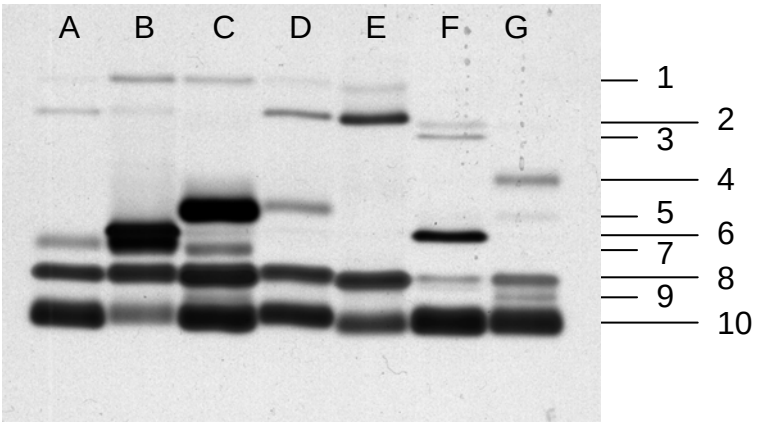


Fig. 2: Native SDS-PAGE of concentrated supernatants of *C. cinerea* cultures of modified Kjalke medium stained with MBTH and DHPPA. *C. cinerea* monokaryons: 218 (lane A), 306 (lane B), AT8 (lane C), FA2222 (lane D), H5 (lane E), JV6 (lane F), PG78 (lane G). Numbers 1 to 10 label laccase bands of different sites.

The zymogram for all strains differed from each other in total number of bands (between 4 and 7), in appearance of bands at different positions within the gel and in strength of bands shared between strains. In total by position, 10 different band types were defined (Fig. 2, Table 3). Only two bands (number 8 and 10) were present in all strains. Deduced from the strength of staining, in all strains either one or both of these bands were major bands of laccase activity. In strains 306, AT8 and JV6, band 5, band 6 and band 7 were other main bands (Fig. 2). For protein identification for all strains, corresponding bands or regions (where in Coomassie-stained gels no visible band existed) were cut from parallel Coomassie-stained gels and treated with trypsin. Peptides were eluted from the gel-slices for LC/MS-MS and Mascot detection analysis.

Table 3 Presence of different isoenzyme bands in the supernatant of different *C. cinerea* strains cultivated in modified Kjalke medium at 25 °C supplemented with 0.1 mM $CuSO_4$

Isoenzyme band*	*Coprinopsis cinerea* **monokaryons**						
	218 (A)	**306 (B)**	**AT8 (C)**	**FA 2222 (D)**	**H 5 (E)**	**JV6 (F)**	**PG78 (G)**
1	+/-	+	+	+/-	+/-	-	-
2	+	(Lcc5) +/-	-	+	Lcc1/5	+	-
3	-	-	-	-	-	+	-
4	-	-	-	-	-	-	+
5	-	-	Lcc9	+	-	-	+/-
6	-	Lcc2/9/10	+/-	-	-	(Lcc2)	-
7	+	Lcc9/10	Lcc10	-	-	-	-
8	Lcc1	Lcc1	Lcc1	Lcc1	Lcc1	Lcc1	Lcc1
9	-	-	Lcc1	-	-	-	+
10	Lcc5	Lcc5	Lcc5	Lcc5	Lcc1/(5)	Lcc5	Lcc5

* The letters A to G refer to lanes and numbers of isoenzyme bands to laccase bands in the gel shown in Fig. 2
+ indicates the presence of the isoenzyme band, +/- indicates a faint isoenzyme band and – means that no band occurred. Grey shaded cells correspond to strong isoenzyme bands. Laccase names of laccases identified by LC/MS-MS analysis are given in the cells. Laccase names in brackets indicate non-significant hits (only 1 peptide with an ion score >42).

The results of the protein identification by LC/MS-MS are shown in Table 4. In the very weak band 1, occurring in strains 218, 306, AT8, FA2222 and H5 (lanes A-E in Fig. 2), no laccase could be determined. In band 2 of strain 206 (lane B in Fig. 2), Lcc1 was detected by one peptide, and in band 2 of strain H5 (lane E in Fig. 2) two laccases (Lcc1 and Lcc5) were detected, whereas for all other strains showing weak activity in the zymogram at this positions in the gel (Fig. 2) no significant hits were found. Likewise for bands at other positions usually no significant hit was found. In contrast, Lcc9 was found in the stronger band 5 of strain AT8 (lane C in Fig. 2) and Lcc2 in band 6 of strain 306 (lane B in Fig. 2), respectively. Bands 6 and 7 of strain 306 (lane B) had a high intensity and overlapped each other (Fig. 2). Therefore, we cut out the piece of the gel in between band 6 and 7 named the fragment 6/7, in which Lcc9 and Lcc10 were found. In the stronger band 7 of strain 306 (lane B in Fig. 2) as well as in band 7 of strain 218 and strain AT8 (lanes A and C in Fig. 2), Lcc10 could be determined only for band 7 in strain 306 (lane B in Fig. 2) and strain AT8 (lane C in Fig. 2). Lcc1 was present in all analysed samples of band 8. Band 9 only present in strain AT8 (lane C in Fig. 2) and strain PG78 (lane G in Fig. 2), gave a hit for Lcc1 in strain AT8, but no identification for strain PG78. The reason for a positive detection of Lcc1 in strain AT8 (lane C in Fig. 2) might be the strong Lcc1 band just above band 9. Lcc5 was detected in all strains of band 10, except for strain H5 (lane E in Fig. 2) where no significant hit for Lcc5 was obtained. However, in band 10 of strain H5 in addition to Lcc5 Lcc1 was detected.

Table 4 Laccases detected by LC/MS-MS and Mascot peptide search

Band	Total protein score	Protein NCBI number	MW (Da) ExPASy	IP	Peptide sequence	Ion score
2 - 306	45	Lcc5 gi|115371523	57476	4.71	R. DVVAVGTEAGQGDTIIR.F	45
2 – H5	184	Lcc1 gi|115371515	58385	5.17	R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R	88 70
	250	Lcc5 gi|115371523	57476	4.71	R.YATADEVEPDTPR.L R SAGGSPNFVDPVR.R R.DVVAVGTEAGQGDTIIR.F	69 46 95
5 – AT8	319	Lcc9 gi|115371531	56724	5.62	R.INVVNDLNDPTMLR.Q R.RPGGPETDIAIVNVQR.N R.AIPNVGSNNLPNFSSGGINSAILR.Y	67 51 76
6 – 306	284	Lcc2 gi|115371517	55465	6.19	R.SAVLAGATQPTVQFPGPVIQGNK.N K.NSFFAINVIDALTDPTMLR.T R.ANPNIGTTGFVGGVNSAILR.Y	55 78 46
6 – JV6	49	Lcc2 gi|115371517	55465	6.19	R.SAGSSTYNFANPVR.R	49
6/7 – 306	220	Lcc9 gi|115371531	56724	5.62	R.RPGGPETDIAIVNVQR.N R.AIPNVGSNNLPNFSSGGINSAILR.Y	51 54

Band	Total protein score	Protein NCBI number	MW (Da) ExPASy	IP	Peptide sequence	Ion score
	188	Lcc10 gi115371533	57765	5.31	R.GPLVIYDDNDPYK.N R.YSFILEANQPVGNYWIR.A R.SAGSDTYNYVNPVR.R	49 45 57
7 – 306	247	Lcc10 gi115371533	57765	5.31	R.GPLVIYDDNDPYK.N R.NLPDFSSGGINSAILR.Y R.SAGSDTYNYVNPVR.D	66 70 68
7 – AT8	213	Lcc10 gi115371533	57765	5.31	R.NLPDFSSGGINSAILR.Y R.SAGSDTYNYVNPVR.R	50 46
8 – 218	375	Lcc1 gi|115371515	58385	5.17	R.AGILVNGVHGPLIR.G R.YVGGPAAELSIVNVEQGK.K R. YSFVLDANQPVDNYWIR.A R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R	56 52 44 67 76
8 – 306	448	Lcc1 gi|115371515	58385	5.17	R.AGILVNGVHGPLIR.G R.YVGGPAAELSIVNVEQGK.K R.YVGGPAAELSIVNVEQGKK.Y R.YSFVLDANQPVDNYWIR.A R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R R.SAGSSTYNFVNPVKR.D	60 55 70 49 78 58 51
8 – AT8	586	Lcc1 gi|115371515	58385	5.17	R.YVGGPAAELSIVNVEQGK.K R.YVGGPAAELSIVNVEQGKK.Y R.YSFVLDANQPVDNYWIR.A R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R R.SAGSSTYNFVNPVKR.D R.DVVSLGVTGDEVTIR.F	84 70 65 78 56 96 83
8 – FA2222	359	Lcc1 gi|115371515	58385	5.17	R.AGILVNGVHGPLIR.G R.YSFVLDANQPVDNYWIR.A R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R R.SAGSSTYNFVNPVKR.D	45 48 70 59 62
8 – H5	289	Lcc1 gi|115371515	58385	5.17	R.YVGGPAAELSIVNVEQGKK.Y R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R R.SAGSSTYNFVNPVKR.D	61 67 71 50
8 – JV6	229	Lcc1 gi|115371515	58385	5.17	R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVKR.D	77 60
8 – PG78	335	Lcc1 gi|115371515	58385	5.17	R.YVGGPAAELSIVNVEQGK.K R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVKR.D	62 83 66
9 – AT8	398	Lcc1 gi|115371515	58385	5.17	R.AGILVNGVHGPLIR.G R.YVGGPAAELSIVNVEQGK.K R.YVGGPAAELSIVNVEQGKK.Y R.YSFVLDANQPVDNYWIR.A R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVK.R R.SAGSSTYNFVNPVKR.D	61 52 54 55 68 46 59
10 – 218	475	Lcc5 gi|115371523	57476	4.71	R.INVVNNLDDDTMLR.Q R.YSVVVNADQAIGNYWIR.A R.AEPNIGDTGLVGTSGGGVNSAILR.Y R.YATADEVEPDTPR.L R.DVVAVGTEAGQGDTIIR.F	55 73 62 71 101
10 – 306	298	Lcc5	57476	4.71	R.YATADEVEPDTPR.L	88

Band	Total protein score	Protein NCBI number	MW (Da) ExPASy	IP	Peptide sequence	Ion score
		gi|115371523			R.DVVAVGTEAGQGDTIIR.F	68
10 – AT8	587	Lcc5 gi|115371523	57476	4.71	R.INVVNNLDDDTMLR.Q R.RPGNTEGDIAVVNVEK.D R.YSVVVNADQAIGNYWIR.A R.AEPNIGDTGLVGTSGGGVNSAILR.Y R.YATADEVEPDTPR.L R.DVVAVGTEAGQGDTIIR.F	96 94 61 65 75 119
10 – FA2222	365	Lcc5 gi|115371523	57476	4.71	R.YSVVVNADQAIGNYWIR.A R.DVVAVGTEAGQGDTIIR.F R.AEPNIGDTGLVGTSGGGVNSAILR.Y	62 101 46
10 – H5	216	Lcc1 gi|115371515	58385	5.17	R.NGLAGTFANGVNSAILR.Y R.SAGSSTYNFVNPVKR.D	76 83
	164	Lcc5 gi|115371523	57476	4.71	R.YATADEVEPDTPR.L	68
10 – JV6	340	Lcc5 gi|115371523	57476	4.71	R.INVVNNLDDDTMLR.Q R.YATADEVEPDTPR.L R.DVVAVGTEAGQGDTIIR.F	87 76 102
10 – PG78	345	Lcc5 gi|115371523	57476	4.71	R.INVVNNLDDDTMLR.Q R.YSVVVNADQAIGNYWIR.A R.AEPNIGDTGLVGTSGGGVNSAILR.Y R SAGGSPNFVDPVR.R R.DVVAVGTEAGQGDTIIR.F	67 47 80 46 84

Band corresponds to the isoenzyme band (number) and lane (strain) of the PAGE gel shown in Fig. 2; **Total score** is a value indicating the quality of this hit; **MW** is the molecular weight in Daltons as calculated by the pI/MW tool of ExPASy (www.expasy.org) for the known amino acid sequence, **IP** is the predicted isoelectric point as calculated by the Mascot software, **Peptide sequence** gives the peptide found by Mascot software analysis for the identified protein; **Ion score** gives the score for a single peptide (only peptides with a score >43 are shown).

E. Discussion

In this study, I have shown that laccase secretion in monokaryotic *C. cinerea* strains depends on the cultivation medium and the cultivation temperature. Different isoenzymes were secreted by the monokaryotic strains which were identified to be Lcc1, Lcc2, Lcc5, Lcc9 and Lcc10, occurring in different amounts and varying compositions at 25 °C in the glucose-based modified Kjalke medium.

1. Temperature and medium effect

At standard cultivation temperature of 37 °C in YMG/T medium, only in the monokaryotic strain 306 a reasonable activity of 0.53 U/ml occurred, whereas for the other strains no or only very low activity of 0.1 to 0.3 U/ml was found. When decreasing the temperature to 25 °C, only in strains 218 and PG78 an increase in enzymatic activity was observed. For

modified Kjalke, where 4 of the tested strains produced at 37 °C enzymatic amounts above 0.1 U/ml, a decrease of the cultivation temperature to 25 °C lead to a tremendous increase in laccase activity for most of the strains. The results suggest that both, medium composition and temperature play a role in laccase production in the tested strains. A combination of temperature and media effect was previously observed by Tong et al. (2007) for laccase production by *Trametes* sp. cultivated in shaken cultures using copper and o-toluidine as inducer. With the lower temperature at 28 °C highest laccase activity was yielded in a glucose medium, while cultivation at the higher temperature of 37 °C in cellobiose medium shielded in highest laccase activities.

For *Trametes modesta* and *Cyathus bulleri* the optimal temperature for laccase production is described to be 30 °C (Nyanhongo et al. 2002, Vasdev et al. 2005). For *P. ostreatus* and *T. versicolor* the highest laccase activity was recorded by Snajdr and Baldrian (2007) to be 30 °C and 35 °C, respectively, although there are studies indicating that the optimal growth temperature for *T. versicolor* might be lower (Xavier et al. 2007).

In comparison to these observations, *C. cinerea* showed higher activities in modified Kjalke medium at the low temperature of 25 °C with an increase in the laccase activity of over 100fold as compared to 37 °C; although the fungal growth for *C. cinerea* is found to be optimal at 37 °C (Kües 2000). This leads to the conclusion that there could be an inducing effect of temperature on the production of laccase in *C. cinerea*.

2. Isoenzyme

Fungi secrete laccase isoenzymes under different nutritious and inductive conditions (Munoz et al. 1997, Téllez-Téllez et al. 2005). For the native production of laccases in basidiomycete fungi, the nutrient composition of the growth medium is very important, as this might have an impact on the secretion pattern of active enzymes (Pointing et al. 2000, Teerapatsakul et al. 2007). Dong et al. (2005), for example incubated *Trametes gallica* in different media under shaken and static conditions. This showed that the maximal activity and isoenzyme patterns of the supernatant depend on the used media as well as on the incubation condition (shaken or static). Alltogether, (Dong et al. 2005) found 20 different laccase bands with three bands occurring in all types of cultures. Whether the different laccase bands depend on different genes (isoenzymes) or exist due to different post-translational modifications (isoforms) was not investigated (Dong et al. 2005). Another *Trametes* fungus*, T. versicolor* grown in liquid culture showed different isoenzyme patterns of the secreted laccases LacI and LacII depending on the lignocellulosic wastes used for the cultivation (Moldes et al. 2004). Also two different isoenzymes are present in the strain *Trametes* sp. AH28-2, which

were differently induced by aromatic inducers (Xiao et al. 2004). In the white-rot fungus *P. ostreatus* three isoenzymes were found in the supernatant, as well as the corresponding gene transcripts (*pox1*, *poxa1b*, *poxc*) expressed within the cells (Palmieri et al. 2000). All in all, five laccase isoenzymes were found by Palmieri et al. (1997) in *P. ostreatus* and up to five laccase isoforms in *P. sajor-caju* (Lo et al. 2001). For *P. pulmonarius* three different laccase isoforms were detected, of which two were either present in induced or non-induced cultures and only one present in both types of cultures (De Souza et al. 2004). Moreover intracellular activity was found in *P. ostreatus* strains, where at least two isoforms are present (Téllez-Téllez et al. 2005).

Bollag and Leonowicz (1984) tested several ascomycetes, basidiomycetes and deuteromycetes for their ability to produce laccase in liquid medium with and without induction by 2,5-xylidine. New isoforms were found in basidiomycete cultures when induced with 2,5-xylidine, whereas for *Botrytis cinerea*, *Rhizoctonia praticola* and *Podospora anserina* the same isoenzyme bands as in the non-induced cultures were found. This is at least partially due to the lower number of laccase genes generally present in ascomycetes as compared to basidiomycetes (Hoegger et al. 2006). For cultures of the basidioymcete *P. cinnabarinus* no effect in isoenzyme pattern of 2,5-xylidine induced cultures was observed (Eggert et al. 1996).

Previously, expression of different laccases in *C. cinerea* was only determined through detection of mRNA transcripts in mycelial samples (Navarro-González 2008) and cDNA isolation of vegetative mycelium (Yaver et al. 1999). Navarro-Gonzáles (2008) found in the mycelium of the strains PUK22, OU3-1 and PS002-1 transcripts for the eight different genes *lcc2*, *lcc3*, *lcc4*, *lcc5*, *lcc9*, *lcc11*, *lcc12* and *lcc17* respectively, of which only *lcc2*, *lcc3*, *lcc4*, *lcc11* and *lcc17* were found expressed in all three strains. Further investigations on mRNA production in fruiting with the self-compatible strain AmutBmut revealed six further laccase genes (*lcc1*, *lcc7*, *lcc10*, *lcc13*, *lcc14* and *lcc16*) transcribed in cap and stipe tissue. AmutBmut was also cultivated in liquid YMG/T medium at 28 °C and 37 °C to test laccase production in liquid cultures, but no laccase activities were detected with simple YMG/T medium and only up to 0.1 U/ml upon addition of 0.2 mM $CuSO_4$ (Navarro-González 2008). This contributes to our finding where almost all *C. cinerea* strains grown at 37 °C gave no or very low laccase activities in YMG/T medium supplemented with 0.1 mM $CuSO_4$.

Interestingly, the two main laccases (Lcc1 and Lcc5) secreted by the monokaryotic strains in liquid cultures with modified Kjalke medium in this work are only marginally expressed in the mycelium of the *C. cinerea* strains grown on solid YMG/T medium tested in the work of Navarro-González (2008). Nevertheless, for a better comparison between isoenzyme pattern

in liquid cultures and transcripts of laccase genes in mycelium and fruiting bodies of *C. cinerea*, either the same medium or the same strain should be used.

Overall from the presented secretion data, it seems as Lcc1 and Lcc5 are universal laccases in *C. cinerea*, while other secreted laccases (Lcc2, Lcc9 and Lcc10) might be rather strain-specific. According to the amino acid sequence, laccase Lcc1 is mostly related to laccases Lcc6, Lcc7 and Lcc11, and Lcc5 to Lcc9, Lcc10 and Lcc15, whereas Lcc2 cluster separately with Lcc3, Lcc12, Lcc13 and Lcc4 (Kilaru et al. 2006a). Currently it is not known, how much the physiological relatedness reflects similarities in enzymatic properties and specific biological functions. However, biochemical data of Lcc1 from Kilaru (2006) and data on Lcc5, Lcc6 and Lcc7 shown in chapter 5 suggest that the enzymes are not absolutely replaceable with each other. Lcc1 is more effective than the other characterised *C. cinerea* laccases and more stable than Lcc6 and Lcc7. Thus, Lcc1 is possibly evolutionary favoured over Lcc6, Lcc7 and Lcc11 or Lcc1 is more adapted in expression to the environmental cultivation conditions applied. In contrast, Lcc5 being inferior to Lcc1 in enzymatic kinetics for various substrates, but is much better in stability towards organic solvents indicating that enzymes can be differentially adapted to different environmental situation with the possible effect of an better or broader adaptation to different substrates. Anyhow, the actual biological functions remains to be elucidated for all *C. cinerea* laccases.

Since we performed an isoenzmye analysis at a specific point of cultivation, namely at the day of highest enzymatic activities, we do not know whether the isoenzyme pattern might change over the time. In a preliminary study, we observed differences over the time in the band pattern in laccase zymograms of supernatants of the *C. cinerea* strains AT8 and AmutBmut (data not shown). Further work regarding time dependences of laccase secretion may give a better idea about the expression of different enzymes during the fungal culture. A study on the effect of different phenolic inducers on secretion pattern might show the need for production of different isoenzymes by the fungus to attack the specific phenolic compound.

II. Optimisation of recombinant laccase production in *Coprinopsis cinerea*

A. Abstract

Laccases from basidiomycetes have various industrial applications, e.g. in the paper and pulp and in the wood industries. Overexpression of these enzymes in ascomycetes does not give satisfactory yields and, often, the enzyme show altered properties. Therefore, we use the basidiomycete *Coprinopsis cinerea* as a potential host for high level production of laccase from efficient promoter gene constructs in liquid cultures. The *gpdII* promoter of *Agaricus bisporus* was found most efficient in driving laccase production from cloned *C. cinerea* laccase genes. Various laccase genes have by now been expressed in *C. cinerea* (strain FA2222) under the control of the *gpdII* promoter. However, yields of enzymatic activities differed between the genes. Alteration of growth conditions (media, temperature, host strain) increased laccase yields in submerged cultures. By media screening we could double the laccase yields and this was further enhanced by factors of more than 30% though studying effects of different glucose concentrations. Cultivation at different temperatures showed for transformants of three different laccase genes that higher laccase yields could be obtained at 25 °C compared to the standard cultivation temperature of *C. cinerea* at 37 °C. Increase in laccase activity at 25 °C was partially due to a background activity since we observed some laccase activity also in cultures of the control transformant without an extra laccase gene at 25 °C, although not at 37 °C. Another *C. cinerea* strain (LN118) that had no natural laccase activity at 25 °C transformed with the laccase gene *lcc1* gave lower laccase yields at 25 °C than the FA2222 *lcc1* transformant, but had a 3 fold increase in laccase activity at 37 °C compared to the best production conditions defined for the recombinant *C. cinerea* strain FA2222. Highest activities of more than 10 U/ml were achieved with recombinant production of *lcc1* with the strain LN118 at 37 °C in a glucose-based production medium.

B. Introduction

The secretion of laccases, compared to other ligninolytic enzymes [manganese peroxidases (MnP) and lignin peroxidases (LiP)], has been considered to be constitutively in basidiomycete fungi (Aro et al. 2005). However, laccase secretion can be enhanced by several phenolic (ferulic acid, vanillin), aromatic (2,5-xylidine, ferulic acid) and alcoholic (methanol, ethanol) compounds acting as inducers (Leonowicz et al. 2001, De Souza et al. 2004, Meza et al. 2007). High yields of native laccase can be produced by basidiomycete systems. Culture supernatants with laccase activities comprise mostly of more than one laccase isoenzyme since basidiomycetes are a group of higher fungi which often possess large laccase gene families, so for example *Coprinopsis cinerea* that has 17 different laccase genes (Kilaru et al. 2006a). Although, laccases of basidiomycetes are the most studied ones, only a few paper deal with recombinant expression of laccase genes in basidiomycetes (Alves et al. 2004, Kajita et al. 2004, Kilaru et al. 2006b). Overexpression of laccases is normaly attempted in heterologous ascomycetous hosts, such as *Aspergillus* species and yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*). Whereas yields of homologous expressed proteins in these ascomycetous fungi are often high, amounts of expressed heterologous proteins can be quite small (Gouka et al. 1997, Radzio and Kück 1997, Conesa et al. 2001). In general, protein yields can be obtained in filamentous ascomycetes of up to 1-2 g/l from heterologously expressed (Maras et al. 1999) and around 30-40 g/l of homologous expressed genes (Schmidt 2005).

For the recombinant expression of basidiomycete laccases, usually heterologous ascomycetous hosts are used (Hong et al. 2002, Larrondo et al. 2003, Kiiskinen et al. 2004, Hong et al. 2007). However, to our best knowledge highest yields in any recombinant laccase production was achieved with the homologous expression of the *lac1* gene from the basidiomycete *Pycnoporus cinnabarinus* (Alves et al. 2004). Yields of recombinant laccase expression in heterologous systems are in the mg/l range with 5 mg/l for transformants of *Ceriporiopsis subvermispora* laccase gene *lcs* in *A. niger* (Larrondo et al. 2003) to 135 mg/l for *T. versicolor lcc1* or *C. cinerea lcc1* in *A. oryzae* (Yaver et al. 1999, Guo et al. 2006). In contrast, highest yields of up to 1.2 g/l were achieved in homologous over-expression of *Pycnoporrus cinnabarinus* laccase *lac1* (Alves et al. 2004). In standard cultivation conditions in yeast extract-malt extract-glucose (YMG) medium, Kilaru (2006) achieved protein amounts of around 25 mg/l for *C. cinerea* Lcc1 (which corresponds to 1.6 U/ml) when recombinantly expressing the gene *lcc1* in *C. cinerea* under control of the *Agaricus bisporus gpdII* promoter (Kilaru et al. 2006b).

The aim of this study was to increase the yield of the homologous expressed *lcc1* gene in *C. cinerea* strain FA2222 (Kilaru et al. 2006b) by optimisation of the cultivation medium and temperature. As extracellular protein degradation is a common problem in heterologous production (Maras et al. 1999) and might be also a problem in homologous expression, a *C. cinerea* strain with low proteolytic activity (P. Rittershaus, personal communication) was used as a host for recombinant expression of the *lcc1* gene.

C. Material and Methods

1. DNA transformation and *C. cinerea* strains used

Different laccase overexpressing transformants of *C. cinerea* strain FA2222 were obtained from Kilaru et al. (2006b) and used in this study for control puropses: the *lcc1*- (pYSK7, clone 26), *lcc5*- (pYSK20, clone 11) and *lcc7*-transformant (pYSK28, clone 38) as well as the corresponding control (pCc1001, clone 1).

DNA transformation was performed with the *C. cinerea* strain LN118 (*A42 B42*, *ade-2*, *trp-1.1,1.6*) after the protocol of (Granado et al. 1997) with 1 µg of the plasmid pCc1001 (trp^+, Binninger et al. 1987) and the plasmid pYSK7 (Kilaru et al. 2006b) containing the *lcc1* gene and the constitutive *gpdII* promoter of *Agaricus bisporus*. The transformed protoplasts were plated onto regeneration agar plates supplemented with 0.5 mM ABTS (2,2`-azino-bis [3-ethylbenzthiazoline-6-sulfonate], AppliChem GmbH, Darmstadt, Germany) and clones were picked for further growth on minimal medium (Granado et al. 1997). Positive *lcc1*-transformants were tested in liquid YMG cultures for their ability to produce laccase and the best clone (LN7-2) was used with a control transformant (pCc1001 in LN118) in this work for optimisation of media conditions.

2. Fungal cultures with spore inoculum

The fungi were grown on YMG-agar (per litre: 4 g yeast, 10 g malt extract, 10 g glucose, 10 g agar) plates at 37 °C until the mycelium reached the border of the Petri dishes. Sterile ddH_2O was poured onto the plate and the mycelium containing the asexual spores was scraped with a spatula from the agar. The solution was filtered over a sterile funnel filled with glass wool, which keeps back the fungal hyphae. A Thoma counting chamber was used to determine the spore concentration in the eluate. Pre-cultures inoculated with spore suspensions were done in 500 ml flasks, filled with 50 ml of modified Kjalke or YMG medium and the spore concentration was set to a final concentration of 10^6 spores/ml. The flasks

were incubated for 4 days at 37 °C as standing cultures. Pre-cultures were homogenised by an Ultra-Turrax® for around 30 sec at 8000 rpm (rotations per minute) and 30 sec at 9500 rpm. For the main-cultures, 500 ml flasks were filled with 100 ml of either BSM (per litre (Braun-Lüllemann et al. 1997): 5 g glucose, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g KCl, 0.65 g L-asparagine, 10 mg $FeSO_4$ x $7H_2O$, 8 mg $Mn(CHCOO)_2$ x $4H_2O$, 2 mg $ZnNO_3$ x $4H_2O$, 50 mg $Ca(NO_3)_2$ x $4H_2O$, 3 mg $CuSO_4$ x $5H_2O$), MM (per litre: 10 g glucose, 2 g L-asparagine, 1 g KH_2PO_4, 2.25 g Na_2HPO_4, 0.29 g Na_2SO_4, 0.5 g $(NH_4)_2C_4H_4O_6$, 0.25 g $MgSO_4x7H_2O$, 40 µg thiamine), Moore (per litre (Moore 1969): 10 g glucose, 1.35 g KH_2PO_4, 0.12 g $MgSO_4$ x $7H_2O$, 1.5 g NH_4Cl, 1.45 g Na_2HPO_4, 0.29 g Na_2SO_4, 2 g L-asparagine, 0.4 mg thiamine), modified Kjalke (per litre (Kjalke et al. 1992): 10 g yeast extract, 20 g glucose, 0.5 g $CaCl_2$ x $2H_2O$, 2 g KH_2PO_4, 50 mg $MgSO_4$ x $7H_2O$), Raulin (per litre: 33.4 g glucose, 45 mg $FeSO_4$ x $7H_2O$, 2.65 g NH_4NO_3, 0.4 g $(NH_4)_3PO_4$, 0.16 g $(NH_4)_2SO_4$, 45 mg $ZnSO_4$ x $7H_2O$, 0.4 g KCO_3, 2.65 g tartaric acid ($C_4H_6O_6$), 0.26 g $MgCl_2$), YMG (per litre: 4 g yeast extract, 10 g malt extract, 10 g glucose) medium. All media used for the main-culture were supplemented with 0.1 mM $CuSO_4$ and inoculated with 5 ml of the homogenised pre-culture. Cultivation took place at 25 °C or 37 °C on a rotary shaker at 120 rpm.

3. Glucose determination

The glucose concentration was determined indirectly with the Glucose (HK) Assay Kit from Sigma (Sigma-Aldrich Chemie GmbH, Munich, Germany). Glucose was enzymatically phosphorylated and afterwards oxidised with NAD (nicotinamide adenine dinucleotide) to 6-phosphogluconate. During the oxidation, NAD is reduced to NADH, which correlates directly with the glucose concentration and which was measured at 340 nm with a spectrophotometer. Glucose standards (Sigma, G 3285) were measured with the same method in concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625 g/l, respectively. The supernatant samples of the fungal cultures were diluted to fit in the NADH absorption curve for the glucose standards.

4. Ergosterol measurement

For determination of the biomass dry weight from the same flask, the culture was homogenised by an Ultra-Turrax as described above. 15 ml were transferred into a 50 ml Falcon tube, centrifuged at 4000 rpm (1735 g) for 10 min (centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was discarded and the tubes were stored at -20 °C

until further use. The remaining homogenised culture was used for calculation of the biomass dry weight (see Determination of the mycelial dry weight).

The frozen biomass was lyophilised and ergosterol determination was performed according to Nielsen and Madsen (2000). The dry samples were mixed with 10% KOH in methanol with 200 ppm BHT (2,6-di-*tetra*-butyl-4-methyl-phenol) and 5 µg/ml cholesterol as an internal standard. The samples were mixed with glass beads to fragment the lyophilised mycelium. When the biomass was uniformly suspended, the samples were incubated at 60 °C for 3 h and after cooling to room temperature centrifuged for 15 min at 1735 *g* and 4 °C. 2 ml of the supernatant were transferred into a new tube and 2 ml of hexane was added. The samples were mixed and phase separation was achieved by addition of 4 ml dH_2O. The tubes were shaken for at least 30 min, centrifuged at 2000 rpm at 4 °C for 15 min and 1 ml of the hexane phase was transferred into an e-cup. The solution was completely dried in a vacuum centrifuge and stored at -20 °C until further use.

For derivatisation, the samples were dissolved in 10 µl pyridine and 50 µl BSTFA [N,O-bis(trimethylsily)trifluoroacetamide]. Samples were incubated at RT for 30 min, dried and dissolved in 150-300 µl of toluene for subsequent GC-MS analysis (6890N, Agilent Techn., Network GC Systems, Santa Clara CA, USA).

5. Determination of the mycelial dry weight (DW)

The volume of the fungal culture was measured and poured into a Büchner filter containing a cellulose filter whose dry weight was measured in advance. The filter together with the wet biomass was placed in an oven at 80 °C and the filter was weighed every day until the weight was stable. The DW is calculated by the following equation:

$$DW[g/L] = \frac{M_{filter+fungal\ biomass} - M_{filter}}{V_{fungal\ culture}}$$

6. Enzyme assays

Laccase activity was determined in 100 mM sodium acetate buffer (pH 5.0) at room temperature with ABTS (2,2`-azino-bis [3-ethylbenzthiazoline-6-sulfonate], AppliChem GmbH, Darmstadt, Germany). The conversion of ABTS was observed spectroscopically at 420 nm (ε = 36000 M^{-1} cm^{-1}) (Matsumura et al. 1986). One unit of enzyme activity was defined as the amount of substrate in µmol transformed per min and the activities are given in U per volume.

Proteolytic activity was determined with azocasein as a substrate. Azocasein was dissolved in 50 mM KH_2PO_4 (pH 6.2) to a final concentration of 0.25% (w/v). 100 µl of sample was mixed with 500 µl of the azocasein solution and incubated for 30 min at 37 °C. Not cleaved azocasein was precipitated with 400 µl of trichloracetic acid (20%, TCA) and precipitated protein is separated by centrifugation. The absorption of the supernatant was measured in a UV/VIS-spectrophotometer (DU 800 V, Beckman Coulter Inc., Krefeld, Germany) at 340 nm. One unit is defined as the change of absorption at 340 nm per min and given in U/L.

D. Results

1. Media supporting growth for recombinant laccase Lcc1 production

Different media were tested for their ability to support production of laccase with the *lcc1* transformant of *C. cinerea* FA2222. The pCc1001 transformant of strain FA2222 was used in parallel as control. The tested media were inoculated with a homogenised pre-culture (YMG) and incubated for 12 days at 37 °C. Laccase activity was measured every second day and the dry weight (DW) was determined after the last day of measurement. Only the complex media YMG and modified Kjalke gave good growth conditions and best biomass production (data not shown) for the *lcc1* transformant and the control transformant and, therefore, high laccase activities in the supernatant (see Fig. 1). The pH of the media with the highest laccase production (modified Kjalke and YMG) shifted from initially pH 6.3 to around pH 8.7 to 9.1 for both the *lcc1* transformant and the control transformant pCc1001, whereas for all other media the pHs remained between pH 4.4 and 7.5 (the pH was measured only at the end of the experiment at day 12 of cultivation; data not shown).

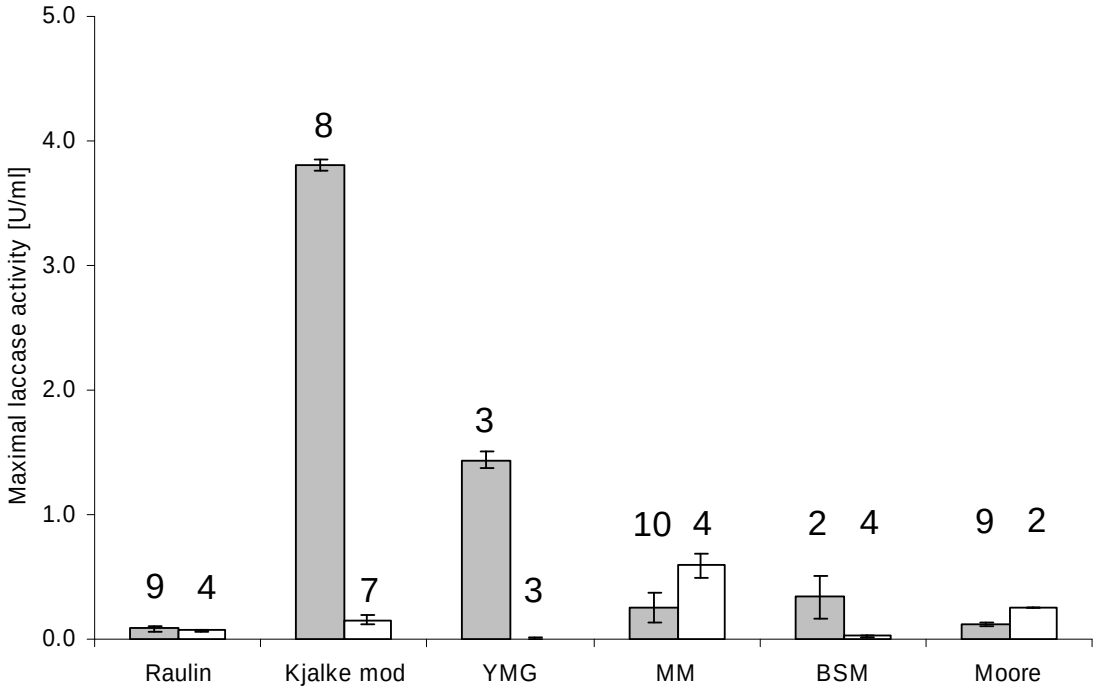


Fig. 1: Maximal laccase activity of the *C. cinerea* FA2222 *lcc1* transformant (grey column) and the control transformant pCc1001 (white column) cultivated in different media at 37 °C. Values are means of two different cultures and the bars above and below the mean value indicate the laccase activity of each of both cultures. The days of maximum laccase activities during the 12 day-long cultivation period is indicated by the respective number above the column.

To study the effect of the modified Kjalke medium on the *lcc1* transformant in more detail, different glucose concentrations were tested: 1%, 2%, 3% and 4%. Growth was observed by measuring the dry weight (DW) of the mycelial cultures and by meaning its ergosterol content. The DW of the cultures increased steadily from day 1 onwards until the maximal DW values of 6.1 g/l at day 4, 10.2 g/l at day 5, 14.2 g/l at day 6 and 16.3 g/l at day 7 were reached for the media supplemented with 1%, 2%, 3% and 4% glucose, respectively. After reaching the maximum DW in cultures with 1-3% glucose, the DW slowly declined, whereas the DW in cultures with 4% glucose remained more stable with high DW of 15-16 g/l over the time (Fig. 2A).

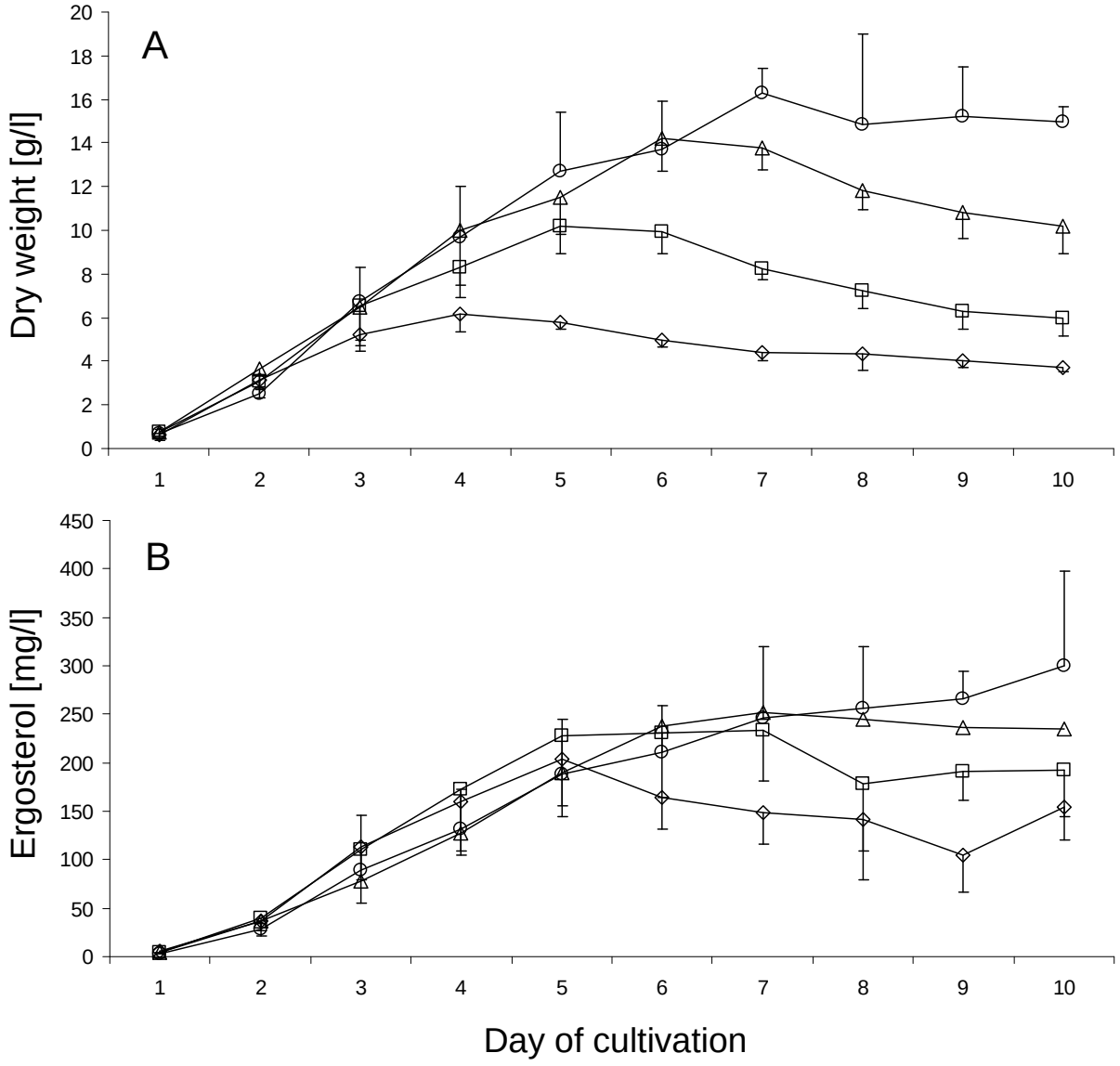


Fig. 2 Growth curves of the *C. cinerea* FA2222 *lcc1* transformant at 37 °C with respect to (**A**) dry weight (DW) and (**B**) ergosterol content in modified Kjalke medium cultures with 1% (diamonds), 2% (squares), 3% (rectangles) and 4% glucose (circles), respectively. Bars above or below the lines indicate the standard deviation derived from at least 3 points of measurement.

It is known that filamentous fungi produce polysaccharides as nutrient storage (Chaumeton et al. 1993, Burns et al. 1994) which rest at the outer part of the fungal hyphae and, therefore, could account for higher biomass values. To control this, the ergosterol content of the fungal cultures was determined as part of the cell membrane of living fungal cells. Irrespectively of the glucose content, the ergosterol content in the cultures was similar until day 5. Afterwards, it started to decline at day 6 for cultures with 1% and 2% glucose and on day 7 for cultures with 3% glucose. In contrast, for the media supplemented with 4% glucose, no decrease in its ergosterol content was observed at later times of cultivation (Fig. 2B). The maximum ergosterol content of the different cultures increased with higher glucose concentration from 136 mg ergosterol per litre to 156, 168 and 200 mg ergosterol/l for 1%, 2%, 3% and 4% glucose in the modified Kjalke medium, respectively. However, the relative enhancement between 1% and 4% glucose was lower for the values of the ergosterol content (1.5 fold increase) compared to the value of the DW (2.7 fold increase). The calculated correlations between ergosterol and DW with respect to the initial glucose content in the culture medium from 1% to 4% glucose are given in Table 1.

Highest laccase activities of 3.3 U/ml were obtained in modified Kjalke medium with 3% glucose at day 7 of cultivation at 37 °C (Fig. 3A). The maximum laccase activities in cultures with other glucose concentrations occurred at day 5, 6 and 9 with activities of 2.1, 2.5 and 3.1 U/ml for cultures with 1%, 2% and 4% glucose in the medium, respectively (Fig. 3A). However, DW-specific laccase activity showed a different pattern. The highest yield of 362 U/gdw (gram dry weight) was obtained in modified Kjalke medium with 1% glucose

Table 1 Maximum values for laccase activities and biomass data of the *C. cinerea* FA2222 *lcc1* transformant cultures in modified Kjalke medium supplemented with different glucose amounts

Maximal values	Initial glucose concentration in the modified Kjalke medium			
	1% glucose	2% glucose	3% glucose	4% glucose
Laccase activity [U/ml]	**2.1** (± 0.5)	**2.5** (± 0.9)	**3.3** (± 1.3)	**3.1** (± 0.9)
Laccase activity [U/gdw]	**362** (± 83)	**254** (± 106)	**245** (± 115)	**198** (± 41)
Laccase activity [U/mg ergosterol]*	**7.3** (± 2.1)	**7.7** (± 5.4)	**9.8** (± 4.5)	**9.2** (± 1.3)
Dry weight [g/l]	**6.1** (± 0.8)	**10.2** (± 1.3)	**14.2** (± 1.5)	**16.3** (± 1.1)
Ergosterol content [mg/l]	**136** (± 40)	**156** (± 35)	**168** (± 35)	**200** (± 66)
Biomass ratio [mg ergosterol/gdw]	**55** (± 14)	**45** (± 15)	**32** (± 10)	**27** (± 10)

Number in brackets is the standard deviation derived from at least 3 different parallel cultures. * Maximal l values were determined for day 2 of cultivation onwards.

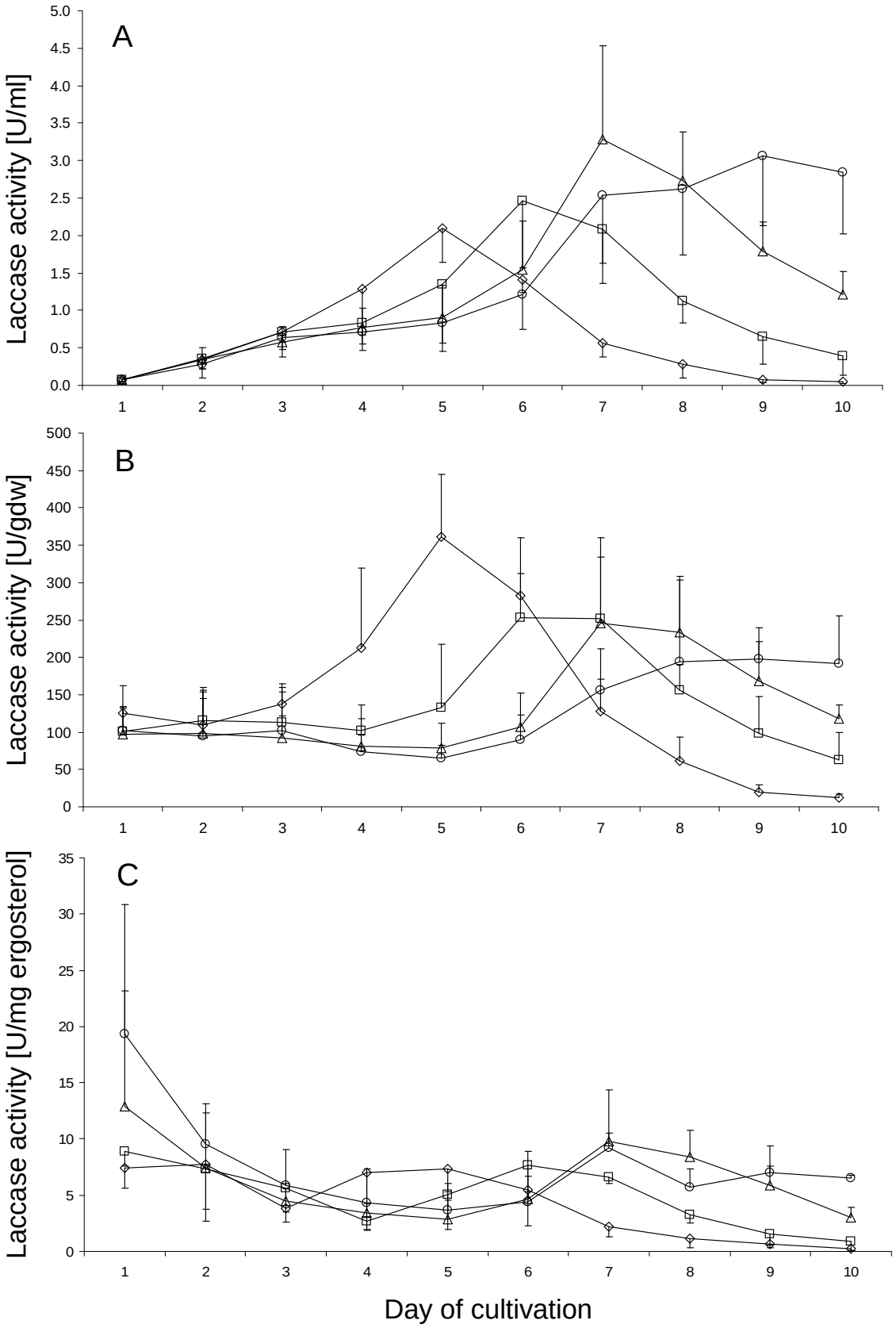


Fig. 3 Laccase activity of the *C. cinerea* FA2222 *lcc1* transformant grown in modified Kjalke medium at 37 °C with different glucose concentrations [1% (diamonds), 2% (squares), 3% (rectangles) and 4% (circles)] is given as (**A**) volumetric activity, (**B**) activity compared to the mycelial dry weight and (**C**) activity per ergosterol content. Bars above or below the lines indicate the standard deviation. Mean values were calculated from at least 3 different parallel cultures.

(Fig. 3B). In media with 2% and 3% glucose, the yield had an average value of 250 U/gdw and in media with 4% glucose almost 200 U/gdw. The time points of the peak activities for the DW-specific laccase activity were identical with the highest laccase-ergosterol relations, except for cultures of modified Kjalke medium with 4% glucose. Ergosterol-specific activities in cultures with 1% and 2% glucose with 7.3 U/mg ergosterol at day 5 of cultivation and 7.7 U/mg ergosterol at day 6 of cultivation, respectively, were relatively comparable to each other. Likewise, the ergsoterol-specfic activities in cultures with 3% and 4% glucose with 9.8 and 9.2 U/mg ergosterol, respectively, at day 7 of cultivation were relatively comparable to each other (Fig. 3B, C and Table 1).

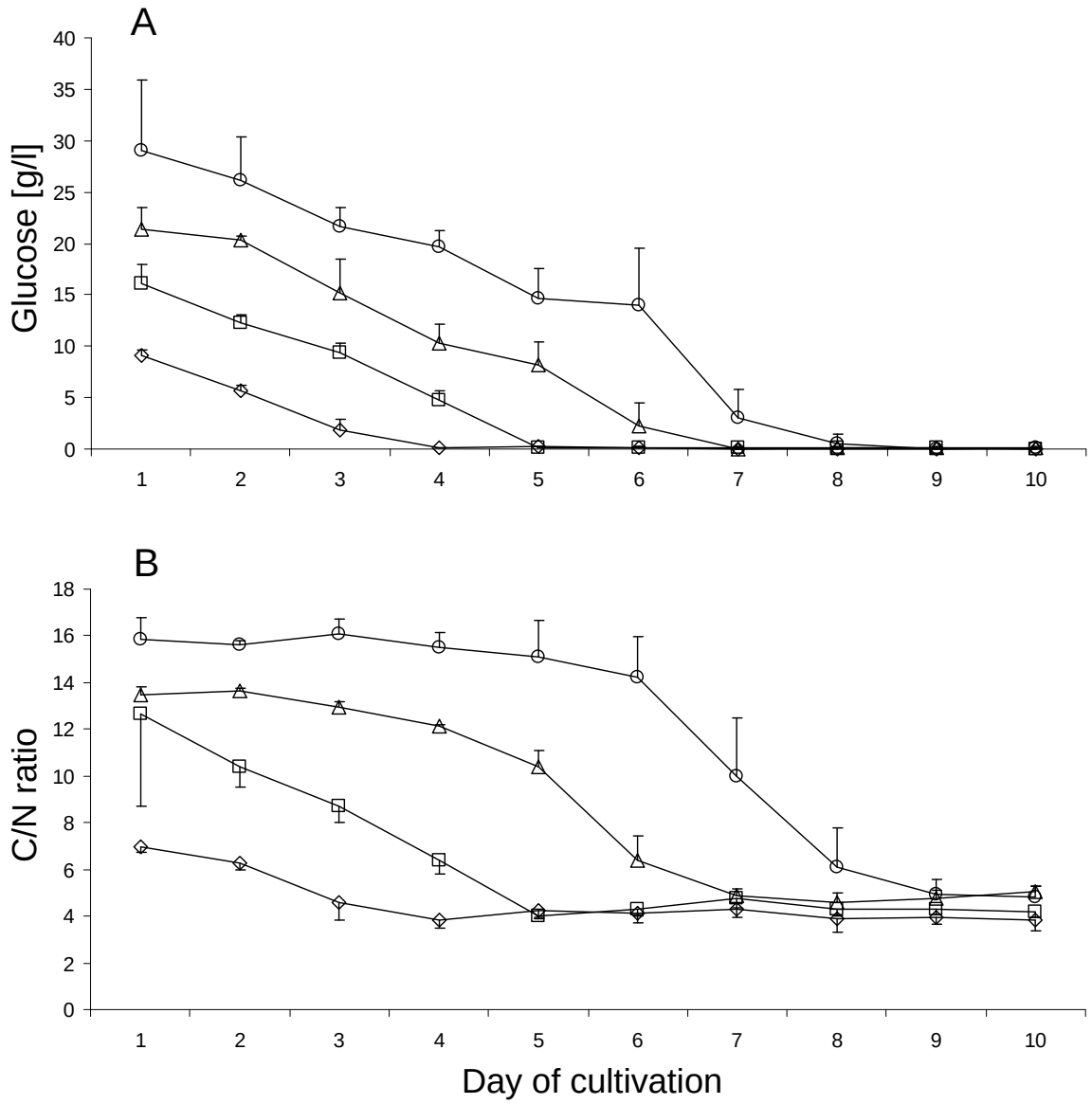


Fig. 4 Glucose concentration (**A**) and C/N ratio (**B**) of the supernatant of the *C. cinerea* FA2222 *lcc1* transformant grown at 37 °C in modified Kjalke medium with different initial glucose concentrations [1% (diamonds), 2% (squares), 3% (rectangles) and 4% (circles)]. Bars above or below the lines indicate the standard deviation derived from 3 cultures analysed in parallel.

Since glucose as part of the modified Kjalke medium was altered in concentration, the glucose content of the supernatant was observed via determination of the glucose in the cultures with different initial glucose concentrations over the whole cultivation periods (Fig. 4A). Glucose depletion in the supernatant occurred on day 4, 5, 7 and 8 for media with 1%, 2%, 3% and 4% glucose, respectively. All cultures showed a similar metabolisation rate of the glucose with around 3.7 to 3.9 g of glucose per day of cultivation as calculated from the slope of the regression lines of the actual glucose concentration over the time (Fig. 4A, for the calculated values see Table 2).

Table 2 Glucose metabolisation rate of the *C. cinerea* FA2222 *lcc1* transformant cultivated at 37 °C in modified Kjalke medium with different initial glucose concentrations in the modified Kjalke medium

Parameters for the regression line*	**Initial glucose concentration in the modified Kjalke medium**			
	1% glucose	**2% glucose**	**3% glucose**	**4% glucose**
Range of days used for calculation	3	4	6	7
Slope of regression line [g glucose/day]	-3.7	-3.7	-3.9	-3.9
R^2 of regression line+	0.999	0.994	0.975	0.938

*Range of days used for calculation: A linear best-fit-line was calculated with Excel (Excel 2002, Microsoft) from the measurement values of glucose concentration at day 1 to day 3 of cultivation (cultures with 1% initial glucose concentration), to day 4 of cultivation (cultures with 2% initial glucose concentration), to day 6 of cultivation (cultures with 3% initial glucose concentration) and to day 7 of cultivation (cultures with 4% initial glucose concentration). +The coefficient of determination (R^2) as a quality measure of the regression line approaching to 1 indicates a high quality.

In addition to glucose, other C- and N-sources in the supernatant were observed via the general C/N ratio. The C/N ratio of the media started with values of around 7 to 16 for cultures with the different glucose concentrations of 1% to 4 % glucose (Fig. 4B). In cultures with an initial content of 1% and 2% glucose, the C/N ratio declined clearly from day 1 of cultivation onwards, whereas for cultures with 3% and 4% initial glucose a delay of 3 to 4 days in a rapid decrease of the C/N ratio was observed. In cultures with 3% and 4% initial glucose, a rapid decline in the C/N ratios occurred from day 4 to 7 and day 6 to 9, respectively (Fig. 4B). At the end, all cultures had the same low C/N-ratio of around 4, which was reached at days 4, 5, 7 and 9 of cultivation for the cultures with 1%, 2%, 3% and 4% initial glucose, respectively.

2. Temperature as an important growth parameter

To determine the temperature effect on the laccase activity of the *C. cinerea* FA2222 *lcc1* transformant, three different temperatures were tested for cultivation of the strain in YMG and in modified Kjalke medium. In YMG only small effects by the temperature on maximum yields of laccase activities were seen when cultivating the *lcc1* transformant at 25 °C, 30 °C and 37 °C. In all cultures, irrespectively of the growth temperature, maximum laccase activities in the supernatant were around 1.1 to 1.5 U/ml although the days of maximum laccase activity differed (maximum laccase activity at 25 °C of 1.5 U/ml at day 9 of cultivation, at 30 °C of 1.1 U/ml at day 5 of cultivation and at 37 °C of 1.1 U/ml at day 4 of cultivation). In contrast, in modified Kjalke medium a tremendous increase in laccase activities was observed at a cultivation temperature of 25 °C compared to the higher temperatures (Fig. 5). At 30 °C and 37 °C, almost the same maximal laccase activity was observed with measured values of 2.2 U/ml and 2.7 U/ml, respectively. At 25 °C, a three fold increase as compared to the higher growth temperatures was noted with a highest measured laccase activity of 7.0 U/ml at day 10 of cultivation.

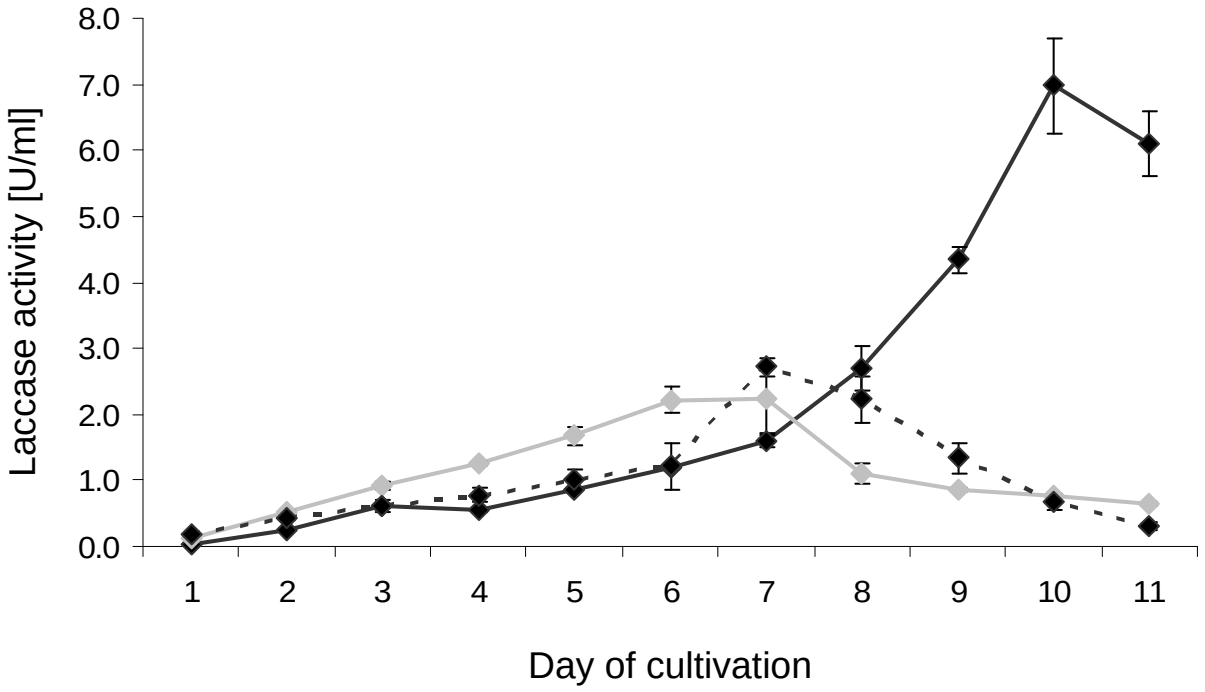


Fig. 5 Laccase activity of the *C. cinerea* FA2222 *lcc1* transformant (filled diamond) cultivated in modified Kjalke medium with 2% glucose at different temperatures: 25 °C (black solid line), 30 °C (grey solid line) and 37 °C (black dashed line). Bars above or below the lines indicate the standard deviation derived from 3 replicate cultures.

Temperature effects on laccase activities were further studied in modified Kjalke medium with 2% glucose with FA2222 transformants Lcc1, Lcc5 and Lcc7, and, in addition, with a FA2222 pCc1001 transformant carrying no extra laccase gene. For all strains, higher enzymatic activities were observed at a cultivation temperature of 25 °C as compared to 37 °C (Fig. 6). In this series of experiments, transformant Lcc1 produced as maximum 9.1 U/ml laccase at 25 °C (day 10 of cultivation), transformant Lcc5 produced 31.1 U/ml (day 10 of cultivation), transformant Lcc7 produced 6.8 U/ml (day 8 of cultivation) and control transformant pCc1001 produced 3.5 U/ml (day 9 of cultivation). The difference in the laccase activity of the *lcc5* transformant between 25 °C and 37 °C was 2.5 U/ml, respectively, and thus comparable in value to the difference of 3.5 U/ml measured between cultures of transformant pCc1001 at 25 °C and 37 °C, respectively. In cultures of the *lcc1* transformant and the *lcc7* transformant a difference in laccase activity between 25 °C and 37 °C of 6.3 and 6.7 U/ml was observed, respectively, and thus almost twice as high as for the control transformant pCc1001. It is thus very likely that transformants Lcc1 and Lcc7 produced at 25 °C about 5 U/ml and 3 U/ml of recombinant laccase, respectively, in addition of about 3.5 U/ml of background activity from native production. In case of transformant Lcc5, about 90% of the obtained laccase yields at 25 °C is expected to be from recombinant production.

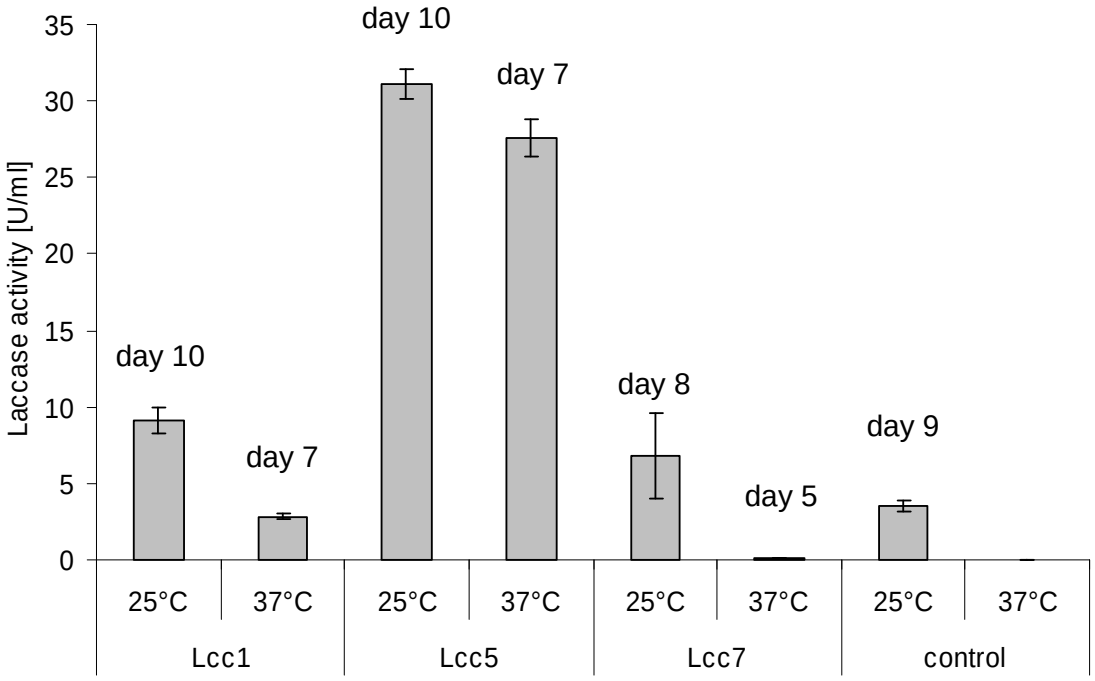


Fig. 6 Maximum laccase activity of the *C. cinerea* FA2222 *lcc1*, *lcc5* and *lcc7* transformant as well as the control transformant (pCc1001) cultivated at 25 °C and 37 °C in modified Kjalke medium supplemented with 2% glucose. Bars above or below the lines indicate the standard deviation derived from 3 replicate cultures.

C. cinerea strain FA2222 was so far used for recombinant production by its failure to secrete laccase into a culture medium when grown at 37 °C (Kilaru et al. 2006 and Table 2 in section 4.I of this study). The above results indicate that recombinant enzyme production is better at 25 °C. However, at 25 °C not all of the laccase activity found in the culture supernatants might be due to the enzyme coming from recombinant production. Although the impurities might be low, for purification of an enzyme from a culture supernatant for example, a situation of high enzyme production with no impurities of related other enzymes, is preferable. Therefore, another strain than FA2222 producing no natural laccase activity at a culture temperature of 25 °C would be required. Comparison of different *C. cinerea* strains at different culture temperatures for laccase production suggested that the monokaryon LN118 could be such ideal strain for recombinant laccase production (see section 4.I). LN118 showed negligible laccase activities (0.00-0.11 U/ml) in YMG and modified Kjalke medium at 25 °C and 37 °C (see section 4.I) and, thus, was transformed with the *lcc1* containing plasmid pYSK7 (Kilaru 2006) in co-transformation with the *trp1*$^{+}$ plasmid pCc1001. Positive clones were obtained after complementation of the *trp1* tryptophan auxotrophy of monokaryon LN118 and selection of *trp1*$^{+}$ transformants on regeneration medium (Fig. 7). Three clones were tested for best laccase production in YMG medium at 37 °C. The range of maximum laccase production was between 0.7 and 1.5 U/ml. The LN118 clone LN7-2 (named after LN118, pYSK7, clone 2) was found to be the best laccase producer in liquid culture and, therefore, the clone was used for further experiments.

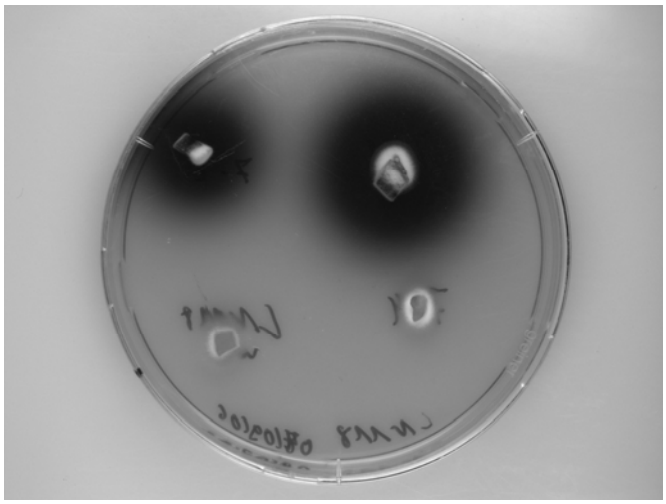


Fig. 7 Positive clones of *C. cinerea* monokaryon LN118 co-transformed with the *lcc1* containing plasmid pYSK7 and the *trp1*$^{+}$ plasmid pCc1001 (upper two colonies), a clone containing only pCc1001 (lower right colony) and the native monokaryotic strain LN118 (lower left colony) on YMG agar plates supplemented with ABTS. The brownish red halo around that colonies indicate that laccase was secreted and the colourless substrate ABTS was converted by the enzyme into a coloured product.

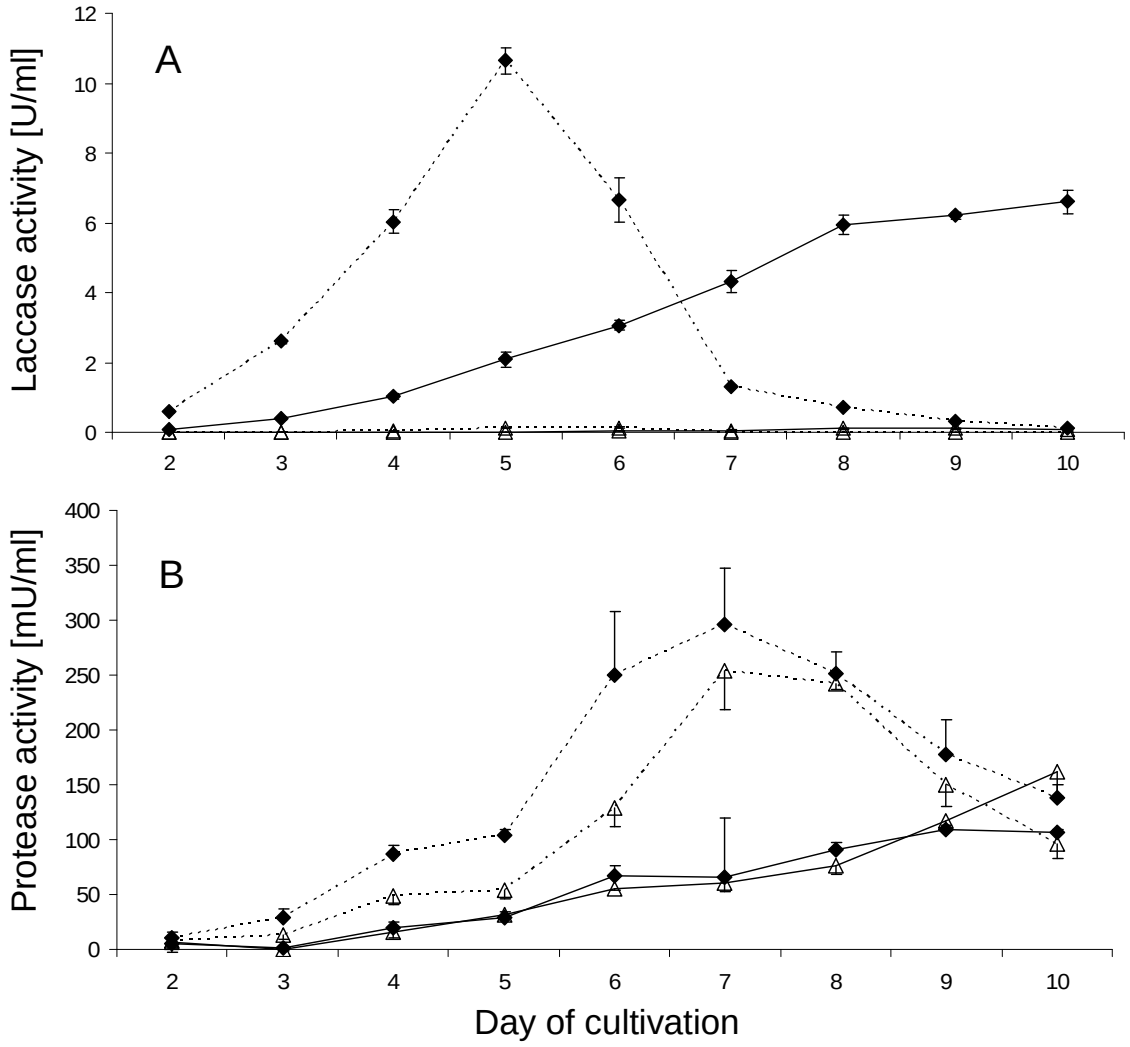


Fig. 8 Laccase activity (**A**) and protease activity (**B**) in cultures of LN7-2 (*lcc1* transformant of monokaryon LN118; filled diamond) and a control transformant (pCc1001 in LN118; open triangle) grown in modified Kjalke medium supplemented with 2% glucose at 25 °C (solid line) and 37 °C (dashed line). Bars above or below the lines indicate the standard deviation derived from 3 parallel cultures.

LN7-2 and a corresponding control transformant (pCc1001 in LN118 without further laccase gene) were cultivated in modified Kjalke medium at 25 °C and 37 °C, respectively. The laccase activities in the cultures were measured every day from day 2 of cultivation onwards over a period of 10 days (Fig. 8A); only for LN7-2 at 25 °C cultivation and measurements were prolonged for 4 further days, because maximum activity of laccase was not reached before or at day 10 (data not shown in Fig. 8). The maximum laccase activity of LN7-2 of 8.0 U/ml was reached at day 11 of cultivation at 25 °C and, afterwards, the laccase activity decreased to 2.3 U/ml at day 14 of cultivation. Maximum laccase activity of LN7-2 at 37 °C was reached with 10.8 U/ml at day 5 of cultivation. For comparison, the control transformant (pCc1001) showed minor activities at both tested temperatures of up to maximum 0.1 U/ml during the whole cultivation period of 10 days (Fig. 8A). It is interesting to note that, unlike

for the FA2222 *lcc1* transformant (Fig. 5), for the LN118 *lcc1* transformant the total laccase yields were better at 37 °C than at 25 °C.

In the series of temperature experiments with the LN118 transformants, next to the laccase activities in the culture supernatant also proteolytic activities were measured (Fig. 8). The protease activity for LN7-2 and the control transformant showed a similar pattern to each other for the two temperatures. At 37 °C, highest activity occurred at day 7 of cultivation two days after the peak of the laccase activities were obtained with protease values of 296 mU/ml for the *lcc1* transformant and 254 mU/ml for the control transformant. At 25 °C, protease activities in the culture supernatants of both transformants were steadily increasing within the 10 days of parallel cultivations to levels of 162 mU/ml for the control transformant and the levels of 106 mU/ml for LN7-2 at day 10 of cultivation. Upon prolonged cultivation of LN7-2 at 25 °C, the *lcc1* transformant showed the highest proteolytic activity of 240 mU/ml at day 14 of cultivation three days after the laccase activity peak. Accordingly, the maximum protease activity to be obtained at 25 °C seems to be in the same range as the maximum activities obtained at 37 °C - 254 mU/ml and 296 mU/ml for the control transformant and LN7-2, respectively, at day 7 of cultivation (Fig. 8B).

Proteolytic activities in the supernatants might negatively influence the amount of laccase activities by degradation of parts of secreted enzymes. To test whether the proteolytic activity possibly had an impact on the degradation of the laccase in the culture broth, the supernatants of the *lcc1* transformant with the highest proteolytic activity (from day 7 of cultivation for 37 °C cultures and from day 14 of cultivation for 25 °C cultures) were used. One part of the supernatant (1 ml in total) was boiled for 15 min to deactivate any protease activity, supplemented with purified Lcc1 (see chapter 5) to a final amount of 2 U/ml and incubated at 25 °C. The other part of the supernatant, with a natural laccase activity of around 6.8 U/ml and 1.2 U/ml for the 25 °C and 37 °C culture supernatants, respectively, was directly incubated at 25 °C. The laccase activities in the sample were measured at different times of incubation (Fig. 9). Overall, the decrease in lacacse activity within the samples over the time followed the same pattern, regardless of whether a supernatant was obtained from a culture grown at 25 °C or a culture grown at 37 °C and regardless of whether a supernatant was fresh or heat-inactivated with subsequent addition of external laccase (Fig. 9). Within the first 6 h of incubation, the activity loss in all samples was little with about 11 to 20%. After 24 h, the laccase activity of the fresh supernatants from 37 °C and 25 °C cultures retained 41% and 47% of the laccase original activity, respectively (Fig. 9). The heat-inactivated supernatant from cultivations at 25 °C and 37 °C with added

laccase Lcc1 after 24 h of incubation showed remaining activities of 61% and 79%, respectively. After further incubation up to in total 48 h, laccase activities were still present in all samples with 16 and 18% of activity in the native supernatants and denaturised supernatant with added Lcc1 from 37 °C cultivation of clone LN7-2 and with 32 and 35% of activity in the native supernatant and the denaturised supernatant with added Lcc1 from 25 °C cultivation of clone LN7-2.

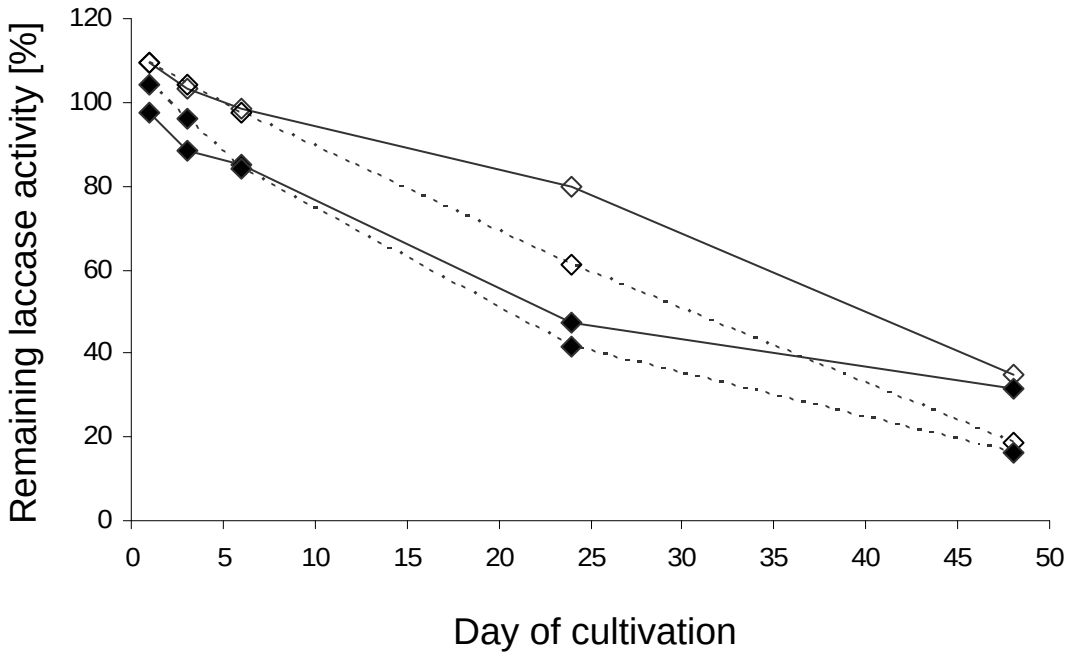


Fig. 9 Decrease of the laccase activity in denaturised (open diamond) and fresh (filled diamond) supernatant from LN7-2 cultures of modified Kjalke medium supplemented with 2% glucose from cultivations of the LN7-2 transformant at 25 °C (solid line) and 37 °C (dashed line) incubated at room temperature. At indicated times, residual laccase activties were determined in percentage of the original activity. Note that the values were calculated from each a single sample.

E. Discussion

Defining optimal culture conditions for the native, but also recombinant production of enzymes is important to increase the yield of the production of interest and to understand the requirements of the organism for such production. In this study, the recombinant production of the laccase Lcc1 from the basidiomycete *C. cinerea* was studied in transformants of these species under different culture conditions (media and temperature) in shake flask cultures.

1. Media effects on laccase production

When different *C. cinerea* media described in the literature were tested for growth and enzyme production in liquid cultures, it was observed that this dung-inhabiting fungus prefers complex media, such as the rich complex YMG and modified Kjalke media, as compared to the semi-complex (BSM) and synthetic (MM, Moore and Raulin) media (Fig. 1). As earlier described in section 4.I, the media might have a tremendous effect on native production of laccase in *C. cinerea* strains. This effect can also be seen in Fig. 1, where the control pCc1001 transformant without an extra laccase gene showed no activity in YMG and low activity in BSM and modified Kjalke medium. In comparison, the laccase transformant Lcc1 yielded high enzymatic yields in the YMG and even higher yields in modified Kjalke medium and also some activity in BSM. In contrast, in Raulin, MM and Moore medium, laccase activities of the control were as high or higher as for the *lcc1* transformant. Growth of the fungal strains was generally better in modified Kjalke medium than in the YMG medium and this probably bases on the higher amounts of glucose and yeast extract in the modified Kjalke medium.

From reports in the literature, the monosaccharide glucose seems to be the best carbon source for *C. cinerea*, besides the disaccharides maltose, lactose and fructose (Madelin 1956, Moore 1969), showing that hexose-based sugars are preferred by the fungus. In other basidiomycete fungi, it was already shown that glucose might have an impact on laccase secretion. In *Trametes pubescence* an increase in the glucose concentration from 1% to 4% increased the laccase activity 5 times, whereas a further augmentation in glucose concentration lowered the final laccase activity (Galhaup et al. 2002). This repression of laccase commonly secretion by high glucose concentration may be due to an energy saving effect, which occurs in fungi (Ronne 1995). Tavares et al. (2005) observed that a glucose decrease of <0.8% increased laccase activity in 2,5-xylidine and copper induced *Trametes versicolor* cultures of more than 3-fold.

In this study with *C. cinerea* transformants, modified Kjalke medium was found best for recombinant laccase production, and YMG medium second best (Fig. 1). Although we used a *lcc1* transformant with a constitutive promoter for the overexpression of the laccase Lcc1, an effect of medium concerning the yield of laccase activity was already previously observed. Kilaru et al. (2006b) found that the medium YMG gave much better laccase yields than minimal medium.

Both the YMG and the modified Kjalke media contain relatively high amounts of glucose together with nitrogen supplied from yeast-extract. In order to study the effect of the relative concentration on growth and laccase production of the FA2222 *lcc1* transformant, modified Kjalke medium was prepared with different glucose amounts (1%, 2%, 3% and 4%). The peak of the DW was reached for all tested glucose concentrations one day before the highest laccase activity was achieved. In contrast, the peak of the ergosterol content appeared at around the same time with the highest laccase yields. When comparing the peaks of the DW and ergosterol values (Fig. 2A and B) the trend of increase and subsequent decrease in DW and ergosterol per l of culture seems to be similar. However, the ratios of ergosterol and DW [mg ergosterol/g DW] for the used media are different and clearly depend on the glucose amount added to the culture medium. The ratio ergosterol to DW slightly increased over the time with highest ratios of 55, 45, 32 and 27 mg ergosterol/g DW at the end of the cultivation in modified Kajlke medium with 1%, 2%, 3% and 4% glucose, respectively (Fig. 10). Assuming that the ergosterol content and/or the DW specify the growth of the fungus, their ratio should be more or less stable over the whole culture. In the literature, the ergosterol to DW ratio of basidiomycetes are given for agar plate cultures, mushrooms and solid state fermentation (SSF) on wood or other materials, but for liquid cultures only few studies are available (Barajas-Aceves et al. 2002, Silva et al. 2005, Niemenmaa et al. 2008). Barajas-Aceves et al. (2002) for example cultivated twenty white-rot strains in liquid culture and obtained values of around 2.38 to 13.06 mg ergosterol/g DW after 7 days of incubation. When studying three species in more detail over a growth period of 14 days, the ergosterol to DW values increased for all cultures during the incubation reaching stationary values of around 5, 13 to 20 mg ergosterol/g DW at the end of the cultivation for the tested strains of *Phanerochaete chrysosporium*, *Coriolopsis gallica* and *Bjerkandera adusta*, respectively. In this study, the *lcc1* transformant of *C. cinerea* strain FA2222 showed also in all cultures a steady increase in the ergosterol to DW ratio (Fig. 10).

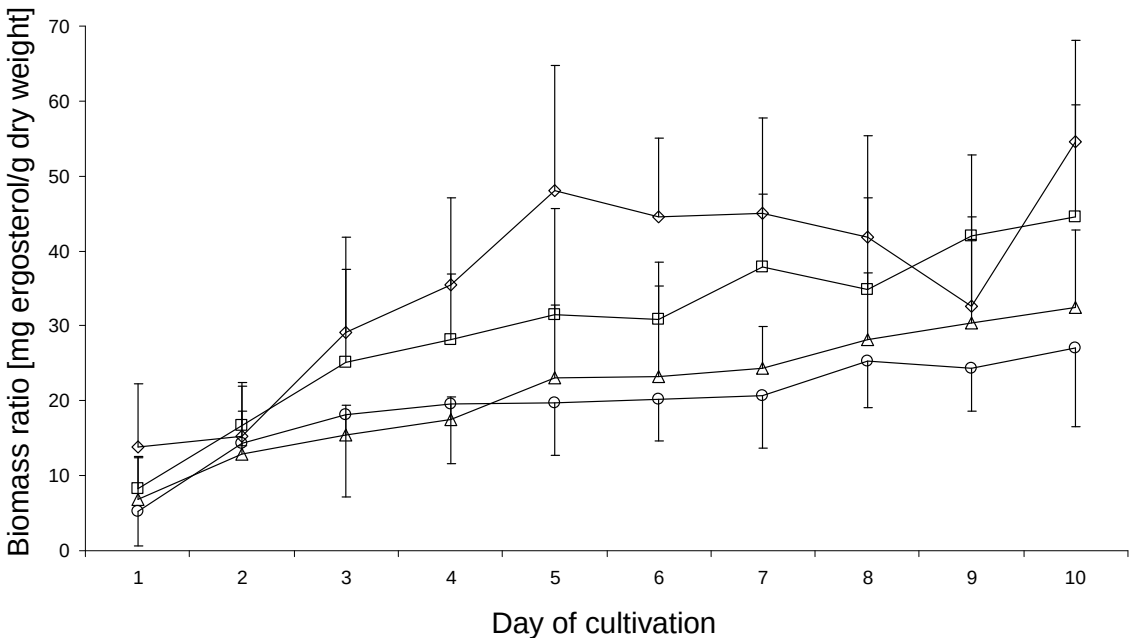


Fig. 10 Biomass ratio [mg ergosterol per g dry weight] of the *C. cinerea* FA2222 *lcc1* transformant grown in modified Kjalke medium at 37 °C with different glucose concentrations [1% (diamonds), 2% (squares), 3% (rectangles) and 4% (circles)]. Bars above or below the lines indicate the standard deviation. Mean values were calculated from at least 3 parallel cultures.

To better understand the relationship between the ergosterol content and the DW in the different cultures, a regression for the values obtained over the whole cultivation time might be useful to compare both types of values. This was done previously for nine *Lentinula edodes* strains in a combined analysis of the values for all strains leading to a slope of 4.1 mg ergosterol/g DW for the calculated linear regression line with a relatively good regression coefficient of 0.87 (n>40; n= number of single values analysed) (Silva et al. 2005). In another study with several white and brown rot fungi, values of 0.6 to 3.9 mg ergosterol/g DW and regression coefficients of 0.235 to 0.969 (for each fungus: n=5) were obtained (Niemenmaa et al. 2008), but the very low number of analysed samples per fungus makes even the ratios for ergosterol/DW with highest regression coefficients insecure in reliability. In this study with n=30-40 per cultivation condition, linear regressions of the values over the whole culturing periods of the *lcc1* transformant lead to correlation values of 19.7, 16.0, 12.0 and 10.9 mg ergosterol/g DW with regression coeffiecients of 0.717, 0.832, 0.863 and 0.939 for the cultures with 1%, 2%, 3% and 4% glucose, respectively (Fig. 11). The results here in this study therefore showed a good reliable correlation for ergosterol to DW ratios for cultures with 2%, 3% and 4% glucose, whereas the regression coefficient for 1% was comparatively low and the calculated ergosterol to DW ratio is less reliable. Comparing the data for the different glucose concentrations strongly supports an effect of the sugar concentration in the medium on the ergosterol/DW ratios. With increasing sugar

concentrations, the ergosterol to DW ratios decrease. The reason for the lower ergosterol/DW ratios at higher glucose concentration is probably due to the higher amount of polysaccharides in cultures with high glucose concentrations. Secretion of polysaccharides out of the cells and deposition of the material on the hyphal surface would cause a higher absolute dry weight per cellular unit than without such depositions. In contrast, ergosterol being present in the cellular membranes of fungi (Niemenmaa et al. 2008), has only a limited space per cellular unit to distribute.

In any case, the growth curves deriving from either the ergosterol content or the DW show similar overall patterns (see Fig. 2) and, therefore, are both adequate to describe the growth of the *lcc1*-transformant. Accordingly, one might either correlate laccase activities in the culture supernatant as an indication of the specific laccase production with the ergosterol content in the cultures and/or with the DW values of the cultures.

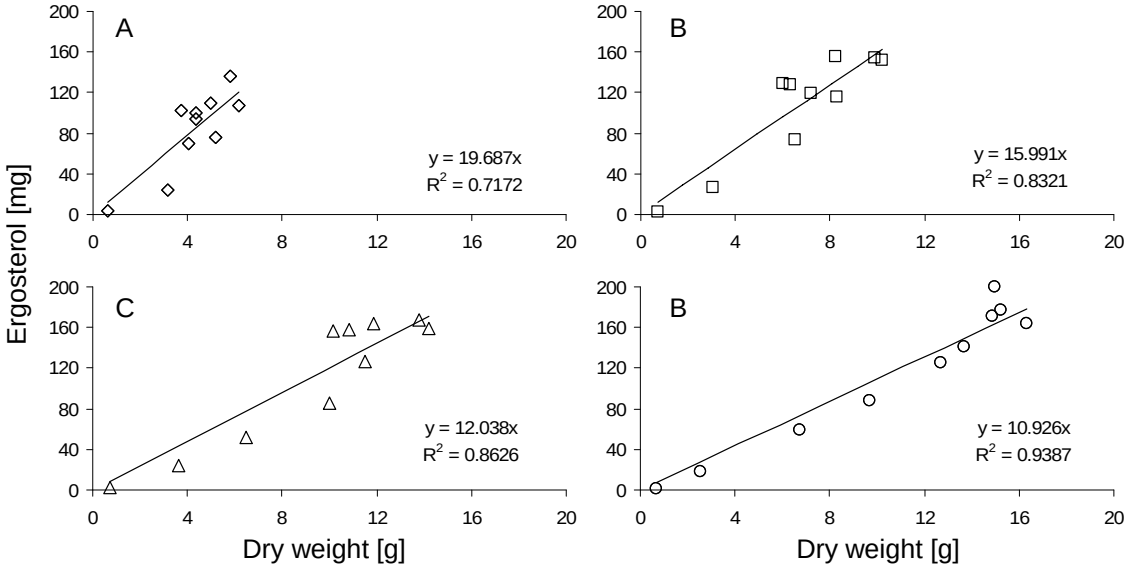


Fig. 11 Correlation between the ergosterol contents and the dry weights of the *lcc1* transformant cultivated at 37 °C in Kjalke mod medium with 1% (open diamonds), 2% (open squares), 3% (open triangles) and 4% glucose (open circles). Linear regression lines were calculated with Excel (Excel 2.0, Microsoft) for each glucose concentration. Linear equation and the respective coefficient of determination (R^2) are given in each graph.

Laccase activities in the modified Kjalke medium with the different glucose concentrations showed a similar pattern at the beginning of the periods of cultivation over the first 3 to 5 days of cultivation (Fig. 3A). The highest volumetric activities [U/ml] where reached over the time one after the other for increasing glucose concentrations at day 5 of cultivation for cultures with 1% glucose, at day 6 of cultivation for cultures with 2% glucose, at day 7 of cultivation for cultures with 3% glucose and at day 9 of cultivation for cultures with 4%

glucose. Also, the biomass specific activities [U/mg ergosterol and U/g DW] increase for the different cultures with increasing glucose concentration in the media (Fig. 3). In the experimental phase, due to the usage of the constitutive *A. bisporus gpdII* promoter (Harmsen et al. 1992, Kilaru and Kües 2005) for the overexpression of *lcc1*, a correlation between active biomass and laccase should be present. In Fig. 3B and C, one can observe that the calculated DW-specific laccase activity varies much more than the calculated ergosterol-specific laccase activity, although the standard deviations for some of the ergosterol-based values are quite high. As shown in Table 1, the calculated ergosterol-based maximum laccase activities are comparable to the volumetric ones. Both types of values define the same order of glucose concentration in the medium (3%>4%>2%>1%, see Table 1) for the highest laccase production. Accordingly from this observation, one might conclude that the biomass-specific laccase activity as determined for the ergosterol content is the more reliable estimation of culture parameter than the DW-based specific lacacse activity. From the order of best laccase production in the medium, one would for future work suggest to use 3% glucose in the modified Kjalke medium for batch cultures.

Interesting is the decline of the laccase activity curves at later stages of cultivation (Fig. 3A) in cultures with 1%, 2% and 3% glucose as compared to the more or less stable laccase activity curve at the later stages of cultivation in the 4% culture. The peaks of the volumetric activities in the culture with 1%, 2% and 3% glucose occur at the same day as highest biomass yields were obtained, namely days 4, 5 and 6, respectively. After this point of highest laccase activity, no additional laccase is possibly produced in the cultures and, furthermore, the resident laccase will be degraded or inactivated slowly over the time. The longer residing laccase activity in cultures with 4% glucose might be due to a stabilising effect of components in the supernatant (e.g. polysaccharides) or, although the stationary growth phase will have been reached (Fig. 2), the fungus still produces laccase compensating the concomitant degradation of the enzyme. Therefore, modified Kjalke medium with 4% glucose might be considered to be a better production medium than the media with lower glucose concentrations. Nevertheless, in continuous cultivation, where a steady-state-point between growth, substrate and product is seeked, cultures with the highest production rate at a constant biomass level should be used.

2. Temperature effects on laccase secretion

Heterologous expression of the laccase encoding *lcc1* gene of *T. versicolor* in *Pichia methanolica* could be optimised by changing the cultivation temperature from 30 °C to 20 °C

with an increase in laccase activity by the factor of 1.7 (Guo et al. 2006). Also Hong et al. (2002) were able to increase the laccase yields of a heterologous expression of *lcc1* from *T. versicolor* in *P. pastoris* by decreasing the temperature from 30 °C to 20 °C. Heterologous expression of *lcc2* of *T. versicolor* in *Saccharomyces cerevisiae* gave 16fold higher laccase yields at 19 °C as compared to 28 °C in liquid cultivation (Cassland and Jonsson 1999). Also In this study, the decrease of the cultivation temperature from 37 °C to 25 °C lead to an increase in laccase activity of the *C. cinerea* FA2222 *lcc1* transformant cultivated in modified Kjalke medium supplemented with 2% glucose (Fig. 5). However, also the pCc1001 control transformant, as well as the *lcc5* and *lcc7* transformant, showed a higher laccase activity when cultivated at 25 °C (Fig. 6). Usage of a *lcc1* transformant of monokaryon LN118 as another another *C. cinerea* strain for laccase production did not lead to a higher laccase activity when cultivated at 25 °C, but instead when cultivated at 37 °C. The maximum activity at 25 °C with 8.0 U/ml was around 3 U/ml lower than the maximum activities of 10.8 U/ml obtained at 37 °C. The result described here and the results presented earlier in section 4.I of this thesis indicate that strains of different genetic background (compare Kertesz-Chaloupková et al. 1998 for strain origins) will behave quite variably in enzyme production. In consequence, any new strain of *C. cinerea* for laccase production will again have to be tested for enzyme production at different temperature.

3. Protease production and effect on laccase activties

In cultures of the LN118 *lcc1* transformant, next to laccase activities, proteolytic activties were measured (Fig. 8). Most importantly, the proteolytic activities for 25 °C and 37 °C cultures increased with decreasing laccase activity (Fig. 8), but the proteases from the 25 °C and 37 °C culture supernatant seem not to degrade the homologously expressed laccase (Fig. 9). As there is no degradation due to proteolytic activity in the supernatant, no protease-deficient strains appear to be needed for the recombinant production of Lcc1 by *C. cinerea*. Since culture supernatants from both cultivation temperatures, 25 °C and 37 °C, were tested for their effects on proteolytic inactivation of Lcc1 and no major differences were detected (Fig. 9), differences in degradation of produced laccase will not account for the different amounts of laccase activities detected in the cultures grown at the different temperature. In the future, more LN118 transformants will have to be tested in order to determine whether higher laccase production at 37 °C from laccase genes cloned behind the *A. bisporus gpdII* promoter is common in this strain as it is at 25 °C for all so far tested FA2222 transformants.

III. References

Anh DH, Ullrich R, Benndorf D, Svatos A, Muck A, and Hofrichter M (2007) The coprophilous mushroom *Coprinus radians* secretes a haloperoxidase that catalyzes aromatic peroxygenation. Applied and Environmental Microbiology 73:5477-5485

Alves AMCR, Record E, Lomascolo A, Scholtmeijer K, Asther M, Wessels JGH, and Wösten HAB (2004) Highly efficient production of laccase by the basidiomycete *Pycnoporus cinnabarinus*. Applied and Environmental Microbiology 70:6379-6384

Ardon O, Kerem Z, and Hadar Y (1996) Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. Journal of Biotechnology 51:201-207

Aro N, Pakula T, and Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiology Reviews 29:719-739

Arora DS and Gill PK (2001) Effects of various media and supplements on laccase production by some white rot fungi. Bioresource Technology 77:89-91

Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiology Reviews 30:215-242

Barajas-Aceves M, Hassan M, Tinoco R, and Vazquez-Duhalt R (2002) Effect of pollutants on the ergosterol content as indicator of fungal biomass. Journal of Microbiological Methods 50:227-236

Binninger DM, Skrznia C, Pukkila PJ, and Casselton LA (1987) DNA-mediated tranformation of the basidiomycete *Coprinus cinereus*. Embo Journal 6:835-840

Bollag JM and Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Applied and Environmental Microbiology 48:849-854

Braun-Lüllemann A, Majcherczyk A and Hüttermann A (1997) Degradation of styrene by white-rot fungi. Applied Microbiology and Biotechnology 47:150-155

Burns PJ, Yeo P, Keshavarz T, Roller S, and Evans CS (1994) Physiological studies of exopolysaccharide production from the basidiomycete *Pleurotus* sp *florida*. Enzyme and Microbial Technology 16:566-572

Claus H (2004) Laccases: structure, reactions, distribution. Micron 35:93-96

Cassland P and Jonsson LJ (1999) Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Applied Microbiology and Biotechnology 52:393-400

Chaumeton JP, Chauveau C, and Chavant L (1993) Water-soluble polysaccharides excreted by mycelium of higher fungi - relationship with taxonomy and physiology. Biochemical Systematics and Ecology 21:227-239

Conesa A, Punt PJ, van Luijk N, and van den Hondel CAMJ (2001) The secretion pathway in filamentous fungi: A biotechnological view. Fungal Genetics and Biology 33:155-171

D'Souza DT, Tiwari R, Sah AK, and Raghukumar C (2006) Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. Enzyme and Microbial Technology 38:504-511

D'Souza TM, Merritt CS, and Reddy CA (1999) Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Applied and Environmental Microbiology 65:5307-5313

De Souza CGM, Tychanowicz GK, De Souza DF, and Peralta RM (2004) Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. Journal of Basic Microbiology 44:129-136

Dong JL, Zhang YW, Zhang RH, Huang WZ, and Zhang YZ (2005) Influence of culture conditions on laccase production and isozyme patterns in the white-rot fungus *Trametes gallica*. Journal of Basic Microbiology 45:190-198

Eggert C, Temp U, and Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. Applied and Environmental Microbiology 62:1151-1158

Faraco V, Pezzella C, Miele A, Giardina P and Sannia G (2009) Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. Biodegradation 20:209-220

Galhaup C, Wagner H, Hinterstoisser B, and Haltrich D (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme and Microbial Technology 30:529-536

Gouka RJ, Punt PJ, and van den Hondel CAMJ (1997) Efficient production of secreted proteins by *Aspergillus*: Progress, limitations and prospects. Applied Microbiology and Biotechnology 47:1-11

Granado JD, Kertesz-Chaloupková K, Aebi M, and Kües U (1997) Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Molecular & General Genetics 256:28-36

Guo M, Lu FP, Du LX, Pu J, and Bai DQ (2006) Optimization of the expression of a laccase gene from *Trametes versicolor* in *Pichia methanolica*. Applied Microbiology and Biotechnology 71:848-852

Harmsen MC, Schuren FHJ, Moukha SM, Vanzuilen CM, Punt PJ and Wessels JGH (1992) Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. Current Genetics 22:447-454

Havlis J and Shevchenko A (2004) Absolute quantification of proteins in solutions and in polyacrylamide gels by mass spectrometry. Analytical Chemistry 76:3029-3036

Herpoel I, Moukha S, Lesage-Meessen L, Sigoillot JC, and Asther M (2000) Selection of *Pycnoporus cinnabarinus* strains for laccase production. FEMS Microbiology Letters 183:301-306

Hoegger PJ, Navarro-González M, Kilaru S, Hoffmann M, Westbrook ED, and Kües U (2004) The laccase gene family in *Coprinopsis cinerea* (*Coprinus cinereus*). Current Genetics 45:9-18

Hong F, Meinander NQ, and Jonsson LJ (2002) Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. Biotechnology and Bioengineering 79:438-449

Hong YZ, Zhou HM, Tu XM, Li JF, and Xiao YZ (2007) Cloning of a laccase gene from a novel basidiomycete *Trametes* sp 420 and its heterologous expression in *Pichia pastoris*. Current Microbiology 54:260-265

Hou HM, Zhou JT, Wang J, Du CH, and Yan B (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochemistry 39:1415-1419

Ikehata K, Buchanan ID, and Smith DW (2004) Extracellular peroxidase production by *Coprinus* species from urea-treated soil. Canadian Journal of Microbiology 50:57-60

Kajita S, Sugawara S, Miyazaki Y, Nakamura M, Katayama Y, Shishido K and Iimura Y (2004) Overproduction of recombinant laccase using a homologous expression system in *Coriolus versicolor*. Applied Microbiology and Biotechnology 66:194-199

Kertesz-Chaloupková K, Walser PJ, Granado JD, Aebi M, and Kües U (1998) Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genetics and Biology 23:95-109

Kiiskinen LL, Kruus K, Bailey M, Ylosmaki E, Siika-aho M, and Saloheimo M (2004) Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology-SGM 150:3065-3074

Kilaru S (2006) Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology. Cuvillier Verlag, Göttingen, Germany

Kilaru S and Kües U (2005) Comparison of *gpd* genes and their protein products in basidiomycetes. Fungal Genetics Newsletters 52:18-24

Kilaru S, Hoegger PJ and Kües U (2006a) The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. Current Genetics 50:45-60

Kilaru S, Hoegger PJ, Majcherczyk A, Burns C, Shishido K, Bailey A, Foster GD and Kües U (2006b) Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Applied Microbiology and Biotechnology 71:200-210

Kjalke M, Andersen MB, Schneider P, Christensen B, Schulein M, and Welinder KG (1992) Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. Biochimica et Biophysica Acta 1120:248-256

Kollmann A, Boyer FD, Ducrot PH, Kerhoas L, Jolivalt C, Touton I, Einhorn J, and Mougin C (2005) Oligomeric compounds formed from 2,5-xylidine (2,5-dimethylaniline) are potent enhancers of laccase production in *Trametes versicolor* ATCC 32745. Applied Microbiology and Biotechnology 68:251-258

Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiology and Molecular Biology Reviews 64: 316-353

Kües U, Richardson WVJ, Tymon AM, Mutasa ES, Göttgens B, Gaubatz S, Gregoriades A, and Casselton LA (1992) The combination of dissimilar alleles of the *Ao* and *Aß* gene complexes, whose proteins contain homeodomain motifs, determines sexual development in the mushroom *Coprinus cinereus*. Genes & Development 6:568-577

Larrondo LF, Avila M, Salas L, Cullen D, and Vicuna R (2003) Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper-activated apoprotein and complex isoform patterns. Microbiology 149:1177-1182

Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, and Rogalski J (2001) Fungal laccase: properties and activity on lignin. Journal of Basic Microbiology 41:185-227

Lewis D (1961) Genetical analysis of methionine suppressors in *Coprinus*. Genetical Research 2:141-155

Lo SC, Ho YS, and Buswell JA (2001) Effect of phenolic monomers on the production of laccases by the edible mushroom *Pleurotus sajor-caju*, and partial characterization of a major laccase component. Mycologia 93:413-421

Lomascolo A, Cayol JL, Roche M, Guo L, Robert JL, Record E, Lesage-Meessen L, Ollivier B, Sigoillot JC, and Asther M (2002) Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. Mycological Research 106:1193-1203

Madelin MF (1956) Studies on the nutrition of *Coprinus lagopus* Fr., especially as affecting fruiting. Annals of Botany 20:307-330

Mansur M, Suarez T, and Gonzalez AE (1998) Differential gene expression in the laccase gene family from basidiomycete I-62 (CECT 20197). Applied and Environmental Microbiology 64:771-774

Maras M, van Die I, Contreras R, and van den Hondel CAMJ (1999) Filamentous fungi as production organisms for glycoproteins of bio-medical interest. Glycoconjugate Journal 16:99-107

Matsumura E, Yamamoto E, Numata A, Kawano T, Shin T, and Murao S (1986) Structures of the laccase-catalyzed oxidation products of hydroxy benzoic acids in the presence of ABTS (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)). Agricultural and Biological Chemistry 50:1355-1357

May G, Le Chevanton L and Pukkila PJ (1991) Molecular analysis of the *Coprinus cinereus* mating type A factor demonstrates an unexpectedly complex structure. Genetics 128:529–538

Meza JC, Auria R, Lomascolo A, Sigoillot JC, and Casalat L (2007) Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. Enzyme and Microbial Technology 41:162-168

Moore D (1969) Sources of carbon and energy used by *Coprinus lagopus* sensu Buller. Journal of General Microbiology 58:49-&

Munoz C, Guillen F, Martinez AT, and Martinez MJ (1997) Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. Applied and Environmental Microbiology 63:2166-2174

Mutasa ES, Tymon AM, Göttgens B, Mellon FM, Little PFR, and Casselton LA (1990) Molecular organization of an A-mating type factor of the basidiomycete fungus *Coprinus cinereus*. Current Genetics 18:223-229

Myasoedova NM, Chernykh AM, Psurtseva NV, Belova NV, and Golovleva LA (2008) New efficient producers of fungal laccases. Applied Biochemistry and Microbiology 44:73-77

Navarro-González M (2008) Growth, fruiting body development and laccase production of selected coprini. Georg-August University of Göttingen, Göttingen, Germany

Niemenmaa O, Uusi-Rauva A, and Hatakka A (2008) Demethoxylation of [(OCH_3)-C-14]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria* (*Postia*) *placenta*. Biodegradation 19:555-565

Nielsen KF and Madsen JO (2000) Determination of ergosterol on mouldy building materials using isotope dilution and gas chromatography-tandem mass spectrometry. Journal of Chromatography A 898:227-234

Nyanhongo GS, Gomes J, Gubitz G, Zvauya R, Read JS, and Steiner W (2002) Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresource Technology 84:259-263

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, and Sannia G (1997) A novel white laccase from *Pleurotus ostreatus*. Journal of Biological Chemistry 272:31301-31307

Palmieri G, Giardina P, Bianco C, Fontanella B, and Sannia G (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Applied and Environmental Microbiology 66:920-924

Pointing SB, Jones EBG, and Vrijmoed LLP (2000) Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia 92:139-144

Prasad KK, Mohan SV, Rao RS, Pati BR, and Sarma PN (2005) Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochemical Engineering Journal 24:17-26

Pukkila PJ (1996) Production and analysis of meiotic mutants in *Coprinus cinereus*. In: Bos CJ (Ed.) Fungal Genetics: Principles and Practice. Dekker, New York, 363-370

Radzio R and Kück U (1997) Synthesis of biotechnologically relevant heterologous proteins in filamentous fungi. Process Biochemistry 32:529-539

Ronne H (1995) Glucose repression in fungi. Trends in Genetics 11:12-17

Schmidt FR (2005) Optimization and scale up of industrial fermentation processes. Applied Microbiology and Biotechnology 68:425-435

Silva EM, Machuca A, and Milagres AMF (2005) Evaluating the growth and enzyme production from *Lentinula edodes* strains. Process Biochemistry 40:161-164

Snajdr J and Baldrian P (2007) Temperature affects the production, activity and stability of ligninolytic enzymes in *Pleurotus ostreatus* and *Trametes versicolor*. Folia Microbiologica 52:498-502

Stajic M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E, and Vukojevic J (2006) Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. Enzyme and Microbial Technology 38:65-73

Tavares APM, Coelho MAZ, Coutinho JAP, and Xavier AMRB (2005) Laccase improvement in submerged cultivation: induced production and kinetic modelling. Journal of Chemical Technology and Biotechnology 80:669-676

Tavares APM, Coelho MAZ, Agapito MSM, Coutinho JAP, and Xavier AMRB (2006) Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. Applied Biochemistry and Biotechnology 134:233-248

Teerapatsakul C, Abe N, Bucke C, Kongkathip N, Jareonkitmongkol S, and Chitradon L (2007) Novel laccases of *Ganoderma* sp KU-Alk4, regulated by different glucose concentration in alkaline media. World Journal of Microbiology & Biotechnology 23:1559-1567

Téllez-Téllez M, Sánchez C, Loera O, and Díaz-Godínez G (2005) Differential patterns of constitutive intracellular laccases of the vegetative phase of *Pleurotus* species. Biotechnology Letters 27:1391-1394

Tong PG, Hong YZ, Xiao YZ, Zhang M, Tu XM, and Cui TJ (2007) High production of laccase by a new basidiomycete, *Trametes* sp. Biotechnology Letters 29:295-301

Vasdev K, Dhawan S, Kapoor RK, and Kuhad RC (2005) Biochemical characterization and molecular evidence of a laccase from the bird's nest fungus *Cyathus bulleri*. Fungal Genetics and Biology 42:684-693

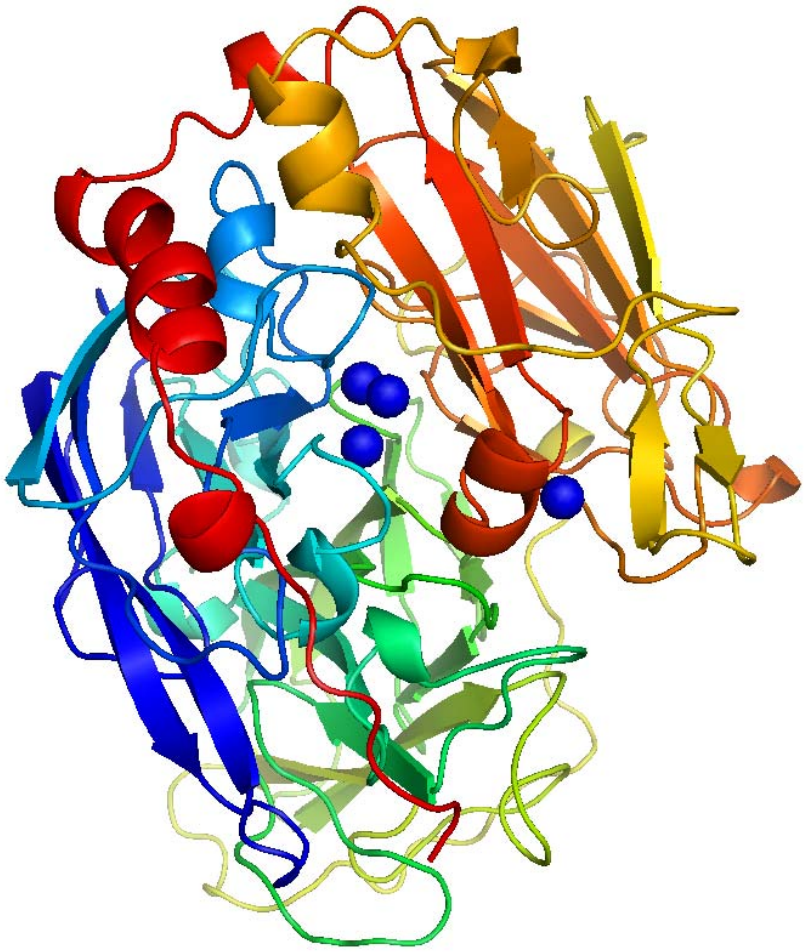
Xavier AMRB, Tavares APM, Ferreira R, and Amado F (2007) *Trametes versicolor* growth and laccase induction with by-products of pulp and paper industry. Electronic Journal of Biotechnology 10:444-451

Xiao YZ, Chen Q, Hang J, Shi YY, Xiao YZ, Wu J, Hong YZ, and Wang YP (2004) Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes* sp AH28-2. Mycologia 96:26-35

Yaver DS, Overjero MD, Xu F, Nelson BA, Brown KM, Halkier T, Bernauer S, Brown SH, and Kauppinen S (1999) Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase Lcc1. Applied and Environmental Microbiology 65:4943-4948

Chapter 5

Purification and characterisation of three new laccases from the basidiomycete *Coprinopsis cinerea*



I. Abstract

Laccases are oxidoreductases containing 4 copper atoms involved in the transport of electrons from a substrate to a oxygen molecule, which is reduced to water. The reaction mechanism for laccases is known and also structural features of several representatives of this class of enzymes have been elucidated. Laccases are widespread in nature and have distinct biochemical characteristics. In this study, three new laccases (Lcc5, Lcc6 and Lcc7) from *Coprinopsis cinerea* were recombinantly expressed in the same organism. The molecular and biochemical characteristics of the enzymes were studied and compared with the already characterised *C. cinerea* enzyme Lcc1. Inhibition experiments and stability tests with different laccase inhibitors and solvents were performed. The molecular weight for all laccases was within the range of 61-63 kDa and N-glycosylation amount accounts for 2-5% of the total weight. Different isoform bands of the purified laccases were separated by isoelectric focusing (IEF). The isoelectric points (pI) of the isoforms were in the range of 3.5 and 3.7 for Lcc5 and Lcc6, and from 4.1 to 4.2 for Lcc7. The optimal pH for the standard substrate ABTS varied from 3.0 to 5.0 for laccases Lcc7 and Lcc5, respectively, whereas optimal temperature of 50 °C was found to be the same for all tested laccases. Compared to the earlier characterised Lcc1, the laccases tested in this study showed a lower catalytical efficiency for the substrates ABTS, DMP, guaiacol but not for syringaldazine (SGZ), which was most effectively oxidised by Lcc7. Lcc1 described by Kilaru (2006) in a previous study, was the most stable laccase in contact with different laccase inhibitors compared to Lcc5, Lcc6 and Lcc7. Lcc1 remained more than 50% of its activity when incubated with 10 mM NaF. Nevertheless, Lcc5 was the most stable laccase when incubated in acetonitrile (ACN) and ethanol (EtOH), where more than 50% of its initial activity could be detected after incubation of 2 h in 90% ACN and 4 h in 90% EtOH, respectively.

Unlike for enzymes Lcc6 and Lcc7, stable crystals were obtained for Lcc5. The crystallographic structure of Lcc5 is presented in this work and the distance of the copper atoms towards their amino acid ligands is calculated.

II. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are blue multi-copper oxidases that bind four copper atoms at 3 copper binding sites, whereas the T1 and T2 sites contain one copper atom each and the T3 site two copper atoms (Messerschmidt et al. 1989). The oxidation of substrates, which are mono-, di- and polyphenols as well as aromatic amines (Hoegger et al. 2007), is fulfilled by the enzyme via a one electron reduction at the mononuclear T1 site. At the trinuclear T2/T3 cluster, which binds oxygen molecules, reoxidation occurs via a four electron reduction of dioxygen to water. Spectroscopic features of the laccases determined by UV/Vis or electron paramagnetic resonance (EPR) differ between the copper centres. The copper at the T1 site also known as 'blue copper' shows an absorption band in UV/Vis light at about 600 nm and an EPR spectrum. The T2 copper ('normal copper') shows no intense characteristic in the visible spectrum, but comprise a copper like EPR signal. In contrast, the T3 copper pair displays an absorption maximum at 330nm but no EPR signal (Thurston 1994, Solomon et al. 1996).

Usually, laccases have a molecular weight (MW) of approximately 60-85 kDa and are glycosylated to about 10-13% (Thurston 1994). Their isoelectric point (pI) is in the acidic pH range of 3.0 to 5.0 (Kilaru 2006), but also isolectric points of above 6.0 were reported such as for the laccase of *Trametes hirsuta* which has a pI of 7.4 (Shin and Lee 2000). For biochemical characterisation of the laccases four standard substrates are used, which are listed in reviews describing laccase properties: ABTS, 2,6-dimethoxyphenol (DMP), guaiacol and syringaldazine (SGZ) (Baldrian 2006, Kilaru 2006). The optimal pH for oxidation varies among the substrates with values of 2.0 to 5.0, 3.0 to 8.0, 3.0 to 7.0 and 3.5 to 7.0 with median values of 3.0, 4.0, 4.5 and 6.0 for the substrates ABTS, DMP, guaiacol and SGZ, respectively (reviewed by Baldrian 2006). Nevertheless, also different other substrates have been tested up to now showing the broad substrate variety of phenolic compounds and aromatic amines laccases can oxidise (Messerschmidt 1998, Baldrian 2006).

This broad substrate range makes laccase an enzyme to be used in various applications, such as bleaching of paper pulp, production of wood composites and decolourisation of textile dyes, as well as for bioremediation of toxic and recalcitrant compounds (Felby et al. 1997, Kharazipour et al. 1997, Majcherczyk et al. 1998, Johannes et al. 1998, Pointing 2001, Mai et al. 2004, Novotny et al. 2004, Fackler et al. 2008).

Previous work described that *Coprinopsis cinerea* possesses 17 different laccase genes that divide into two subfamilies (Kilaru et al. 2006). The reason for this high number of laccase genes is not known, but one explanation might the that different laccases with various properties are required for different functions.

Up to know, only the *C. cinerea* laccase Lcc1 was purified and studied regarding its biochemical and structural characteristics (Yaver et al. 1999, Schneider et al. 1999, Kilaru et al. in prep.). In this study, three further laccase genes of *C. cinerea* were recombinantly expressed in the *C. cinerea* strain FA2222 under the control of the *Agaricus bisporus gpdII* promoter as previously described (Kilaru et al. 2006). The laccases Lcc5, Lcc6 and Lcc7 were purified via Fast Protein Liquid Chromatography (FPLC) and characterised regarding optimal reaction conditions (pH, temperature), stability and enzymatic kinetics.

III. Material and Methods

A. Fungal culture

Laccase transformants overexpressing laccase genes *lcc5*, *lcc6* and *lcc7* from *C. cinerea* from a previous study (Kilaru 2006) were used for production of the corresponding protein. The clones with the highest laccase activities were grown on YMG-agar (yeast extract: 4 g/l; malt extract: 10 g/l; glucose: 4 g/l; agar: 10 g/l) plates at 37 °C until the mycelium reached the border of the Petri dish. Sterile dH_2O was poured onto the plate and the mycelium together with the asexual spores was scraped with a spatula from the agar. The solution was filtered using a sterile funnel filled with glass wool to hold back the fungal hyphae. A Thoma counting chamber was used to determine the spore concentration in the eluate. Pre-cultures were prepared in 500 ml flasks with 5 x 10^7 spores in 50 ml of modified Kjalke medium (after Kjalke et al. 1992, per litre: 10 g yeast extract, 20 g glucose, 0.5 g $CaCl_2$ x $2H_2O$, 2 g KH_2PO_4, 50 mg $MgSO_4$ x $7H_2O$). The flasks were incubated for 4 days at 37 °C as stationary (standing) cultures. Pre-cultures were homogenised by an Ultra-Turrax (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 sec at 8000 rpm and 30 sec at 9500 rpm. For the main-cultures, 500 ml flasks were filled with 100 ml of modified Kjalke medium supplemented with 0.1 mM $CuSO_4$ and inoculated with 5 ml of homogenised pre-culture. All cultures were incubated at 37 °C on a rotary shaker at 120 rpm until high laccase activity was detected in the supernatant.

B. Purification

At the day of highest laccase activities, the supernatants of the fungal cultures were collected by filtration with a suction filter through Whatman No. 1 paper (Whatman, Dassel, Germany). The pH of the eluate was adjused to 6.4 with either HCl or NaOH and the laccase containing supernatant was cleared from mycelial fragments by centrifugation at 10000 g

(Beckman J2-HS, Beckman Coulter, Krefeld, Germany) and 4 °C for 30 min. To eliminate small particles able of blocking the FPLC-columns, the supernatant was filtrated with a PALL filter cassette of 1 MDa (OMEGA™ membrane, Pall Cooporation, USA). All chromatographic steps were performed with a GE Healthcare (formerly Amersham Biosciences, Freiburg, Germany) ÄKTA FPLC chromatographic system at pH 6.4. Two anion exchanging DEAE-Sepharaose Fast Flow (diethylaminoethyl group) columns with diameters of 50 mm and 26 mm were used with a volume of 150 ml and 90 ml, respectively. A hydrophobic interaction chromatography (HIC) column step (Phenyl-Sepharose Fast Flow (high sub), Ø 26 mm, 80 ml) completed the purification.

Table 1 Chromatographic steps during laccase purification performed with the Äkta FPLC system (Amersham Bioscience)

Purification step	Chromatographic system	Loading buffer (LB)	Elution buffer (EB)	Gradient EB*
1st DEAE-Sepharose	Anion exchanger	20 mM KH_2PO_4	20 mM KH_2PO_4 + 1 M NaCl	0-100% EB in 5 column volumes
2nd DEAE-Sepharose				5-50% EB in 12 column volumes
Phenyl-Sepharose	Hydrophic interaction	20 mM KH_2PO_4 + 1 M $(NH_4)_2SO_4$	20 mM KH_2PO_4	0-100% EB in 5 column volumes

* Gradient EB is the percentage of the elution buffer (EB) used for the chromatographic steps.

After each chromatographic run, according to their laccase activity as determined with ABTS as a substrate (see Biochemical characterisation) fractions were pooled and desalted using an Amicon ultrafiltration cell (Millipore GmbH, Eschborn, Germany) with a 10 kDa YM filter. Before loading the HIC column, a pooled laccase containing solution was resalted with the appropriate loading buffer (LB: 20 mM KH_2PO_4 + 1 M $(NH_4)_2SO_4$, Table 1).

Amounts of N-glycosylation of proteins were determined by deglycosylation with a peptide-N^4-(N-acetyl-beta-glucosaminyl)asparagine amidase (PNGase F, New England BioLabs Inc., Frankfurt Main, Germany). 20 µg of protein was incubated for 10 min at 100 °C in 1x denaturation buffer (0.5% SDS, 1% ß-mercaptoethanol), then 1/10 volume of the 10x G7 buffer (0.5 M $NaPO_4$, pH 7.5), 1/10 volume of 10% NP-40 and 1500 U of PNGase F were added to the reaction mixture. After incubation at 37 °C for 60 min the deglycosylated protein samples were frozen at -20 °C and stored until further usage.

Purified laccase Lcc1 used for comparison with the laccases processed in this work was obtained from a previous study (Kilaru 2006).

C. Molecular characterisation

1. Electrophoresis

For separation of proteins, polyacrylamide gel-electrophoresis (PAGE) was performed with 4% stacking and 12% resolving gels: **4%**: 2.5 ml of 0.5 M Tris pH 6.8, 1.3 ml acrylamide (37%), 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS (ammonium peroxodisulfate, Sigma-Aldrich, Steinheim, Germany), 10 µl TEMED (N,N,N',N'-tetramethylethylenediamine, Amersham Bioscience AB, Uppsala, Sweden) and filled up to 10 ml with dH_2O. **12%**: 2.5 ml of 1.5 M Tris pH 8.8, 4.0 ml acrylamide (37%), 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS, 5 µl TEMED and filled up to 10ml with dH_2O.

The loading buffer contained 0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.025% bromphenol blue (w/v). Proteins were reduced by 10 mM DTT and denatured by boiling for 5 min. To determine the size of the denatured proteins, a molecular weight marker (#SM0431, Fermentas GmbH, St. Leon-Rot, Germany) was used. Separation was performed at a constant current of 15 mA until the samples reached the resolving gel and continued at 25 mA for separation in the resolving gel. In native electrophoresis, the proteins were neither reduced nor denatured.

2. Isoelectric focusing (IEF)

IEF was performed using non-denatured PAGE with a precast Clean Gel (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) rehydrated in carrier ampholytes (Pharmalyte: pH 2.5 – 5.0 and Ampholine: pH 4.0 – 6.5). Supernatant of fungal cultures and the purified laccase samples (40 mU for native staining and 5 µg for Coomassie staining) were loaded onto filter paper pads, placed on the rehydrated gel and the run was performed on a Multiphor II system (Amersham-Pharmacia, Munich, Germany). A low-pI marker kit (pH 2.8-6.5; Amersham-Pharmacia, Munich, Germany) was used to determine the pI values. Native staining was performed as described below with 5 mM MBTH (3-methyl-2-benzothiazolinon-hydrazone hydrochloride) and 5 mM DHPPA (3,4-dihydroxyhydrocinnamic acid).

3. Staining

For Coomassie staining, the gel was fixed in 12% TCA (trichloroacetic acid) (w/v) for at least 1 h and stained in a colloidal Coomassie staining solution (10% phosphoric acid (v/v), 10% ammonium sulfate (w/v) and 0.12% Coomassie Brilliant Blue G250 (Serva electrophoresis

GmbH, Heidelberg, Germany) (w/v) in water/methanol (80/20, v/v) solution) overnight. The gel was washed with water until the background level was reduced for an adequate band to background ratio. Silver-staining was performed according to (Blum et al. 1987).

For native laccase staining, gels were washed in 100 ml of 100 mM sodium acetate buffer (pH 5.0) for at least 10 min and then incubated with 5 mM MBTH (3-methyl-2-benzothiazolinon-hydrazone hydrochloride) and 5 mM DHPPA (3,4-dihydroxyhydrocinnamic acid) in the same buffer.

To analyse the amount of the laccases in the supernatant, fluorescence staining was performed after the protocol of Lamanda et al. (Lamanda et al. 2004). Gels were fixed in 10% acetic acid (v/v), 30% EtOH (v/v) overnight and stained afterwards with 1 µM RuBP [ruthenium II tris (bathophenantroline disulfonate)] (Rabilloud et al. 2001). The gel was washed twice with dH_2O and scanned using a fluorescence reader FLA-5100 (Fujifilm, Düsseldorf, Germany) with 50 µm resolution. 16 bit reader files were converted to TIF-format using AIDA image analyser (v4.10.020, Raytest, Straubenhardt, Germany). The calibration curve for the determination of the protein amount was obtained by applying BSA standards (1.2 µg, 1.0 µg, 0.7 µg, 0.4 µg and 0.2 µg of protein per lane) separated parallel to the samples on the same gel. An AIDA image analyzer was used for gel processing and calculating total protein. The following parameters were used: baseline determination at search level 1, smooth parameter 10 pixel and search level 0 for peak determination (not smooth). Integration parameters for BSA bands were set manually to prevent any distortion of the protein measurements by BSA contaminations. The resulting calibration curve (R^2 = 0.9675 for Lcc5 and R^2 = 0.9881 for Lcc6 and Lcc7) was used for calculation of laccase amounts in the purified laccase solutions. The corresponding laccase bands in the supernatant samples were used for determination of the secretion amount [mg/l] of laccases in the fungal samples.
Silver-staining was performed according to (Blum et al. 1987).

4. Protein identification

Protein bands of the Coomassie-stained gel were cut with a razor blade and gel pieces were digested as described by Havlis and Shevchenko (2004) with some modifications. Briefly, gel pieces were washed twice with water for 15 min and subsequently once with 50 % ethanol for destaining. Gel pieces were dehydrated with 100 % acetonitril for 20 min and dried in a

vacuum centrifuge (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) for 10 min. Subsequently the gel pieces were soaked in 2 µM trypsin in 50 mM ammoniumbicarbonate buffer (pH 8) at 4 °C for 1 h and digested for 30 min at 58 °C. Peptides were extracted from the gel pieces on a shaker first with 150 µl 5% (v/v) formic acid for one hour, followed by two extractions with 150 µl 5 % formic acid, 50% acetonitril. Extracts were combined, dried in the vacuum centrifuge to total dryness and stored at -20 °C until further proceeding. For further processing, peptides were dissolved in 15 µl of 5 % formic acid and 3 times 4 µl were injected to a 12 cm capillary column packed with 3 µm paticles of Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) for peptide separation. Analysis of the peptides was performed by ESI-LC-MS (HP 1100 Agilent; Esquire 3000, Bruker Daltonik). Each sample was analysed in a mass range of the ion trap from 200 to 1500 m/z. Analysis of the raw data was done with Daltonic DataAnalysis version 3.0 (Bruker Daltonic GmbH; Bruker Daltonic esquire 5.0). Proteins were identified by searching the data against a database of the annotated genome of *C. cinerea* using local Mascot software (www.matrixscience.com). The following settings were used for the database search: fixed modification: carbamidomethylation; variable modification: oxidation; peptide mass tolerance: 1.4 Da; peptide charge: 1+,2+ and 3+; MS/MS tolerance: 0.4 Da; missed cleavages allowed: 1. For final identification and predicted function protein BLAST against NCBI database, PFAM (http://pfam.sanger.ac.uk/), and Interpro (http://www.ebi.ac.uk/interpro/) were used.

D. Biochemical characterisation

Laccase activity was determined in 100 mM sodium acetate buffer (pH 5.0) at room temperature with the following substrates: ABTS (2,2‘-azino-bis-[3-ethylbenzthiazoline-6-sulfonate]), DMP (2,6-dimethoxyphenol), guaiacol (2-methoxyphenol) and syringaldazine (SGZ, 4-hydroxy-3,5-dimethoxybenzaldehyde azine). The conversion of the substrate was monitored spectroscopically at 420 nm (ε = 36000 M^{-1} cm^{-1}), 468 nm (ε = 49600 M^{-1} cm^{-1}), 436 nm (ε = 6400 M^{-1} cm^{-1}) and 526 nm (ε = 65000 M^{-1} cm^{-1}) for ABTS, DMP, guaiacol and syringaldazine, respectively (Matsumura et al. 1986, Wariishi et al. 1992, Slomczynski et al. 1995, Eggert et al. 1996). One unit of enzyme activity was defined as the amount of enzyme oxidising 1 µmol substrate per min.

The spectrum of the purified and completely oxidised laccases was determined with a Beckman DU800 spectrophotometer (Beckman Coulter GmbH, Krefeld, Germany).

1. Optimal reaction conditions

The optimal pH of the laccases was determined for the above listed substrates in the following buffers: universal buffer pH 2.0, 3.0 – 12.0; McIlvaine buffer pH 2.5, 3.0 – 7.0; acetate buffer pH 3.6, 4.1 – 5.6; phosphate buffer pH 5.8, 6.4, 7.0, 7.6 and 8.0; borat buffer pH 7.8, 8.3 – 9.8 (Rauen 1964). For pH stability, approximately 150 mU laccases were incubated in 1 ml universal buffer (2.0 – 12.0) up to 24 h, samples were taken in defined time intervals and activity was measured with ABTS in 100 mM sodium acetate buffer (pH 5.0). For the optimal temperature of the laccases, activity was determined at 10 °C, 20 °C – 80 °C in 100 mM sodium acetate buffer (pH 4.6) with ABTS as a substrate. The temperature was measured inside a reference cuvette to guarantee a correct determination of the temperature. The thermal stability was analysed by incubating the laccases in 100 mM sodium acetate buffer (pH 4.6) at 10 °C, 30 °C, 50 °C and 70 °C over a period of 3 h. Samples were taken at different time intervals and the laccase activity was determined in 100 mM sodium acetate buffer (pH 5.0) with ABTS as a substrate at room temperature.

2. Enzyme kinetics

Michaelis-Menten constant Km, Vmax and catalytic efficiency (kcat/Km) were defined with laccases Lcc5, Lcc6, Lcc7 and Lcc1 for all the above mentioned substrates. The substrates were applied in concentrations around the estimated Km value and measurement points of at least eight different substrate concentrations were chosen for the determination of the Km and Vmax value according to the linear regression after Lineweaver-Burk (SOFTmax® PRO, Molecular Devices Corporation, Sunnyvale, CA).

3. Stability of laccases against inhibitors and in organic solvents

Inhibitors for laccase activity were dissolved in sodium acetate buffer (pH 5.0, 100 mM) to different concentrations. The purified laccases were incubated for 2 min in the inhibitor solution and activity was measured with ABTS as described above. To test the stability of the laccases against organic solvents, 50 µl of laccases were mixed with 450 µl of acetonitrile (ACN) and ethanol (EtOH) to final concentrations of 9%, 18%, 45% and 90% v/v. The mixture was incubated and measured with ABTS as described above over a defined time period (1 h, 2 h, 4 h and 24 h). Laccase mixed with water served as a control.

4. Dye decolourisation

Different industrial dyes were used to test the ability of the purified laccase for decolourisation. Seven different dyes were kindly provided by the department for Bioprocess Engineering (Prof. Bley) at the Technical University of Dresden: Levafix Blue E-RA gran., Levafix Blue PN-3R, Procion Blue H-EGN 125, Remzol Black B gran. 133%, Remazol Golden Yellow 3R liq 25, Remazol Brilliant Red 5B 150%, Remazol Turquoise Blue G133. Solution of the dyes were prepared according to Böhmer et al. (2006): 250 mg of the dye was dissolved in 50 ml water (5 g/l) followed by addition of 10 g of Na_2SO_4. Thereafter, the pH was adjusted to 12 with 1 M NaOH, the stock solution was heated for 1 h at 80 °C and chilled to room temperature before usage. For decolourisation experiments in water, the dyes were used in a final concentration of approximately 50 mg/l, except for Remazol Golden Yellow which was used in a concentration of 500 mg/l. Laccases were used with a final activity of approximately 2.0 U/mg dye, except for Remazol Golden Yellow where laccases were used in a concentration of 0.2 U/mg dye. In addition to the purified laccases of *C. cinerea*, *Trametes versicolor* laccase (Tv_lac) from Novozymes A/S (Bagsvaerd, Denmark) was used in this experiment. For determination of the decolourisation rate, a spectrum of the dye-laccase solution was monitored with a spectrophotometer (SpectraMax 340PC^{384}, Molecular Devices, Sunnyvale, CA). The decolourisation rate was calculated after the reduction of the absorbance at the dye specific wavelength: λ = 614 nm for Levafix Blue E-RA gran., λ = 586 for Levafix Blue PN-3R, λ = 624 nm for Procion Blue H-EGN 125 and λ = 620 nm for Remazol Turqoise Blue G133.

5. Redox potential determination

The redox potential of the Cu(I) of the purified laccase Lcc5 (34 µM) was determined in 20 mM KH_2PO_4 buffer pH 6.4 at room temperature under anaerobic conditions (N_2) with a Mediator Mix of a total concentration of 1 µM (methyl viologen, neutral red, safranine, anthraquinone-2-sulfonic acid, 2-hydroxy-1,4-naphthoquinone, phenazine, indigo carmine, resorufin, duroquinone, methylene blue, juglone, 1,4-naphthoquinone, N-methylphenazonium methyl sulfate, 1,2-naphthoquinone). Stepwise oxidation and reduction were performed by anaerobic titration with 10 mM hexacyanoferrat(III) and 5 mM ascorbate, respectively. The absorbance at 610 nm was recorded by a S2000 fibre optic spectrometer (Ocean Optics) with a deuterium-tungsten halogen light source (Mikropack) and the potential of the reaction solution was measured using an EMC 50 Ag/AgCl electrode (Sensortechnik,

Meinsberg, Germany). All recorded potentials were corrected for the normal hydrogen electrode (NHE).

IV. Results

A. Purification

The supernatants of the laccase transformants Lcc6, Lcc7 and Lcc5 cultivated in modified Kjalke medium supplemented with 0.1 mM $CuSO_4$ showed laccase activities of 5.1 U/ml, 2.0 U/ml, and 25.0 U/ml when harvested for purification, respectively. Three chromatographic steps were needed for purification of the laccases. An anion exchanger (DEAE Sepharose) column (Ø 50 mm, 150 ml) was used for a preliminary crude purification step (Fig. 1A) followed by a second anion exchanger (DEAE Sepharose) column (Ø 26 mm, 90 ml) (Fig. 1B). These two chromatographic steps resulted in an increase of the specific activity of two times for Lcc5 and more than 3 times for Lcc6 and Lcc7, respectively (Table 2). Finally, a hydrophobic interaction chromatographic (HIC) step, which was performed on a Phenyl Sepharose column (Fig. 1C), yielded a blue coloured laccase solution with specific laccase activities of 795 U/mg, 247 U/mg and 91 U/mg for Lcc5, Lcc6 and Lcc7, respectively. Subsequent purification steps with a high resolution anion exchanger (MonoQ) and size exclusion chromatography (gel filtration) columns could not further improve the purity of the laccase solutions.

Besides the specific activity of the different purification steps, Table 2 shows also the yield defined by the residual laccase activity compared to the activity in the supernatant and it shows the final purification factor, which varied between 3 and 4. The yield of 62%, 43% and 55% for laccases Lcc5, Lcc6 and Lcc7 showed that around half the laccase activity got lost during the purification steps.

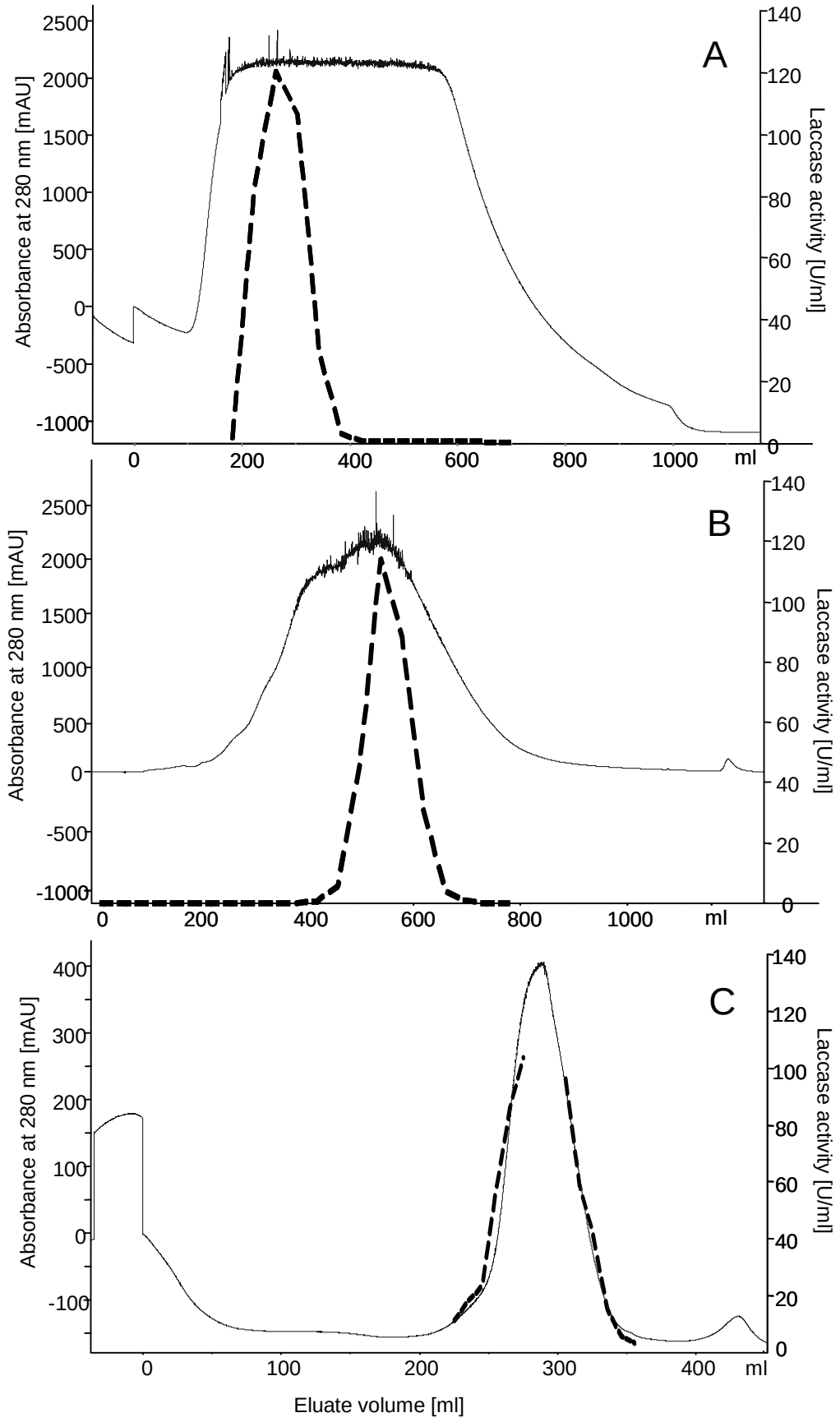


Fig. 1 Chromatographic steps of purification of laccase Lcc6 as an example for the progress of enzyme purification. (**A**) 1st DEAE-Sepharose, (**B**) 2nd DEAE-Sepharose and (**C**) Phenyl Sepharose (HIC) columns. The laccase activity detected in chromatographic fractions (dashed line) is overlaid over the chromatographic results: UV-absorbance (solid grey line).

Table 2 Process of purification of laccases Lcc5, Lcc6 and Lcc7 from 2 l, 3 l and 2 l culture supernatant, respectively

Purification step	Total laccase activity [U]	Total protein amount [mg]	Specific activity [U/mg]	Yield [%]	Purification factor
Lcc5					
Supernatant	56560	211	268	100	1.00
Ultrafiltration	54096	236	229	96	0.86
1st DEAE-Sepharose	47558	158	301	84	1.12
2nd DEAE-Sepharose	27270*	60	452	48	1.69
HIC	34973	44	795	62	2.97
Lcc6					
Supernatant	19479	261	75	100	1.00
Ultrafiltration	18248	246	74	94	0.99
1st DEAE-Sepharose	18312	95	192	94	2.57
2nd DEAE-Sepharose	17255	76	228	89	3.06
HIC	8278	34	247	43	3.30
Lcc7					
Supernatant	4459	198	23	100	1.00
Ultrafiltration	4246	196	22	95	0.96
1st DEAE-Sepharose	3455	81	42	77	1.89
2nd DEAE-Sepharose	2973	44	68	67	3.01
HIC	2434	27	91	55	4.05

* This value is likely due to a pipetting error in sample taking during the process of enzyme purification. Remeasuring the laccase activity of the frozen sample confirmed the result of total laccase activity.

For comparison of the different laccases and the quality of the purification steps, SDS-PAGE with native and with denatured samples was performed. The results of the purification steps are documented for Lcc7 as an example in the reduced SDS-PAGE gel in Fig. 2A. The main band in the supernatant (Fig. 2A lane 1) is the recombinantly produced laccase Lcc7 having a size of ~63 kDa. Purified Lcc5 and Lcc6 had a single band (Fig. 2A lane 7 and 8) indicating a little smaller size of ~62 kDa (see below). The results of the corresponding native SDS-PAGE is shown in Fig. 2B where the three laccases showed different running patterns with one slower migrating band for Lcc7 and one faster migrating band for Lcc7 and two intermediate migrating bands for Lcc6 (Fig. 2B lanes 6 to 8).

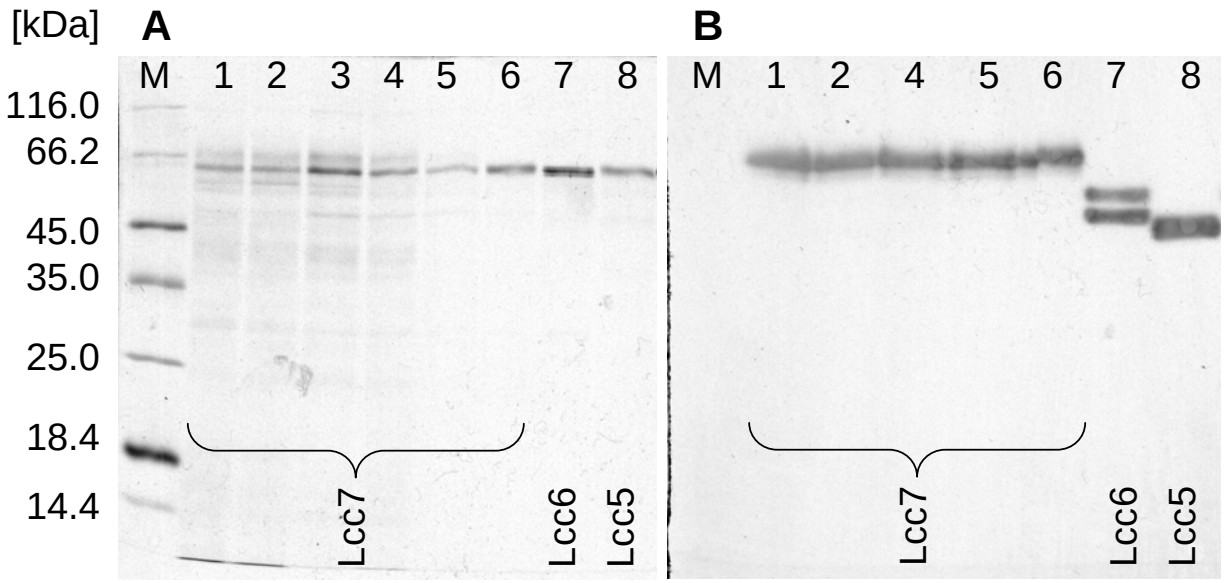


Fig 2 SDS-PAGE and native PAGE of Lcc7 purification steps and of purified Lcc5, 6 and 7 stained with silver (**A**) and native PAGE with MBTH+DHPPA (**B**), respectively. Lanes M: Marker, lanes 1: supernatant, lanes 2: laccase solution after ultrafiltration, lanes 3+4: after 1st DEAE-sepharaose, lanes 5: after 2nd DEAE-sepharose and lanes 6: after HIC. Lanes 7: purified Lcc6. Lanes 8: purified Lcc5. SDS-PAGE lanes 1-4: 1 µg of protein were loaded, lanes 5-8: 0.5 µg of protein were loaded. Native PAGE: 20 mU of laccase activity per lane.

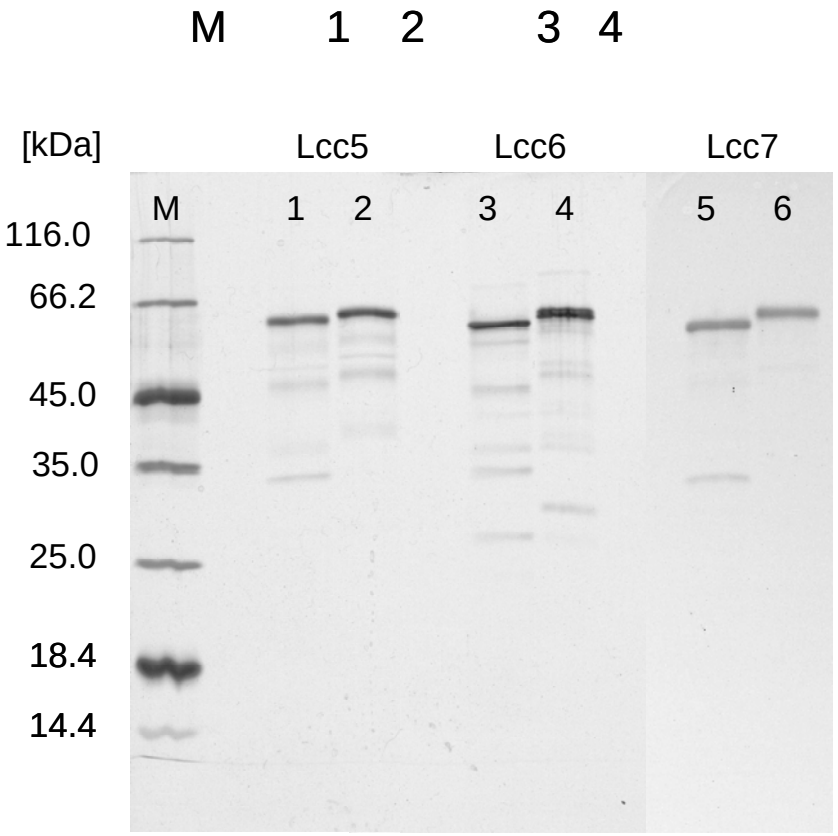
The amount of recombinant laccase secreted into the medium was determined by fluorescence staining with RuBP of a SDS-PAGE gel (not shown). The calculated values are given in Table 3 and vary between the different laccases from 115 mg/l for Lcc5 and around 28 to 27 mg/l for Lcc6 and Lcc7, respectively. The percentage of laccase in the supernatant compared to the total protein amount was also calculated from the fluorescence stained SDS-PAGE giving a very high value of 86% for Lcc5 and somewhat lower fractions of 63% and 62% for Lcc6 and Lcc7, respectively. In the purified laccase samples, faint bands of protein below the main laccase bands occurred (Fig. 3), which accounts for 4%, 5% and 3% of total protein in the purified laccase samples of Lcc5, Lcc6 and Lcc7, respectively. LC/MS-MS analysis of the faint band in the Lcc7 sample suggests that they are at least partially due to degradation products (data not shown).

Table 3 Amount of secreted laccase in liquid cultures of the different laccase transformants

Amount of enzymes	**Lcc5**	**Lcc6**	**Lcc7**
Laccase secreted [mg/l]	115	28	27
% of the laccase band compared to all proteins in the supernatant	86	63	62
% of the main laccase band in the purified solution	96	95	97

B. Molecular characteristics

The MW of the purified laccases and of PNGaseF-treated laccase was compared by SDS-PAGE (Fig. 3) and calculated according to MW values of standard proteins. The MWs of the purified laccases were determined to be 62 kDa, 61-62 kDa and 63 kDa for Lcc5, Lcc6 and Lcc7. respectively. Upon PNGaseF treatment, the molecular weight decreased to 61 kDa, 59 kDa and 60 kDa for Lcc5, Lcc6 and Lcc7, respectively. Accordingly, the N-glycosylation amount of the total molecular weight accounts for 2% for Lcc5, 4% for Lcc6 and 5% for Lcc7.



Enzyme	Native [kDa]	PNGaseF -treated [kDa]
Lcc5	62	61
Lcc6	61-62	59
Lcc7	63	60

Fig. 3 SDS-PAGE of purified laccase Lcc5 (lanes 1 and 2), Lcc6 (lanes 3 and 4) and Lcc7 (lanes 5 and 6) with (lanes 1, 3 and 5) and without (lanes 2, 4 and 6) treatment of PNGaseF. The gel was stained with silver.

Isoelectric focusing (IEF) of the purified laccases showed one major laccase band for all laccases when stained with the laccase substrates MBTH and DHPPA, whereas with Coomassie staining at least two bands became visible for all three of the laccases. Lcc7 showed two bands of similar intensity with pI values of 4.1 and 4.2, whereas the major band for Lcc6 and Lcc5 had pI values of 3.7 and 3.5, respectively, and their minor bands both pI values of 3.6 (Fig. 4).

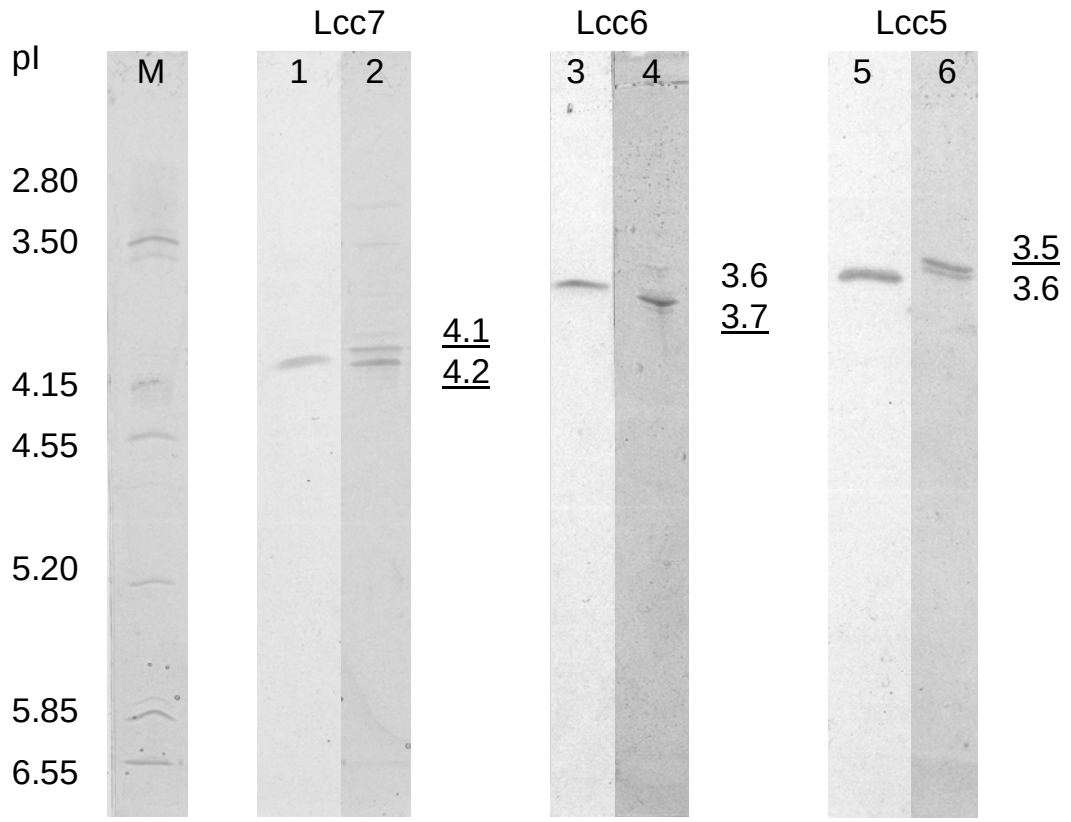


Fig. 4 Isoelectric focusing (IEF) of *C. cinerea* purified laccases Lcc7 (1 and 2), Lcc6 (3 and 4) and Lcc5 (5 and 6). 5 µg proteins were loaded for lanes stained with Coomassie (M, 2, 4, 6) and 40 mU for the lanes stained with the laccase substrate MBTH and DHPPA (1, 3, 5). The pI marker (lane M) was used to calculate unknown pI values of the purified laccases. The pI values of the main bands are underlined. pI values derived from two different IEF experiments and the error is ± 0.1 pI.

The UV/Vis spectrum of the three laccases Lcc5, Lcc6 and Lcc7 (Fig. 5) shows the typical absorbance of the oxidised Cu(I) centre at 610 nm. As in studies with other laccases (Garzillo et al. 2001), the absorption of the T3 Cu(II) at 330 nm was badly resolved. The resolution of the UV/Vis band of the T3 Cu(II) at 330 nm is not visible in the spectra of Lcc5 and Lcc7 in Fig. 5; only in Lcc6 a small shoulder can be seen at about 330 nm.

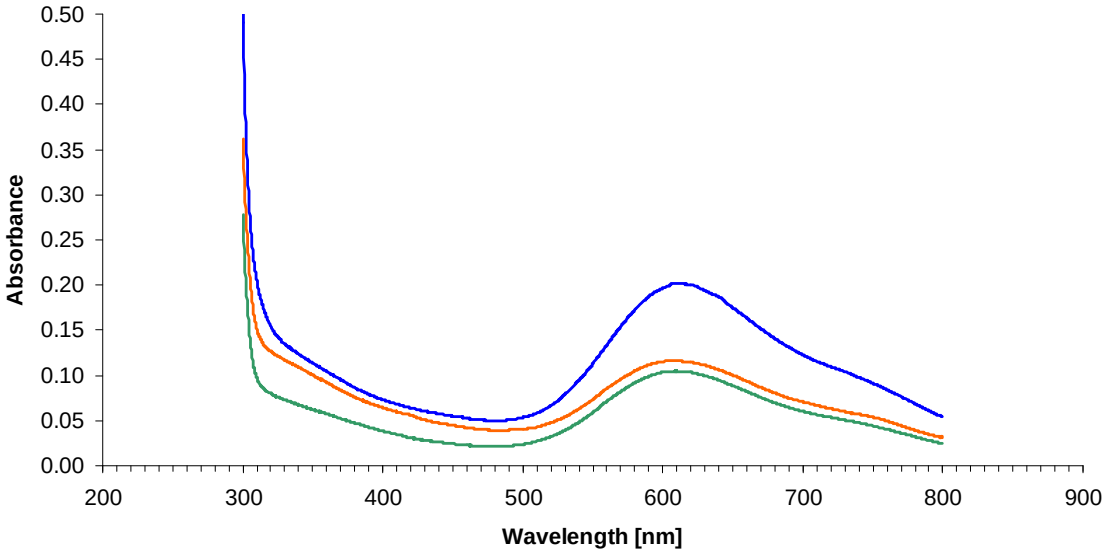


Fig. 5 UV-VIS absorption spectrum of the purified laccases Lcc5 (blue), Lcc6 (red) and Lcc7 (green).

C. Biochemical characterisation using standard laccase substrates

The optimal pH for enzymatic activity of the purified laccases was tested with the universal buffer within a pH range of 2.0 to 12.0 at room temperature using the four most studied laccase substrates: ABTS, DMP, guaiacol and SGZ (syringaldazine). For ABTS, the optimal pH for laccase activity was 5.0, 4.0 and 3.0 for the laccases Lcc5, Lcc6 and Lcc7, respectively. For DMP, guaiacol and SGZ, the optimal pHs were found to vary between 6.0 and 7.0 (Table 4).

The pH stability of the laccases incubated in universal buffer (pH 2.0 to 12.0) was shown to be better in the more alkaline range. More than 50 % of the initial activity was retained in pH ranges of 7.0 to 11.0, 8.0 to 11.0 and 8.0 to 9.0 for laccases Lcc5, Lcc6 and Lcc7, respectively. The optimal temperature revealed to be 50 °C for all tested laccases. The thermal stability (half life) at this temperature varied among the tested laccases from 20 min, 30 min to over 60 min for laccases Lcc7, Lcc5 and Lcc6, respectively.

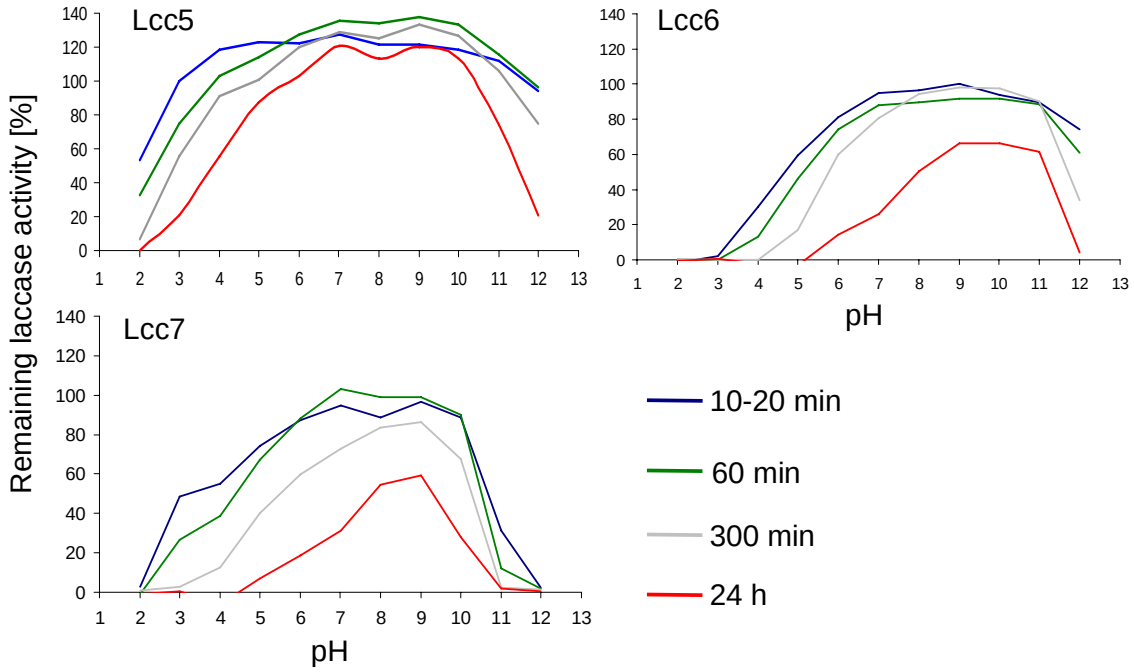


Fig. 6 pH stability of Lcc5, Lcc6 and Lcc7 incubated in universal buffer over a period of 24 h. At different time points the activity was measured in 100 mM sodium acetate buffer at pH 5.0: 10-20 min (blue line), 60 min (grenn line), 300 min (grey line) and 24 h (red line). The remaining activity is calculated against the respective laccase activity at the beginning of incubation.

Table 4 Properties of laccases Lcc5, Lcc6 and Lcc7

Enzyme properties	substrate	Lcc1*	Lcc5	Lcc6	Lcc7
Molecular weight [kDa]		63	62	61-62	63
Amount of N-glycosylation		3%	2%	4%	5%
Optimal pH	ABTS	4.0	5.0	4.0	3.0
	DMP	7.0	6.0	6.0	7.0
	Guaiacol	7.0	7.0	7.0	7.0
	SGZ	7.0	7.0	7.0	7.0
Thermal stability at 50°C	ABTS	60 min	30 min	60 min	20 min
Optimal T	ABTS	50 °C	50 °C	50 °C	50 °C
Optimal pH range for stability	ABTS	pH 5-11	pH 5-11	pH 8-11	pH 8-9
pI value (main band(s) in IEF)		3.8	3.5	3.7	4.1/4.2

Optimal pH and pH stability were determined in universal buffer; pH stability gives the range of pH at which at least half of the activity remains after 24 h and thermal stability gives the time at which at least half of the activity remains at 50 °C. * For comparison, the data for purified Lcc1 as determined by Kilaru (2006) were added.

Michaelis-Menten kinetics were determined for four substrates (ABTS, DMP, guaiacol and SGZ) and the four enzymes (Lcc1, Lcc5, Lcc6 and Lcc7) with substrate concentrations above and below the estimated K_m values (Table 5). At least eight different points of measurement were used for determining the K_m and V_{max} value according to Lineweaver Burk with linear plots whose coefficients of determination (R^2) were between 0.961 and 0.999 for the obtained data (Table 5). Lcc1 had the highest affinity towards the substrates - low Michaelis-Menten constants (K_m) - with values of 656 µM, 199 µM, 855 µM and 289 µM for ABTS, DMP, guaiacol and SGZ, respectively (Table 5). Lcc6 and Lcc7 with high K_m values were more similar to each other than to Lcc1 and Lcc5. Lcc5 had intermediate K_m values, except for the substance guaiacol which showed a very low affinity to Lcc5. Generally, SGZ showed low Km values, compared to the other three substrates, for all tested laccases. Highest V_{max} values were also obtained with SGZ for all tested laccases, except for Lcc6 which showed the highest V_{max} value with ABTS. The catalytical efficiency (k_{cat}/K_m), which is an indicator for the performance of a enzymatic reaction, varied between the subtrates for each laccases. At all, Lcc1 showed the highest catalytical efficiency towards the tested substrates with values of 126 mM/min, 310 mM/min and 14 mM/min for ABTS, DMP and guaiacol, respectively; except for SGZ, where Lcc7 had the highest value with 1413 mM/min (Table 5). Overall, guaiacol showed the lowest catalytical efficiencies with values of around 2 to 14 mM/min and SGZ the highest values ranging from 125 mM to 1413 mM/min.

Table 5 Michaelis-Menten parameters of the purified *C. cinerea* laccases for different substrates

Enzyme	Value determined	ABTS	DMP	Guaiacol	SGZ
Lcc1	K_m [µM]	656	199	855	289
	V_{max} [mU min^{-1}]	58	31	13	156
	R^2	0.981	0.996	0.996	0.999
	k_{cat}/K_m [min^{-1} mM^{-1}]	126	310	14	929
Lcc5	K_m [µM]	831	287	9331	387
	V_{max} [mU min^{-1}]	64	54	16	261
	R^2	0.996	0.999	0.960	0.997
	k_{cat}/K_m [min^{-1} mM^{-1}]	104	111	2	403
Lcc6	K_m [µM]	2683	1522	5644	496
	V_{max} [mU min^{-1}]	395	67	47	218
	R^2	0.976	0.998	0.997	0.996
	k_{cat}/K_m [min^{-1} mM^{-1}]	28	47	2	125
Lcc7	K_m [µM]	3999	1058	4107	399
	V_{max} [mU min^{-1}]	77	178	33	474
	R^2	0.999	0.998	0.994	0.995
	k_{cat}/K_m [min^{-1} mM^{-1}]	15	100	3	1413

K_m and V_{max} values were obtained with Lineweaver-Burk plots and coefficients of determination (R^2) for these two values are given. k_{cat} [min^{-1}] was calculated by dividing V_{max} with the enzyme concentration used in the reaction mixture (1-5 mU) and divided by the Michaelis-Menten constant [K_m] to obtain the catalytical efficiency [k_{cat}/K_m].

Another value to describe the quality of a laccase reaction is its redox potential, which is generally measured for the T1 copper atom in laccases as it is involved in the oxidation of the substrate (Xu 1996, Riva 2006). The oxidation state of the copper atom (CuI/CuII) was determined via the absorbance of the CuII at 610 nm and the oxidation state changed via redox titration with ascorbate (5mM) and hexacyannoferrat (10mM) at a pH of 6.4 (Fig. 7). The reduction and oxidation curves of the redox titration of Lcc5 revealed differences between both steps performed with a transition point for the oxidation curve being around 50 mV lower than for the reduction curves (Fig. 7B). Actually, oxidation and reduction curves should overlap each other, as shown for the determination of the redox potential for the T1 copper of the plant *Rhus vernicifera* laccase (Reinhammar 1972). This was not achieved in this study, although we repeated the measurements. Nevertheless, the graphical determination using either the Nernst plot (Fig. 7A) as well as oxidation and reduction curves (Fig. 7B) revealed an average redox potential of 542 mV with a standard deviation of 20 mV, which is in the range of standard deviations achieved for redox potentials of laccases (Xu et al. 1998, Durão et al. 2008).

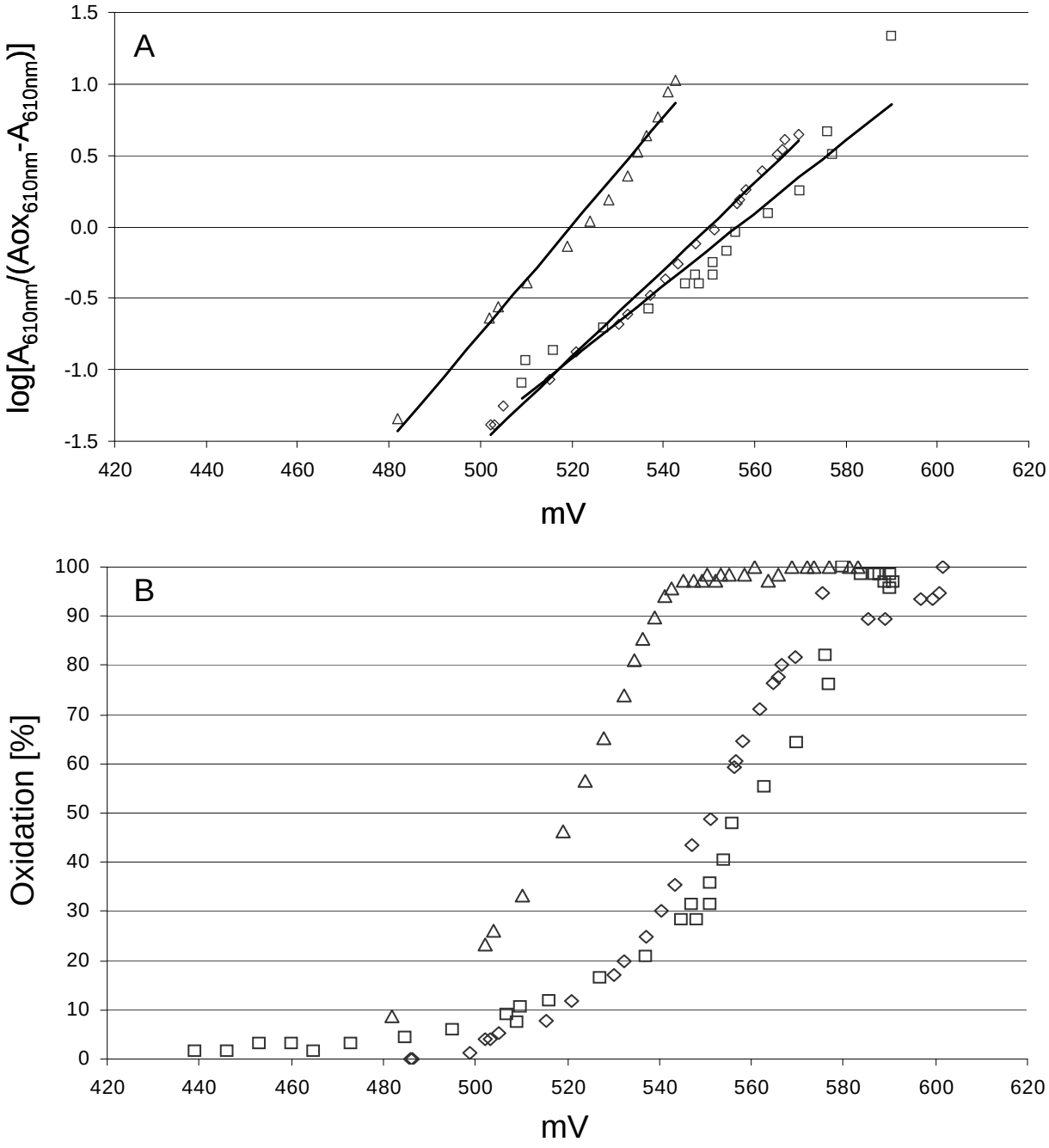


Fig. 7 Reduction (open squares and open diamonds) and oxidation (open triangles) titration of Lcc5. (**A**) Nernst plot of the $\log[A_{610nm}/(Aox_{610nm}-A_{610nm})]$ against the redox potential after Reinhammar (1972), whereas Aox_{610nm} stands for the absorption of the complete oxidised type 1 copper at 610 nm. (**B**) Oxidation of the T1 site (absorption 610 nm) plotted against the redox potential measured with the redox electrode. The values derived from the graphical determination are 551 mV and 556 mV for the reduction and 519 mV for the oxidation curve of Lcc5, respectively.

D. Stability against inhibitors and organic solvents

Inhibition of laccases by various chemicals dissolved in sodium acetate buffer (pH 5.0, 100 mM) revealed differences between the enzymes. The chelator EDTA up to a concentration of 40 mM did not show high inhibition effects in contrast to the compounds binding directly to the copper atoms in the laccases NaN_3 (0.1 mM) and NaF (10 mM), which both reduced the laccase activity of Lcc5, Lcc6 and Lcc7 to more than 50% and 80%, respectively (Table 6). For Lcc1, inhibition by 0.1 mM NaN_3 and 10 mM NaF was lower with values of 27 % and 47% as compared to the other enzymes (Table 6).

Table 6 Inhibition of laccase activity by different chemicals: EDTA, sodium azide, sodium fluorid.

Compound	Concentration [mM]	Inhibition [%]			
		Lcc1	Lcc5	Lcc6	Lcc7
EDTA	0.1	2	5	4	4
	1.0	2	7	4	6
	4.0	12	9	11	7
NaN_3	0.001	2	3	4	4
	0.01	4	14	13	20
	0.1	27	50	51	63
NaF	0.1	9	9	13	8
	1.0	24	38	40	40
	10	47	84	81	83

The stability of the purified laccases in ACN and EtOH was tested for different solvent concentrations (final concentrations: 9%, 18%, 45% and 90%; Fig 8). Compared to water, the instable enzymes Lcc6 and Lcc7 showed an even lower stability in all tested ACN and EtOH concentrations. Even at the low concentration of 9% EtOH as the best situation, only 60% and less than 40% of the activity from Lcc6 and Lcc7 remained after 4 h, respectively. After 24 h, no laccase activity remained in all Lcc6 and Lcc7 solutions, regardless of being just water or containing organic solvents. For Lcc1 and Lcc5 being highly stable in water, incubation at lower ACN and EtOH concentrations (9% and 18%, respectively) had less dramatic effects on protein stability. Lcc1 retained more than 50% of its activity after 4 h in ACN and ethanol at concentrations of up to 18% and up to 45%, respectively. Also after 24 h of incubation, considerable amount of enzymatic activity were still present in samples of up to 18% solvent concentration. Protein Lcc5 had an even better stability in organic solvents than Lcc1. Lcc5 maintained 50% of its activity after 24 h when incubated in up to 18% ACN and 45% EtOH, respectively. Moreover, after 24 h 25% activity was still present in the sample of 90% EtOH concentration. With ACN concentrations of 45% and 90%, after 4 h incubation there was still laccase activity of about 55% and 30%, respectively. However, at these high ACN concentrations, after 24 h all enzymatic activity was lost.

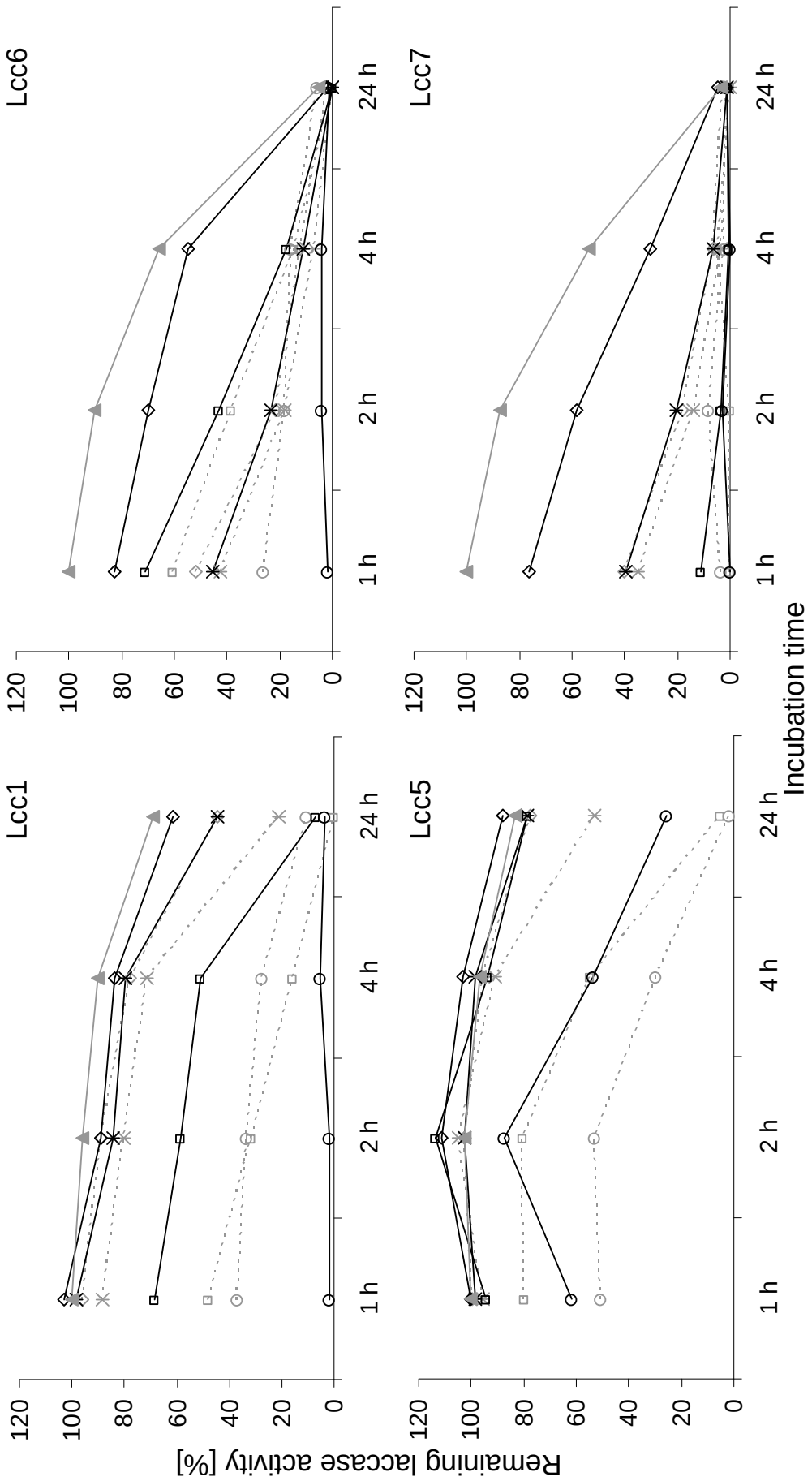


Fig. 8 Stability of the purified laccases Lcc1, Lcc5, Lcc6 and Lcc7 incubated in water (grey solid line with filled triangle) and in different concentrations of acetonitrile (ACN, grey dotted line) or ethanol (EtOH, black solid line): 9% (diamond), 18% (star), 45% (square) and 90% (circle) at room temperature. Laccases with a final concentration of 0.1 to 0.2 U/ml were applied. For calculation of remaining laccase activities in the different solvents over the time, the laccase activity in water present after 1 h of incubation was set to 100%. Averages were determined from two parallel measurements of one incubated samples.

E. Reactions with industrial dyes

Seven different industrial dyes were tested with the four purified *C. cinerea* laccases as well as with a commercial *T. versicolor* laccase (Tv_lac, Novozymes A/S, Bagsvaerd, Denmark). Only four dyes (all blue dyes) could be decolourised to different extends (Fig. 9). Levafix Blue E-RA got completely decolourised after 24 h by all tested laccases. However, Tv_lac showed best results and reacted very fast with the dye directly upon mixing (Fig. 9A). This was also seen for Procion Blue, where during the first measurement (5 min after the start of the experiment) already 30-70% of the dye solution was decolourised; although, Procion Blue could not be degraded completely (Fig. 9C). Levafix Blue RN was degraded by *C. cinerea* laccases to a very low amount, but almost completely by the *T. versicolor* laccase (Fig. 9D). Degradation of the Turqoise dye between 5 min and 24 h was very low with approximately only 2% for all *C. cinerea* laccases, but 20% for Tv_lac (Fig. 9D).

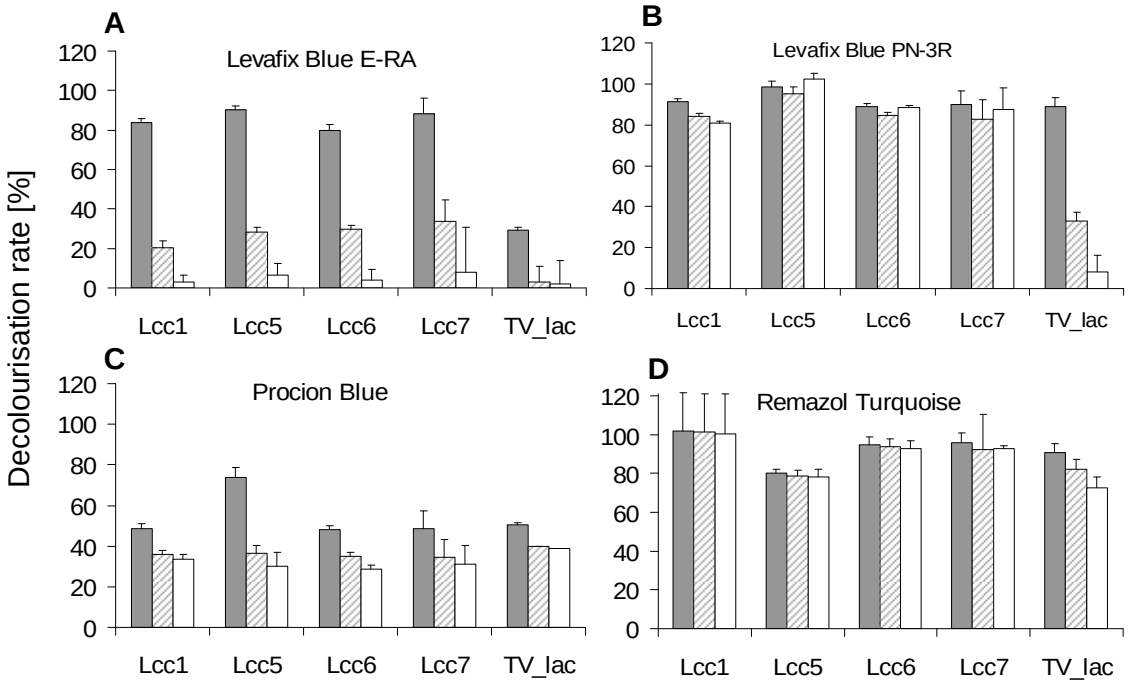
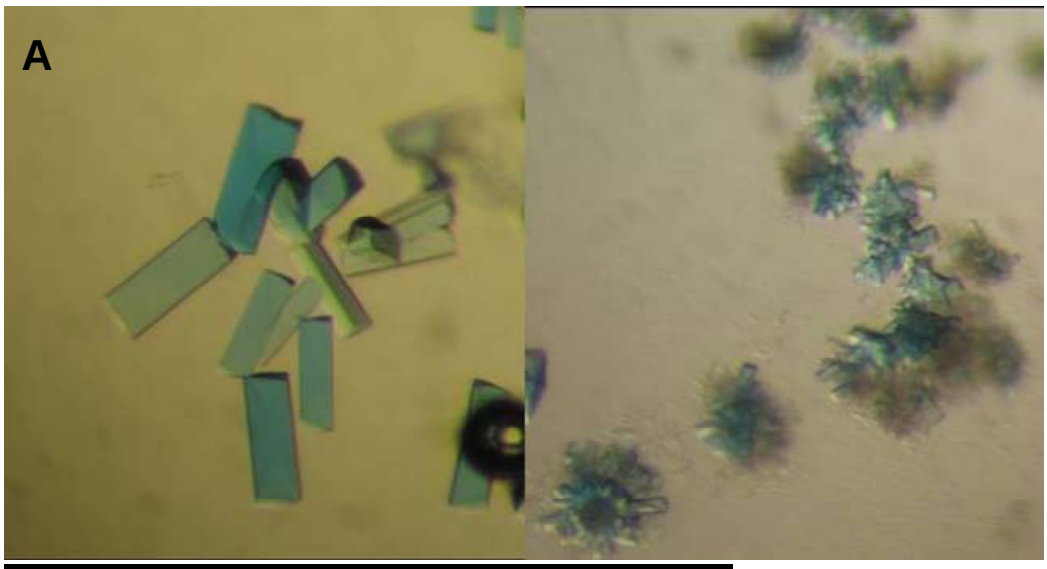
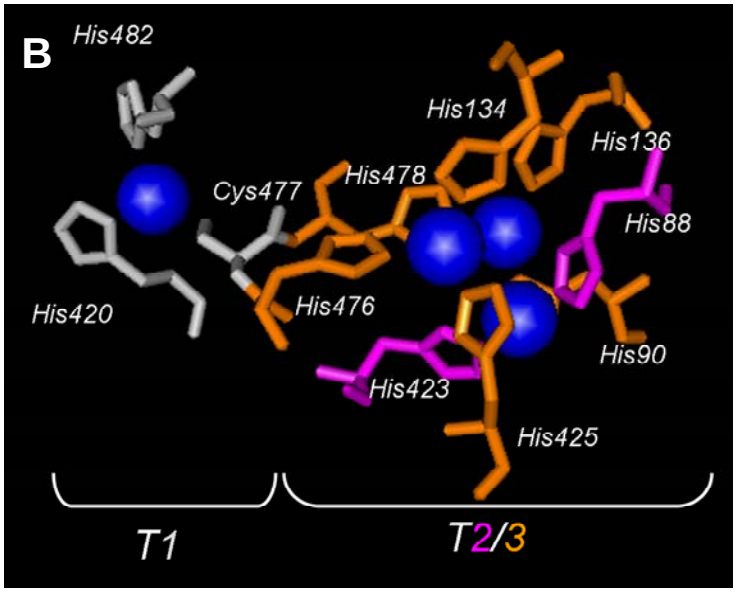


Fig. 9 Decolourisation rates of different textile dyes with four *C. cinerea* laccases (Lcc1, Lcc5, Lcc6 and Lcc7) and a *T. versicolor* laccase (Tv_lac) respectively, each used in a concentration of about 2.0 U/mg dye. Incubation was carried out at room temperature. Decolourisation rates were calculated against a blank sample (dye without enzyme), which was set to 100%, within 5 min after start of the experiment by adding the dye to the respective enzyme buffer solution (time frame required for one measurement in the spectrophotometer, dark grey), after 4 h of incubation (light grey) and after 24 h of incubation (white). Averages and standard deviations were calculated from each three parallel samples. Note that the turquoise dye samples (**D**) with Lcc5 were lower in absorbance already at the beginning of the experiment (5min after addition of the dye) possibly due to a pipetting error.

F. Structural characteristics

Crystals of the purified laccase Lcc5 were produced by Anja Pomowski, who also revealed the crystallographic structure of the complete enzyme Lcc5 (title page of chapter 5, p.213). The structure of the copper clusters, which is presented in Fig. 10B, was generated within this study with the PyMOL software (DeLano 2007) using the structural data provided by Anja Pomowski. We calculated the distances of the copper atoms towards each other and its ligands with the same software. All ligands had similar distances towards their copper atoms of about 2 Å, whereas the cysteine at the T1 site showing the highest distance of 2.2Å. The distances between the three copper atoms distributed over the trinuclear copper cluster T2/T3 were between 3.9 and 4.0 Å.





Copper	Distance Å
T1-Cu(1)	2.1 (HIS420) 2.2 (Cys477) 2.1 (His482)
T2-Cu(4)	2.0 (His423) 1.9 (His88)
T3-Cu(2)	2.0 (His90) 2.0 (His134) 2.1 (His478)
T3-Cu(3)	2.1 (His136) 2.1 (His425) 2.1 (His476)
Cu(2)-(3)	4.7
Cu(2)-(4)	4.0
Cu(3)-(4)	3.9

Fig. 10 (**A**) Crystals obtained from purified Lcc5 (Anja Pomowski, Department of Molecular Structural Biology, Institute for Microbiology and Genetics, Göttingen) and (**B**) the structure of the mono- (T1) and trinuclear (T2/3) copper clusters of Lcc5. The table at the right shows the distances between the amino acid ligands of the copper centres and their relating copper atoms. Image (**B**) of the Lcc5 structure and calculation of the distances were done with the PyMOL software (DeLano 2007).

V. Discussion

A. Molecular structure of enzymes produced by the different *C. cinerea* laccase genes

In this study, three different recombinantly produced *C. cinerea* laccases (Lcc5, Lcc6 and Lcc7) were characterised the first time concerning molecular, biochemical and, in case of Lcc5, also structural features. Furthermore, for the previously characterised enzyme Lcc1 (Kilaru 2006) Michealis-Menten kinetics as well as stability against laccase inhibitors and in organic solvents were determined. The molecular weight of 61-63 kDa determined for the laccases by SDS-PAGE is within the normal range for laccase molecules, although the amount of N-glycosylation (2-5% for the laccases tested in this study, Table 4) is quite low compared to other laccases (Thurston 1994, Kilaru 2006). To the best of my knowledge, only for *P. ostreatus* POXA1, *P. eryngii* Laccase II, *C. cinerea* Lcc1 and two *Fomes sclerodermus* laccase isoforms, similarly low glycosylation amounts of below 5% were detected (Palmieri et al. 1997, Munoz et al. 1997, Papinutti and Martinez 2006, see section 4.I). In the literature, most of the studies describe only the degree of N-glycosylation of laccases (Palmieri et al. 1997, Min et al. 2001, Ko et al. 2001, Nagai et al. 2002, de la Rubia et al. 2002, Saparrat et al. 2002, Xiao et al. 2003), whereas only in few studies the total polysaccharide content was determined (Perie et al. 1998, Schliephake et al. 2000, Galhaup et al. 2002) or the composition of the sugars was studied (Minuth et al. 1978, Lyashenko et al. 2006). O-glycosylation may be present in *C. cinerea* laccases, as suggested by crystal structure of *C. cinerea* type-2-copper-depleted laccase Lcc1 (Ducros et al. 1998). Such extra O-glycosylation could lead to a polysaccharide content which is higher than the values of 3%, 2%, 4% and 5% determined for laccases Lcc1, Lcc5, Lcc6 and Lcc7 by deglycosylation of the N-linked polysaccharides, respectively. The enzymes have 1 to 4 potential N-glycosylation sites (Fig. 11). The theoretical MW of the mature enzymes Lcc1, Lcc5, Lcc6 and Lcc7 (see Fig. 11 for sequences) are 58.4 kDa, 57.5 kDa, 57.5 kDa and 60.0 kDa, respectively. In comparison, the experimentally determined values were 61 kDa, 61 kDa, 59 kDa and 60 kDa, respectively (Table 4, Fig. 3 and Kilaru 2006). Thus, only for Lcc7 the theoretical and experimental MW are similar. The differences of about 2.5 kDa in case of the other threee sequences might be due to sugars still present at the N-glycosylation sites, which are still not cleaved by the PNGaseF enzyme (Lcc1, Lcc5, Lcc6 and Lcc7 have 3, 1, 3 and 4 glycosylation sites, respectively, determined with NetNGlyc1.0 Server, www.cbs.dtu.dk, Fig. 11) or due to O-glycosylation of the protein, which was not analysed in this work. Interestingly, Lcc5, which showed the highest difference between the theoretical and experimental MW

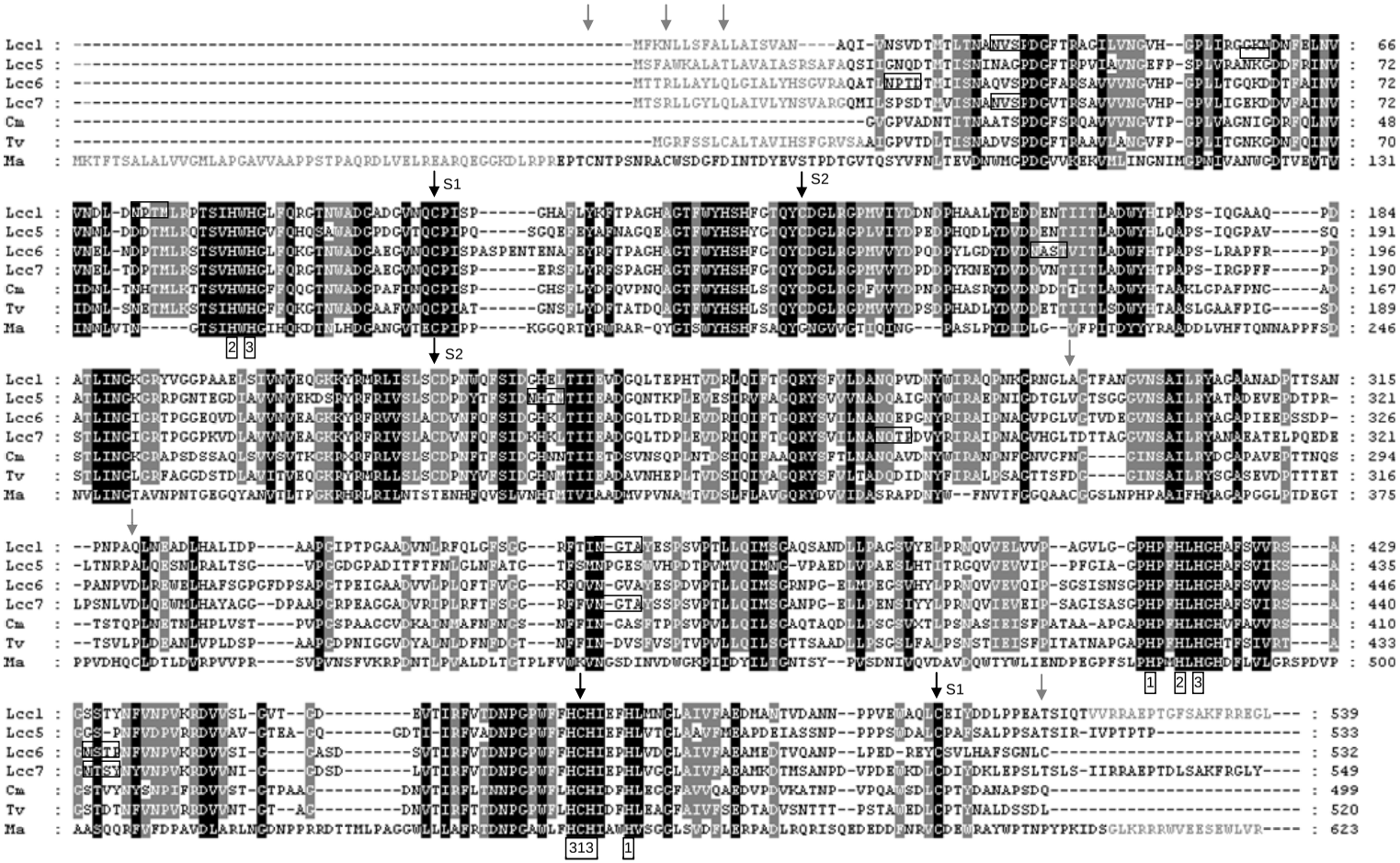


Fig. 11 Alignment of the deduced amino acid sequences of the *C. cinerea* laccases (Lcc1, Lcc5, Lcc6 and Lcc7), the *Cerrena maxima* laccase (Cm: Lyashenko et al. 2006), the *Trametes versicolor* laccase (Tv: Jönsson et al. 1995) and the *Melanocarpus albomyces* laccase (Ma: Kiiskinen and Saloheimo 2004). Cysteines are marked with an arrow either in black (cysteine is present in more than one sequence at this position) or an arrow in grey (cysteine is only present in one sequence at this position). S1 and S2 refer to the cysteines involved in one of the two disulfide bridges revealed by the structure of *C. maxima* (Lyashenko et al. 2006) and Lcc5 (Pomowski et al., unpublished). The cysteine and histidine residues involved in the copper binding are marked with numbers (1, 2 and 3) below the sequences according to their copper site (T1, T2 and T3). Potential glycosylation sites of the *C. Cinerea* laccases are indicated by boxes inside each sequence. Grey amino acids indicate signal sequences at the N-terminal site or cleavage of the C-terminal end (Lcc1: Yaver et al. 1999, Ma: Kiiskinen and Saloheimo 2004; in all other laccases signal peptide was predicted by SignalP 3.0 - Bendtsen et al. 2004)

(ΔMW = 3.5 kDa) among the tested laccases, has only one potential N-glycosylation site. Further studies on the crystal-structures of Lcc5 (see below) might give a better insight into the glycosylation pattern of Lcc5.

SDS-PAGE of denatured purified laccase lead to only one band for all laccases, whereas samples in native PAGE showed two bands for Lcc6, but only one major band in IEF for this laccase (compare Fig. 2 and Fig. 5). Unlike the other *C. cinerea* enzymes, Lcc6 has six cysteines (Fig. 11): One of which (Cys487) should take part in copper binding of the T1 centre. Four others (Cys109, Cys147, Cys234, Cys521) by analogy of structurally characterised enzymes (Lcc1 of *C. cinerea*, Yaver et al. 1999; laccase of *T. versicolor*, Jönsson et al. 1995; laccase of *Cerrena maxima*, Lyashenko et al. 2006d) should contribute to stability due to the formation of disulfide bridges. Cys532 is located at the C-terminal end, eleven amino acids downstream of Cys521, and might alternatively act to Cys521 in disulfide bridging with Cys 147 giving a possible explanation for the two bands of Lcc6 in native gel electrophoresis.

On the other hand, Lcc7 had only one very strong band in native PAGE and IEF, when stained with DHPPA and MBTH, but two bands of similar strength in IEF when stained with Coomassie (Fig. 2 and 4). Likewise, Lcc5 had only one band in native PAGE that resolved into two of different intensity in IEF (Fig. 2 and 4). In case of Lcc6, there was one major band in IEF at a pI of 3.7 and a very faint band at a pI of 3.6. The selective staining of the two bands, however, did not match to the more equal staining of the two bands obtained in native PAGE (compare Fig. 2 and Fig. 4). Finally, Kilaru (2006) presented for enzyme Lcc1 one major band with a pI of 3.8. Theoretical pIs for the native enzymes are 5.17, 4.71, 5.00 and 5.18 for *C. cinerea* laccases Lcc1, Lcc5, Lcc6 and Lcc7, respectively (calculated by the pI/MW tool of ExPASy - www.expasy.org). These pI values differ by 1.0 to 1.4 from the experimental determined values. In the native enzymes, amino acids projected to the surface of the folded proteins should account for the actual pI values. Slight changes in the folding on the enzyme surface might be the reason for obtaining isoforms with different pIs from a given enzyme. Generally, the experimental determined pI values of Lcc1, Lcc5, Lcc6 and Lcc7 are within the range of pI values for known laccases, which differ between pI 2.65 and 3.0 for *Pycnoporus cinnabarinus* laccases (Schliephake et al. 2000) up to values of pI 6.7 for POXA1 and pI 6.9 for POXA1b of *P. ostreatus* (Palmieri et al. 1997, Garzillo et al. 2001) and pI 7.4 for *Trametes hirsuta* laccase (Shin and Lee 2000). Different banding pattern on native PAGE and IEF has been described previously for some other basidiomycete laccases. Purified laccase from *Coriolopsis gallica* for example showed four bands in PAGE analysis,

whereas in IEF only 3 bands were observed (Calvo et al. 1998), meaning that two isoenzymes/isoforms probably have the same pI value. Various reasons can account for the observation of different banding patterns in non-denaturing PAGEs of laccases from a single gene. Different banding might be due to the occurrence of isoforms, different post-translational modifications, differences in the oxidation state of the enzyme, partly proteolysed enzymes or dimers of the same protein (Calvo et al. 1998, Min et al. 2001, Machczynski et al. 2004). From our data, we do not have evidence of dimerisation for any of the four characterised proteins for different post-translational modifications (Fig. 2, 4 and Kilaru 2006). Yaver et al. (1999) reported for Lcc1 recombinantly produced in *Aspergillus oryzae* that the C-terminus was processed – whether this is the case in *C. cinerea* is not known.

B. Efficiency of the laccase production by the different laccase genes

The amount of secreted laccase in the supernatant, which was calculated by dividing the total laccase activity in the supernatant with the specific laccase activity of the purified enzyme (Table 2), varied between transformants with values of 25 mg/l, 32 mg/l, 16 mg/l and 22 mg/l for lacacses Lcc1, Lcc5, Lcc6 and Lcc7, respectively (Table 2 and Kilaru 2006). Higher values for amounts of secreted enzymes where obtained when using fluorescence staining of SDS-PAGEs of the supernatant. For the supernatant of the different laccase transformants values of 81 mg/l, 115 mg/l, 28 mg/l and 27 mg/l for laccases Lcc1, Lcc5, Lcc6 and Lcc7 were determined, respectively. One possible explanation of the discrepancy between values calculated after protein purification and values derived from fluorescence staining might be e.g. too high protein concentration values determined via the Bradford assay in the culture supernatant. Previously it was shown that the Bradford assay might be affected by phenolic compounds produced by fungal organisms (Whiffen et al. 2007, Roberts and Jones 2008). A too high measured protein concentration would lead to redcued values for the specific laccase activity and, thus, to lower secretion amounts. Additionally, calculations of the secreted protein amount from laccase activity and Bradford assay do not include non-active enzymes (e.g. apoenzymes), which would be detected by SDS-PAGE. Thus, the amount of secreted enzymes calculated via the specific activity of the purified enzyme might be smaller than the actual laccase amounts.

Generally, Lcc1 and particularly Lcc5 proved to be much more stable than Lcc6 and Lcc7 (Fig. 8, Table 4). All proteins were recombinantly produced by transformants of *C. cinerea* FA2222 containing the respective introduced laccase gene behind the *A. bisporus gpdII*

promoter (Kilaru 2006). Different transformants of the same construct vary in overall enzymatic activity, due to random integration of the gene into the fungal genome (Kilaru et al. 2006). However, there was a tendency in *C. cinerea* transformation to obtain more positive FA2222 transformants with higher activities in liquid shaken cultures when using the gene *lcc5* than when using genes *lcc1*, *lcc6* and *lcc7* (Kilaru 2006). Always the best transformant of Kilaru (2006) was chosen for this study out of up to 12 clones per gene. Although there will certainly be some difference in total mRNA production from the *A. bisporus* promoter due to the different places of integration, the very different amounts of enzymatic activity obtained from the different transformants will likely be to large parts due to the better overall stability of Lcc1 and Lcc5.

C. Stability of recombinantly produced enzymes

All tested *C. cinerea* lacacses showed similar thermal stability at 50 °C remaining at least 50% of their starting activity after 20 to 60 min (Table 4). This is in the range of fungal laccases, which show a half life at this temperature between several minutes to up to more than two days (reviewed by Baldrian 2006). The pH-stability of fungal laccases is normally higher at a more acidic pH (Leonowicz et al. 1984, Kilaru 2006). Nevertheless, *C. cinerea* seems to have at least four different laccases which are more stable in the alkaline environment (Table 4). Few examples for laccases of basidiomycetes being stable at alkaline pH can be found in the literature. In *Daedalea quercina*, highest laccase stability was observed at pH 6.0 and above with only 3% loss of its activity after 24 h (Baldrian 2004) and *Pleurotus pulmonarius* Lcc2 was stated to be very stable in a range between pH 4 and 10 (De Souza and Peralta 2003). *C. cinerea* has the behaviour to increase the pH of a culture medium up to pH 9.0 (Navarro-González 2008, section 4.II). Accordingly, adaptation of its enzymes in tolerance towards this alkaline pH should be favourable for the fungus. How about the pH range of activity? The optimal pH for activity of the laccases tested in this study was found to be below their optimal pH stability range which was determined to be for *C. cinerea* laccases around pH 7 to 11 for all tested laccases (Table 4). Enzymes of other species are often better active in the acidic pH range which can be seen in the review of Baldrian (2006), who calculated the median for the optimal pH of up to 36 different laccases, showing median values of pH 3.0, 4.0, 4.5 and 6.0 for the substrates ABTS, DMP, guaiacol and SGZ, respectively. Nevertheless, the total range of optimal pH varies drastically among laccases between pH 2.0 to 5.0 for ABTS, pH 3.0 and 8.0 for DMP, pH 2.0 and 7.5 for guaiacol and pH 3.5 to 7.0 for SGZ (Baldrian 2006, Kilaru 2006). In comparison to this, for all four characterised laccases the pH optima for the phenolic substrates (DMP, guaiacol and

SGZ) is in the neutral slightly acidic range (Table 4). Because of the higher stability in an alkaline pH range and since their pH-range for phenolic substrates is in a more or less neutral range, usages where alkali-resistant laccases are needed would be suitable for *C. cinerea* enzymes, e.g. in the treatment of wastewater from dyeing processes (Soares et al. 2006). Contradictory in the acidic pH range, at pH 2 the laccases are very unstable since no activity or only 3% of the activity remained after 20 min for Lcc6 and Lcc7. Also at pH 3, Lcc6 lost more than 96% of the remaining activity after 20 min, whereas Lcc7 remained almost 50% of its activity (data not shown). Such a lower stability as found for enzymes Lcc6 and Lcc7 was already observed for a *Cerrena unicolor* laccase (Gianfreda et al. 1998).

Stability testing is not only restricted to pH and temperature. In several studies in the literature, the characterised laccases are analysed regarding their stability in presence of laccase inhibitors (Table 7) because of the usage of inhibitors in applications were laccases might be used, such as EDTA in the paper and pulp industry (Lapierre et al. 1995) and Naf/NaN_3 in the food industry (Lee et al. 2008). Resistance against known laccase inhibitors for the *C. cinerea* laccases is different compared to other laccases described in the literature, for example *Trametes* and *Pleurotus* laccases which are found to be relatively sensitive against NaN_3 (Table 7). In contrast, the ascomycete *Aspergillus nidulans* laccase was as the *C. cinerea* laccases also very stable against NaN_3 with 49% of the activity remaining (Scherer and Fischer 1998). However, resistance of *A. nidulans* laccase against NaF (66% remaining laccase activity) was better than resistance of all four *C. cinerea* enzymes whereas resistance of the *A. nidulans* enzyme against EDTA was much lower (Table 7). In the industry, a good stability of laccase against inhibitors could be of special interest in industrial processes were fluorides (e.g. food industry) or EDTA (paper industry) are needed.

Table 7 Inhibition of laccases by known inhibitors

Laccases	1 mM EDTA	10 mM NaF	0.1 mM NaN_3*	Reference
Aspergillus nidulans	31%	34%	51%	Scherer und Fischer 1998
Cerrena unicolor	nt	nt	30% (0.02 mM)	Gianfreda et al. 1998
C. cinerea Lcc1	2%	47%	27%	This work, Table 6
C. cinerea Lcc5	7%	84%	50%	
C. cinerea Lcc6	4%	81%	51%	
C. cinerea Lcc7	6%	83%	63%	
Pleurotus pulmonarius	1%	nt	98%	De Souza and Peralta 2003
Trametes sp. AH28-2	0%	nt	100%	Xiao et al. 2003
Trametes sp. 420	Almost no inhibiton	nt	100%	Tong et al. 2007

*Concentrations varying from this value are given in brackets. nt = not tested.

Besides the stability against inhibitor molecules, the stability and reactivity of laccases in organic solvents is interesting for industrial techniques when substrates are not soluble in water, like for degradation of PAHs (Majcherczyk et al. 1998). In the literature, several studies on stability in organic solvents are found. The laccase of the white-rot fungus *P. pulmonarius* for example showed low stability with ACN and EtOH, as only 70% and 64% of the activity remained after incubation in 10% final concentration of the organic solvent, respectively (De Souza and Peralta 2003). *C. unicolor* laccase tested in 50% methanol, 50% ACN and 50% dioxane revealed a half life time of 4 h, 2.8 h and <30 min, respectively. In the respective study, dioxan already showed an inhibition of 70% after 30 min of incubation (Gianfreda et al. 1998). For *Polyporus pinsitus* laccase, residual activity was 45%, 75% and 75% subsequently to mixing the enzymes with (1:1 v/v) EtOH, ACN and dioxane, respectively (d'Acunzo et al. 2004). Compared to these enzymes, Lcc5 showed a very good stability with a half life of 4 h (45% ACN final concentration) and 2 h for ACN concentrations of 45% and 90%. In ethanol of concentrations of up to 45%, Lcc5 showed almost the same activity after 24 h as the control (incubation in water). Only in 90% EtOH a more rapid inhibition was observed, but the enzyme still reached a half life of 4 h. This makes Lcc5 a good candidate for reactions, where organic solvents are needed (Mikolasch and Schauer 2009). To a lesser degree, this can also be deduced for laccase Lcc1 that had also a quite good stability in the organic solvents in contrast to enzymes Lcc6 and Lcc7 (Table 7).

D. Enzymatic kinetics with different substrates

The Michaelis-Menten-kinetics revealed differences between the laccases towards the different substrates tested. Lcc1 and Lcc5 showed K_m values of 656 µM and 831 µM, respectively, which are in the range of fungal laccases (Baldrian 2006, Kilaru 2006). In contrast, the laccases Lcc6 and Lcc7 had higher K_m values of 2683 µM and 3999 µM, respectively, which are more in the upper range and similar to values found for *P. ostreatus* laccases (Kilaru 2006). The phenolic based substrates used in this study (DMP, guaiacol and SGZ) differ in their redox potentials depending on the substituents at the phenolic ring (Lind et al. 1990). In these substrates, the methoxy groups at the phenolic ring reduce the redox potential of the substrate from 0.79 V (vs. NHE) for phenol to 0.54 V for the mono-4-methoxyphenol and 0.42 V for the dimethoxy-phenol DMP (Lind et al. 1990, Jovanovic et al. 1991). With lower redox potentials of tested substrates, the catalytical efficiency of laccases increases as shown here for all tested laccases with guaiacol, DMP to SGZ in increasing order of efficiencies. The best substrate SGZ posses two phenolic rings both substituted with two methoxy groups and seems to have the lowest redox potential for all substrates tested in

this study. The dependence of the enzymatic reaction activity on the redox potential of the substrates guaiacol, DMP and SGZ was already observed for *Trametes pubescens* laccase (Galhaup et al. 2002), *Trametes* sp. LacE (Tong et al. 2007) and *Trametes* sp. strain AH28-2 laccase (Xiao et al. 2003).

The affinity of the laccases towards the phenolic substrates described by the K_m value was in the range for characterised laccases from other fungi (Kilaru 2006), except for Lcc6 and Lcc7, which showed high K_m values for DMP (1.5 mM and 1.1 mM, respectively). Only *A. nidulans* laccases showed higher K_m values for DMP with 1.3 mM for laccase II (Scherer and Fischer 1998) and 3.9 mM for laccase I (Clutterbuck 1972). *A. niger* laccases I did however not oxidise the standard substrates SGZ and ABTS (Scherer and Fischer 1998). It is thus biochemically unclear, in which relation the *A. niger* enzyme stands. Phylogenetic studies of protein sequences suggested that the ascomycete enzymes are different evolutionary developments away from the laccases being typical basidiomycetes' enzymes (Hoegger et al. 2006). The *A. nidulans* enzymes group with other ascomycete enzymes known to act in pigment synthesis (Hoegger et al. 2006).

For a more general characterisation of laccase reactivity, the redox potential of the T1 site of the enzyme of interest can de determined. Because this value depends also on the Michealis-Menten kinetics (shown by Xu et al. 1996), it is a more general value for comparison of the oxidation potential of different laccase. In this study, the redox potential of Lcc5 at pH 6.4 had an average value of 542 mV ± 20 mV. This is in the same range as observed for the *C. cinerea* laccase Lcc1 recombinantly produced in *A. oryzae*, for which in two parallel studies of the same group a redox potential of 540 mV ± 50 mV and 550 mV ± 80 mV was measured using either I_2-NaI and $K_3Fe(CN)_6$-$K_4Fe(CN)_6$ as a redox-couple for redox-titration of the laccase (Yaver et al. 1999, Schneider et al. 1999). Besides the effect of different redox-couples, the pH might also influence the redox potential, e.g. Garzillo et al. (2001) tested native laccases of three different fungi (*P. ostreatus*, *Rigidoporus lignosus* and *Trametes trogii*) and showed that with an increase in pH (pH 5.0 to 7.0) the E° value of the laccases decreased between 10 mV and 50 mV.

Nevertheless, also structural features of the enzyme might have an impact on the redox potential, as different activities of one enzyme towards substrates with similar redox potential were observed (Xu et al. 1996, Garzillo et al. 1998 and 2001). Characterised *Trametes* laccases have, generally, high redox potentials (Klonowska et al. 2002, Saparrat et al. 2002, Shleev et al. 2005), e.g. redox potential of the *Trametes pubescence* laccases LAC1

and LAC2 were 746 and 738 mV, respectively (Shleev et al. 2007). On the other hand, laccases from *P. ostreatus*, *Rigidoporus lignosus* and *Trametes trogii* tested by Garzillo et al. (2001) differed little in their Cu binding site feature, the E° values were quite different (150 mV). The difference between the redox potential of the substrate and the laccase, due to the electron transfer between the molecules, is one criterion for the activity. However, the quality of the oxidation of the substrate via a laccase depends also on how good the laccase accept the electron from the substrate and this reaction takes place at the T1 site.

In laccases, T1 copper is positioned by two histidines and one cysteine. The histidines may also act for electron transfer from the substrate towards the copper (Bertrand et al. 2002). In the nearer region of the T1 site (10 amino acid downstream of the cysteine ligand), either a leucine or phenylalanine is mostly located in fungal laccases (Xu et al. 1996). In contrast, in ascorbate oxidases, the T1 site has a methionine at this position, as has the laccase of *A. nidulans* (Aramayo and Timberlake 1990). This amino acid difference was speculated to be responsible for the higher redox potential of the T1 copper in laccases compared to the ascorbate oxidases (Reinhammar and Malmstrom 1981, Thurston 1994). Eggert et al. (1998) classified the laccases into three classes (1, 2 and 3) for residues methionine, leucine and phenylalanine, respectively. According to this, all *C. cinerea* laccases, predicted from the in total 17 genes in the genome of the fungus (Kilaru et al. 2006), except the product of gene *lcc12* are class 2 enzymes as are the genes coding for laccases of *P. ostreatus pox1* and *pox2*, *A. bisporus lcc1* and *lcc2*, as well as the laccases of *Rigidoporus lignosus*, *Melanocarpus albomyces* and others (Eggert et al. 1998, Zhukhlistova et al. 2008). In the system of Eggert et al. (1998) Lcc12 would be categorised in class 3 and, therefore, as high E^0 laccase. Thus, it would be very interesting to study the redox potential of Lcc12. Unfortunately, expression studies with a construct containing the *C. cinerea* laccase gene *lcc12* under control of the *A. bisporus gpdII* promoter in *C. cinerea* FA2222 failed to give a positive transformant (Kilaru 2006).

Contradictory to the proposal made by Eggert et al. (1998), *Neurospora crassa* laccase with leucine in this axial position has already a high redox potential of 780 mV (Piontek et al. 2002). Also point mutations in laccases of *Myceliophthora thermophila* L513F and *Rhizoctonia solani* L470F in this position did not support this idea, as the redox potential did not alter significantly (Xu et al. 1998). Thus, the differences in the E° values might also be due to conformational changes more far away from the T1-Cu centre. Comparison of *C. cinerea* Lcc1 (550 mV) with *P. ostreatus* POXC (740 mV), whose redox potential values have a 190 mV difference, shows no differences in the 5 Å region near the T1-Cu centre.

This supports the possibility that conformational changes more far away from the Cu centre can have an influence on the redox potential of the enzyme (Garzillo et al. 2001). Piontek et al. (2002) proposed a hydrogen bond between a glutamine (Glu460 in *T. versicolor* laccase) and serine residue (Ser113 in *T. versicolor* laccase) having an indirect effect on the binding distance between the histidine ligand, two amino acid upstream of the glutamine, and the T1 copper. It was also proposed that the redox potential depends on the amino acids building the substrate pocket, as all laccases with high redox potential have conserved amino acid sequences in the T1 and substrate pocket side (Zhukhlistova et al. 2008).

Overall, the laccases might be clustered according to their Michaelis-Menten parameters for two substrates ABTS and DMP into two distinct groups: Lcc1 and Lcc5 show low K_m values with ABTS and DMP, whereas Lcc6 and Lcc7 very high ones. In contrast, for guaiacol, only Lcc1 showed good kinetic parameters. The reason for this discrepancy is still unknown. Regarding differences of amino acid sequences (see Fig. 11) concerning the redox potential a concluding remark towards the *C. cinerea* laccases is hard to give, since we only have measured the redox potential of Lcc5. Ongoing experiments for the determination of the redox potential of the missing *C. cinerea* laccases might help to understand differences in the redox potential coming from different amino acid sequences.

E. Conclusions

In this study, four different C. cinerea laccases were biochemically analysed. Different properties made them candidates for different biotechnological applications. Generally, by a high overall stability and good enzymatic kinetics for the tested substrates laccase Lcc5 would be in many instances a first choice for application. In addition, this enzyme is easily produced in high quantities (Table 3). Also laccase Lcc1 by its high stability against inhibitors and production rates is a promising candidate for usage in biotechnological applications. Lcc6 and Lcc7 are less favourable but further comparative biochemical characterisation may give interesting details to better understand how laccases work and how possibly manipulate amino acid sequences to optimise enzymes for specific biotechnological utilisations.

VI. References

Aramayo R and Timberlake WE (1990) Sequence and molecular structure of the *Aspergillus nidulans ya* (laccase-I) gene. Nucleic Acids Research 18:3415-3415

Baldrian P (2004) Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Applied Microbiology and Biotechnology 63:560-563

Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiology Reviews 30:215-242

Bendtsen DJ, Nielsen H, von Heijne G and Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. Journal of Molecular Biology 340:783-795

Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C and Mougin C (2002) Crystal structure of a four-copper laccase complexed with an arylamine: Insights into substrate recognition and correlation with kinetics. Biochemistry 41:7325-7333

Blum H, Beier H and Gross HJ (1987) Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93-99

Böhmer U, Suhardi SH and Bley T (2006) Decolorizing reactive textile dyes with white-rot fungi by temporary immersion cultivation. Engineering in Life Sciences 6:417-420

Calvo AM, Copa-Patino JL, Alonso O and Gonzalez AE (1998) Studies of the production and characterization of laccase activity in the basidiomycete *Coriolopsis gallica*, an efficient decolorizer of alkaline effluents. Archives of Microbiology 171:31-36

Claus H, Faber G and Konig H (2002) Redox-mediated decolorization of synthetic dyes by fungal laccases. Applied Microbiology and Biotechnology 59:672-678

Clutterbuck AJ (1972) Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. Journal of General Microbiology 70:423-435

d'Acunzo F, Barreca AM and Galli C (2004) Determination of the activity of laccase, and mediated oxidation of a lignin model compound, in aqueous-organic mixed solvents. Journal of Molecular Catalysis B-Enzymatic 31:25-30

de la Rubia T, Linares A, Perez J, Munoz-Dorado J, Romera J and Martinez J (2002) Characterization of manganese-dependent peroxidase isoenzymes from the ligninolytic fungus *Phanerochaete flavido-alba*. Research in Microbiology 153:547-554

De Souza CGM and Peralta RM (2003) Purification and characterization of the main laccase produced by the white-rot fungus *Pleurotus pulmonarius* on wheat bran solid state medium. Journal of Basic Microbiology 43:278-286

DeLano WL (2007) The PyMOL Molecular Graphics System, http://www.pymol.org, DeLano Scientific LLC, Palo Alto, CA, USA

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH and Davies GJ (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Ångström resolution. Nature Structural Biology 5:310-316

Durão P, Chen ZJ, Silva CS, Soares CM, Pereira MM, Todorovic S, Hildebrandt P, Bento I, Lindley PF and Martins LO (2008) Proximal mutations at the type 1 copper site of CotA laccase:

spectroscopic, redox, kinetic and structural characterization of I494A and L386A mutants. Biochemical Journal 412:339-346

Eggert C, Temp U and Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Applied and Environmental Microbiology 62:1151-1158

Eggert C, LaFayette PR, Temp U, Eriksson KEL and Dean JFD (1998) Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. Applied and Environmental Microbiology 64:1766-1772

Fackler K, Kuncinger T, Ters T and Srebotnik E (2008) Laccase-catalyzed functionalization with 4-hydroxy-3-methoxybenzylurea significantly improves internal bond of particle boards. Holzforschung 62:223-229

Felby C, Nielsen BR, Olesen PO and Skibsted LH (1997) Identification and quantification of radical reaction intermediates by electron spin resonance spectrometry of laccase-catalyzed oxidation of wood fibers from beech (*Fagus sylvatica*). Applied Microbiology and Biotechnology 48:459-464

Galhaup C, Goller S, Peterbauer CK, Strauss J and Haltrich D (2002) Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. Microbiology-SGM 148:2159-2169

Garzillo AMV, Colao MC, Caruso C, Caporale C, Celletti D and Buonocore V (1998) Laccase from the white-rot fungus *Trametes trogii*. Applied Microbiology and Biotechnology 49:545-551

Garzillo AM, Colao MC, Buonocore V, Oliva R, Falcigno L, Saviano M, Santoro AM, Zappala R, Bonomo RP, Bianco C, Giardina P, Palmieri G and Sannia G (2001) Structural and kinetic characterization of native laccases from *Pleurotas ostreatus*, *Rigidoporus lignosus*, and *Trametes trogii*. Journal of Protein Chemistry 20:191-201

Gianfreda L, Sannino F, Filazzola MT and Leonowicz A (1998) Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*. Journal of Molecular Catalysis B-Enzymatic 4:13-23

Havlis J and Shevchenko A (2004) Absolute quantification of proteins in solutions and in polyacrylamide gels by mass spectrometry. Analytical Chemistry 76:3029-3036

Hoegger PJ, Majcherczyk A, Dwivedi RC, Svobodova K, Kilaru S, and Kües U (2007) Enzymes in wood degradation. In: Kües U (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Universitätsverlag Göttingen, Göttingen, 383-432

Johannes C, Majcherczyk A and Hüttermann A (1998) Oxidation of acenaphthene by laccase of *Trametes versicolor* in a laccase-mediator system. Journal of Biotechnology

Jönsson L, Sjoström K, Häggström I and Nyman PO (1995) Characterization of A Laccase Gene from the White-Rot Fungus Trametes-Versicolor and Structural Features of Basidiomycete Laccases. Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology 1251:210-215

Jovanovic SV, Tosic M and Simic MG (1991) Use of the Hammett correlation and sigma+ for calculation of one-electron redox potentials of antioxidants. Journal of Physical Chemistry 95:10824-10827

Kharazipour A, Huettermann A and Lüdemann HD (1997) Enzymatic activation of wood fibres as a means for the production of wood composites. Journal of Adhesion Science and Technology 11:419-427

Kilaru S (2006) Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology. Cuvillier Verlag, Göttingen, Germany

Kilaru S, Hoegger PJ, Majcherczyk A, Burns C, Shishido K, Bailey A, Foster GD and Kües U (2006) Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Applied Microbiology and Biotechnology 71:200-210

Kiiskinen LL and Saloheimo M (2004) Molecular cloning and expression in *Saccharomyces cerevisiae* of a laccase gene from the ascomycete *Melanocarpus albomyces*. Applied and Environmental Microbiology 70:137-144

Kjalke M, Andersen MB, Schneider P, Christensen B, Schulein M and Welinder KG (1992) Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. Biochimica et Biophysica Acta 1120:248-256

Klonowska A, Gaudin C, Fournel A, Asso M, Le Petit J, Giorgi M and Tron T (2002) Characterization of a low redox potential laccase from the basidiomycete C30. European Journal of Biochemistry 269:6119-6125

Ko EM, Leem YE and Choi HT (2001) Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. Applied Microbiology and Biotechnology 57:98-102

Lamanda A, Zahn A, Roder D and Langen H (2004) Improved ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. Proteomics 4:599-608

Lapierre L, Bouchard J, Berry RM and Vanlierop B (1995) Chelation prior to hydrogen-peroxide bleaching of kraft pulps - an overview. Journal of Pulp and Paper Science 21:J268-J273

Lee NK, Lee JY, Kwak HG and Paik HD (2008) Perspectives for the industrial use of bacteriocin in dairy and meat industry. Korean Journal for Food Science of Animal Resources 28:1-8

Leonowicz A, Edgehill RU and Bollag JM (1984) The effect of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia praticola* and *Trametes versicolor*. Archives of Microbiology 137:89-96

Lind J, Shen X, Eriksen TE and Merenyi G (1990) The one-electron reduction potential of 4-substituted phenoxyl radicals in water. Journal of the American Chemical Society 112:479-482

Lyashenko AV, Bento I, Zaitsev VN, Zhukhlistova NE, Zhukova YN, Gabdoulkhakov AG, Morgunova FY, Voelter W, Kachalova GS, Stepanova EV, Koroleva GV, Lamzin VS, Tishkov VI, Betzel C, Lindley PF and Mikhailov AM (2006) X-ray structural studies of the fungal laccase from *Cerrena maxima*. Journal of Biological Inorganic Chemistry 11:963-973

Machczynski MC, Vijgenboom E, Samyn B and Canters GW (2004) Characterization of SLAC: A small laccase from *Streptomyces coelicolor* with unprecedented activity. Protein Science 13:2388-2397

Mai C, Kües U and Militz H (2004) Biotechnology in the wood industry. Applied Microbiology and Biotechnology 63:477-494

Majcherczyk A, Johannes C and Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme and Microbial Technology 22:335-341

Matsumura E, Yamamoto E, Numata A, Kawano T, Shin T and Murao S (1986) Structures of the laccase-catalyzed oxidation products of hydroxy benzoic acids in the presence of ABTS (2,2'-

azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)). Agricultural and Biological Chemistry 50:1355-1357

Messerschmidt A (1998) Metal sites in small blue copper proteins, blue copper oxidases and vanadium-containing enzymes. In: Metal Sites in Proteins and Models Redox Centres. Springer Verlag Berlin / Heidelberg, Berlin, 37-68

Messerschmidt A, Rossi A, Ladenstein R, Huber R, Bolognesi M, Gatti G, Marchesini A, Petruzzelli R and Finazziagro A (1989) X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini - analysis of the polypeptide fold and a model of the copper sites and ligands. Journal of Molecular Biology 206:513-529

Mikolasch A and Schauer F (2009) Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Applied Microbiology and Biotechnology 82:605-624

Min KL, Kim YH, Kim YW, Jung HS and Hah YC (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. Archives of Biochemistry and Biophysics 392:279-286

Minuth W, Klischies M and Esser K (1978) Phenoloxidases of ascomycete *Podospora anserina* - structural differences between laccases of high and low molecular weight. European Journal of Biochemistry 90:73-82

Munoz C, Guillen F, Martinez AT and Martinez MJ (1997) Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. Applied and Environmental Microbiology 63:2166-2174

Nagai M, Sato T, Watanabe H, Saito K, Kawata M and Enei H (2002) Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. Applied Microbiology and Biotechnology 60:327-335

Navarro-González M (2008) Growth, fruiting body development and laccase production of selected *Coprini*. Georg-August University of Göttingen, Göttingen, Germany

Nazareth SW and Sampy JD (2003) Production and characterisation of lignocellulases of *Panus tigrinus* and their application. International Biodeterioration & Biodegradation 52:207-214

Novotny C, Svobodova K, Erbanova P, Cajthaml T, Kasinath A, Lang E and Sasek V (2004) Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biology & Biochemistry 36:1545-1551

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A and Sannia G (1997) A novel white laccase from *Pleurotus ostreatus*. Journal of Biological Chemistry 272:31301-31307

Papinutti L and Martinez MJ (2006) Production and characterization of laccase and manganese peroxidase from the ligninolytic fungus *Fomes sclerodermeus*. Journal of Chemical Technology and Biotechnology 81:1064-1070

Perie FH, Reddy GVB, Blackburn NJ and Gold MH (1998) Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens*. Archives of Biochemistry and Biophysics 353:349-355

Piontek K, Antorini M and Choinowski T (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Ångström resolution containing a full complement of coppers. Journal of Biological Chemistry 277:37663-37669

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Applied Microbiology and Biotechnology 57:20-33

Rabilloud T, Strub JM, Luche S, van Dorsselaer A and Lunardi J (2001) Comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. Proteomics 1:699-704

Rauen HM (1964) Biochemisches Taschenbuch, 2. Auflage, Springer Verlag, Germany

Reinhammar B (1972) Oxidation-reduction potentials of the electron acceptors in laccases and stellacyanin. Biochimica et Biophysica Acta 275:245-259

Reinhammar B and Malmstrom BG (1981) "Blue" copper containing oxidases. In: Spiro TG (Ed.) Copper Proteins (Metal Ions in Biology, vol. 3). John Wiley & Sons, New York, 109-149

Roberts P and Jones DL (2008) Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40:1485-1495

Saparrat MCN, Guillen F, Arambarri AM, Martinez AT and Martinez MJ (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. Applied and Environmental Microbiology 68:1534-1540

Scherer M and Fischer R (1998) Purification and characterization of laccase II of *Aspergillus nidulans*. Archives of Microbiology 170:78-84

Schliephake K, Mainwaring DE, Lonergan GT, Jones IK and Baker WL (2000) Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*. Enzyme and Microbial Technology 27:100-107

Schneider P, Caspersen MB, Mondorf K, Halkier T, Skov LK, Ostergaard PR, Brown KM, Brown SH and Xu F (1999) Characterization of a *Coprinus cinereus* laccase. Enzyme and Microbial Technology 25:502-508

Shin KS and Lee YJ (2000) Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*. Archives of Biochemistry and Biophysics 384:109-115

Shleev S, Christenson A, Serezhenkov V, Burbaev D, Yaropolov A, Gorton L and Ruzgas T (2005) Electrochemical redox transformations of T1 and T2 copper sites in native *Trametes hirsuta* laccase at gold electrode. Biochemical Journal 385:745-754

Shleev S, Nikitina O, Christenson A, Reimann CT, Yaropolov AI, Ruzgas T and Gorton L (2007) Characterization of two new multiforms of *Trametes pubescens* laccase. Bioorganic Chemistry 35:35-49

Slomczynski D, Nakas JP and Tanenbaum SW (1995) Production and characterization of laccase from *Botrytis cinerea* 61-34. Applied and Environmental Microbiology 61:907-912

Soares GMB, Amorim MTP, Lageiro M and Costa-Ferreira M (2006) Pilot-scale enzymatic decolorization of industrial dyeing process wastewater. Textile Research Journal 76:4-11

Solomon EI, Sundaram UM and Machonkin TE (1996) Multicopper oxidases and oxygenases. Chemical Reviews 96:2563-2605

Thurston CF (1994) The structure and function of fungal laccases. Microbiology-UK 140:19-26

Tong PG, Hong YZ, Xiao YZ, Zhang M, Tu XM and Cui TJ (2007) High production of laccase by a new basidiomycete, *Trametes* sp. Biotechnology Letters 29:295-301

Wariishi H, Valli K and Gold MH (1992) Manganese(Ii) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium* - Kinetic mechanism and role of chelators. Journal of Biological Chemistry 267:23688-23695

Whiffen LK, Midgley DJ and McGee P.A. (2007) Polyphenolic compounds interfere with quantification of protein in soil extracts using the Bradford method. Soil Biology and Biochemistry 39:691-694

Xiao YZ, Tu XM, Wang J, Zhang M, Cheng Q, Zeng WY and Shi YY (2003) Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. Applied Microbiology and Biotechnology 60:700-707

Xu F, Shin WS, Brown SH, Wahleithner JA, Sundaram UM and Solomon EI (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology 1292:303-311

Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE and Solomon EI (1998) Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. Biochemical Journal 334:63-70

Yaver DS, Overjero MD, Xu F, Nelson BA, Brown KM, Halkier T, Bernauer S, Brown SH and Kauppinen S (1999) Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase Lcc1. Applied and Environmental Microbiology 65:4943-4948

Zhukhlistova NE, Zhukova YN, Lyashenko AV, Zaitsev VN and Mikhailov AM (2008) Three-dimensional organization of three-domain copper oxidases: A review. Crystallography Reports 53:92-109

Chapter 6

Final discussion and prospects

Laccases are enzymes capable of performing various oxidation reactions on different phenolic and aromatic substrates. They mostly occur in the fungal kingdom and are most frequently found and studied in the white-rot fungi and other saprotrophic mostly basidiomycete fungi, which secrete high amounts of these enzymes. By their specific enzymatic properties particularly the enzymes from basidiomycetes are of interest for industrial purposes. According to the varying characteristics of laccases (Baldrian 2006, Kilaru 2006), there exists a broad range of different applications laccases can and have been used for. The utilisation approaches include degradation of toxic aromatic and phenolic compounds, decolourisation of dyes or production of wood composites (section 1.II). Nevertheless, only some of these possible applications have found their way into industrial usage.

Commercially, laccases are usually produced with fungi in submerged fermentation (SmF), where they can grow in a filamentous or pelleted like form (chapter 3). One bottleneck for the use of laccases in industry is the low yield of laccase secretion in natural producing fungal organisms. Although studies were published in which native laccase amounts are in the mg range, as for example in liquid cultures of *Trametes hirsuta* (Koroleva et al. 2002), yields are normally still too low for bulk usage, such as bioremediation and wood composite production. Additionally, the relative slow growth of basidiomycetes in liquid cultures increases fermentation costs for the native laccase production. Thus, for commercial laccases ascomycetes are used as a host for recombinant production in the industry such as for the laccase solution Novozyme® 51003 where an *Aspergillus* species is used as a host for an enzyme originating from *Trametes* sp. (Novozymes A/S, Bagsvaerd, Denmark). However, also native produced laccases can be found on the market such as from *Agaricus bisprous* (ASA Spezialenzyme GmbH, Wolfenbüttel, Germany). Nevertheless of the possibility to use ascomycetous hosts for enzyme production, yields for foreign laccases produced heterologously in filamentous ascomycetes and yeasts as known from many published scientific studies are usually still low (section 2.I). As stated in the literature, several attempts have been conducted to improve laccase yields and reduce production costs. One possibility is the cultivation of the filamentous fungi in solid state fermentation (SSF) with cheap lignocellulosic waste materials as a carrier and substrate (section 2.I).

I. Solid state fermentation (SSF) versus submerged fermentation (SmF) for production of laccases

In this study, industrial SSF cultures of *Pleurotus ostreatus* on wheat straw for mushroom production resulted in laccase and VP (versatile peroxidases) activities of up to 7 U/gds (units per gram dry substrate; comparable to 2 U/ml) and MnP activities of up to 50 U/gds (comparable to 14 U/ml), respectively (section 2.II; Rühl et al. 2008). In a more small laboratory scale, Meza et al. (2007) obtained in SSF cultures of *Pycnoporus cinnabarinus* strain ss3 on sugarcane bagasse an activity of 10 U/gds which was increased to 71 U/gds by addition of ethanol. For *Ganoderma* sp., a much higher laccase activity was achieved with up to 2400 U/gds when cultivated on wheat bran. This yield was further increased by additional carbon and nitrogen sources to values of 10050 U/gds (Revankar et al. 2007). Thus, different studies show that high laccase yields can be obtained with SSF. Nevertheless, also in SmF cultures high yields of laccases are achieved (sections 2.I, 3.I, Couto and Toca-Herrera 2007). Whether SSF or SmF is the favourable technique cannot be decided in general, but varies from fungus to fungus and has to be tested as the cases arise. To gain broader knowledge on this issue, several experiments were conducted on both cultivation types with the same organism.

Different studies compare the growth and enzymatic yields of various filamentous fungi either of ascomycetous or basidiomycetous origin in SSF and SmF cultures, respectively (reviewed by Hölker et al. 2004). Some studies compare the laccase production in both culture techniques conducted with the same strain (Baldrian and Gabriel 2002, Fenice et al. 2003). For example in a study by Téllez-Téllez et al. (2008), *P. ostreatus* biomass and laccase yields were higher in SmF (13.0 AU/ml or 2320 AU/g dry biomass; AU = arbitrary units) compared to SSF (2.4 AU/ml or 540 AU/g dry biomass), which might be due to the high protease activity measured in SSF but lacking in SmF. Nevertheless, *P. ostreatus* had a 50% higher μ_{max} (maximal growth rate) in SSF compared to SmF (Téllez-Téllez et al. 2008). Also in other studies, a higher laccase yield was achieved for *P. ostreatus* in SmF compared to SSF (Ardon et al. 1998, Baldrian and Gabriel 2002). In contrast, for *Lentinula edodes*, *Panus tigrinus* and *P. ostreatus* higher activities were obtained in SSF cultures as compared to SmF (Fenice et al. 2003, Elisashvili et al. 2008, Mazumder et al. 2009).
These data show that production yields of laccases do not only vary between both cultivation techniques (SSF and SmF), but are also influenced e.g. by the substrate used in the SSF, as shown for *P. ostreatus* cultivated on different lignocellulosic waste materials (Elisashvili et al. 2008).

Further, general studies to reveal differences between SSF and SmF were conducted for the industrial important filamentous fungi *Aspergillus niger* and *Aspergillus oryzae*, where the authors studied expression of growth related genes in SSF and SmF (te Biesebeke et al. 2002, 2005a and 2005b, Viniegra-González et al. 2003, Oda et al. 2006). To my knowledge, such analysis are yet missing for basidiomycetes, but approaches exists to gain an insight into differences between secretomes of *P.* ostreatus and *Phanerochaete chrysosporium* cultivated in SmF and SSF (Bernauer et al. 2009, Fragner et al., unpublished).

Primarily, when using bagasse, straw or other lignocellulosic waste materials, SSF may have an economically advantage over SmF (section 2.I). Nevertheless, one bottleneck when using lignocellulosic materials is the purification of the extracellular enzymes, as degradation products (e.g. phenolic compounds) or wash outs from the carrier can hamper the down-stream processing (section 2.II, Rühl et al. 2008). To circumvent this problem, inert carriers can be used, such as a steel mesh or polyurethan foam. When we cultivated *C. cinerea* in an immersion SSF-system (described in section 2.I and Böhmer et al. 2006) for production of laccases, the fungus grew well on the inert carriers perlite and coal. Unfortunately, due to mycelium which was also present in the liquid medium used for the immersion of the carrier, we could not differentiate between laccase activity coming from the mycelium which colonised the carriers or which was present in the liquid medium (data not shown). Future experiments with an improved technical set-up to hinder mycelium reaching the liquid phase of the immersion SSF reactor (RITA®) might help to test the growth and laccase production of *C. cinerea*. Also different laccase isoenzymes and/or isoforms might be expressed in SSF cultures of *C. cinerea* compared to the already studied SmF cultures (section 4.I), as it was done for native laccase production with *P. ostreatus* (Téllez-Téllez et al. 2008).
Currently, in studies of SSF the focus to improve production yields lies mostly on the bioreactor design or process type and not on the fungal organism (Punt et al. 2002). However, future studies should focus on the organism itself to improve our knowledge for usage of filamentous fungi in SSF.

II. Improvement of recombinant laccase production in *Coprinopsis cinerea*

Generally, wildtype strains of filamentous basidiomycetes were used for production of laccases and other ligninolytic enzymes in scientific studies (section 2.I). Nevertheless, also recombinant production of these enzymes in filamentous basidiomycetes exists. There are transformation protocols available for some white-rot fungi such as for *P. ostreatus* (Irie et al. 2001a and 2001b), which was successfully transformed, expressing a homologous manganese peroxidases gene (*mnp2*) under control of the homologous *sdi1*-promoter (Tsukihara et al. 2006). Also laccase genes were successfully transformed into basidiomycetes, such as into the white rot fungi *Pycnoporus cinnabarinus* and *Trametes versicolor* and into the saprotrophic species *Coprinopsis cinerea* where laccase genes were expressed homologously under control of an own or a foreign glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter. Highest yields of 280 U/ml, 1 U/ml and 3 U/ml were reached with transformants of these fungi in liquid cultures, respectively (Alves et al. 2004, Kajita et al. 2004, Kilaru et al. 2006).

Recombinant laccase production in *C. cinerea* and its optimisation has been started in the Göttingen laboratory for Molecular Wood Biotechnology and Technical Mycology by Sreedhar Kilaru (Kilaru 2006). In his work, five promoters, three of them in different fragment length, were tested for their efficiency to homologously express the *C. cinerea* laccase gene *lcc1*. The best promoter was found to be the *gpdII*-promoter of the basidiomycete *A. bisporus* (Kilaru et al. 2006). The clone giving highest laccase yields was used throughout this study for optimisation of laccase activity in the supernatant of shake flask and stirred bioreactor cultures. By changing the media and decreasing the temperature for growth, we could increase the total laccase activity in shake flask cultures up to 3fold (section 3.III). However, the increase in total laccase activity at 25 °C does not result solely from the recombinantly produced enzyme, but to a somewhat small extend from native laccases produced by the fungus at the lower cultivation temperature (section 4.I). Nevertheless, when using a different *C. cinerea* strain for homologous expression of *lcc1* a 3.5 fold increase in recombinant laccase activity could be achieved at the standard cultivation temperature of 37 °C (section 4.II).

In the future, several approaches might be conducted to further increase laccase yields by recombinant production in *C. cinerea* (e.g. further improving cultivation conditions,

optimisation of the production host by selecting better strains for secretion) and to improve laccase properties (e.g. site-directed mutation, randomised mutagenesis).

A. Optimisation of laccase production

The secretion of proteins produced by filamentous fungi occurs most probably at the hyphal tip or the apical region (Wösten et al. 1991, Moukha et al. 1993, Gordon et al. 2000, and reviewed by Conesa et al. 2001). Thus, strains possessing multiple hyphal tips, known as hyperbranching mutants, might positively influence the productivity of filamentous fungi in terms of protein secretion (Bocking et al. 1999, Meyer et al. 2008).

Not only the amount of hyphal tips might play a role, but also the age of the producing hyphae, as it was shown in experiments with *P. chrysosporium*. At the central core area of agar plate colonies of *P. chrysoporium* small hyphae, so called secondary hyphae, showed high production of LiP and MnP (Moukha et al. 1993). In liquid cultures, where *P. chrysosporium* grows in form of mycelial pellets, MnP production occurs mostly at the outer layer of the pellets in the apical part of the hyphae and in chlamydospore like cells present in the subapical region of hyphae in the outer and middle zone of the pellets (Jiménez-Tobon et al. 2003). For the *C. cinerea lcc1* transformant used in this study, we tried to reveal production zones of laccase in the fungal pellets derived from liquid cultures by staining with a laccase substrate. Likely due to the omnipresence of the enzyme throughout the pellet and the cultivation media, no clear differentiation between outer and inner part of the pellet could be determined (data not shown). Whether *in situ* localisation by immunolabelling with antibodies in cross sections of *C. cinerea* pellets might reveal production zones of laccases as it was done for MnP in *P. chrysosporium* pellet (Jiménez-Tobon et al. 2003) has to be tested. It was already shown that polyclonal antibodies are capable to detect the recombinantly produced Lcc1 (Kellner et al. 2007). Also further studies with a *gfp*-expressing transformant (section 3.II) might reveal a better insight into the production pattern of *C. cinerea* pellets and regions of active hyphae. Fig. 1 shows a promising collage of single photos made from a *gfp*-expressing pellet of a *C. cinerea* FA2222 transformant.

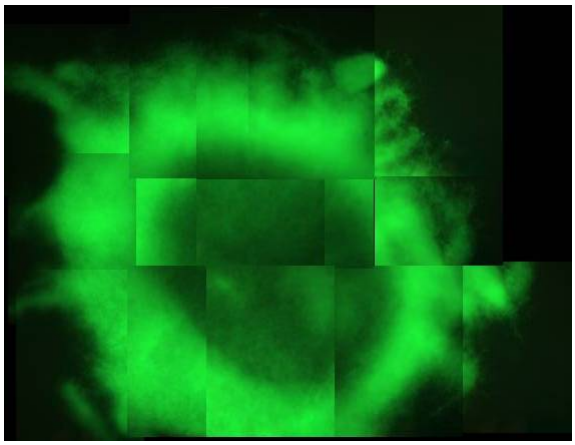


Fig. 1 A pellet of a clone of *C. cinerea* strain FA2222 transformed with the *egfp*-construct pYPH8 (see Fig. 3 in section 3.II) and photographed in single sectors by a CCD camera attached to a microscope (Axioplan 2, Carl Zeiss MicroImaging GmbH, Göttingen).

Several other factors are of interest to enhance protein secretion, such as improvement of the signal sequence of the laccases, and correct glycosylation and folding by the host in case of recombinant protein production (Conesa et al. 2001, Iwashita 2002). For example, O-linked glycosylation is generally important for secretion and N-linked glycosylation for the stability of secreted proteins (Peberdy 1994, Deshpande et al. 2008). Another factor influencing the stability of the secreted proteins are proteases, which might degrade the extracellular enzymes and, thus, abolish an improvement in the secretion amount of an enzyme of interest. Therefore, protease-deficient mutants are of high interest for recombinant production of enzymes in filamentous organisms, such as for example the *Aspergillus* species (Punt et al. 2002, Schmidt 2004). Low or no protease activity might be achieved by applying certain cultivation techniques or genetical modification of the used strain. For example, Xu et al. (2000) described a decrease of protease activity, concomitant with a shift from free mycelium to a pellet growth form, in *A. niger* liquid cultures.

Although *C. cinerea* strains seem to secrete a high amount of different proteases (Lilly et al. 2008, Fragner et al. unpublished) with increasing proteolytic activity in liquid cultures over the time, they seemingly do not degrade homologue proteins, such as the laccase Lcc1 (section 4.II). For heterologous expression this might be different, as shown for *A. niger* (Xu et al. 2000). Nevertheless, this has to be clarified for *C. cinerea*, where studies are in progress for heterologous expression of laccase genes (Grimrath 2007, Spezzia-Mazzocco unpublished). Various *T. versicolor* laccase genes under control of the *A. bisporus gpdII*-promoter were introduced into *C. cinerea* Fa2222, but none of the transformants rise to laccase production. Further studies need to clarify whether this is due to missing protein production or to fast degradation of produced protein.

Different other methods were already tested to improve laccase yields in production processes with filamentous fungi. One of these improvements concerned a better copper transport, as it was shown for *T. versicolor*, where an eightfold increase was obtained when overexpressing a copper-transporting ATPase (*ctaA*) and a copper chaperone protein (*tahA*) (Uldschmid et al. 2002 and 2003).

B. Changing biochemical properties of *C. cinerea* laccases

Laccases of *C. cinerea* secreted naturally into liquid media vary in their composition and amounts (section 4.I), as well as in their properties (chapter 5). Laccases Lcc1 and Lcc5 which are produced natively in higher amounts compared to the other extracellular laccases of the fungus showed better biochemical properties for most tested substrates than laccases Lcc6 and Lcc7, so far not found naturally expressed by monokaryotic strains cultivated in liquid cultures (compare section 4.I and chapter 5). Lcc1 showed best biochemical properties with three of the four substrates and highest stability in presence of the tested inhibitors in comparison to all other tested laccases. Lcc5 was the most stable enzyme in the organic solvents ethanol and acetonitrile (ACN). Nevertheless, also both of the probably not natively produced laccases Lcc6 and Lcc7 showed best properties for some of the tested characteristics: Lcc6 had a high thermal stability and Lcc7 better kinetic properties with the laccase substrate syringaldazine (SGZ) than all other laccases.

Unfortunately up to know, it was only possible to determine the redox potential for the purified laccases Lcc5. The value for Lcc5 of around 540 mV (chapter 5) is similar to the redox potential of the already heterologously produced Lcc1 of *C. cinerea* (540-550 mV; Yaver et al. 1999, Schneider et al. 1999). Whether the laccases Lcc6 and Lcc7 are also in this range has to be clarified in the future. Worth mentioning is that Lcc5 was the most stable laccase tested in the organic solvents ACN and ethanol with remaining 50% of its activity after incubation for 24 h either in 18% ACN or 45% ethanol (chapter 5).

Both laccase properties, stability in organic solvents and redox potential, are in focus of optimisation studies to improve the quality of laccases (Hu et al. 2007, Zumárraga et al. 2008, Festa et al. 2008). Randomised mutagenesis might give rise to new laccases with interesting properties. For example Zumárraga et al. (2007) could improve the stability of the temperature tolerant laccase from *Myceliophthora thermophila* in the organic solvents ACN and ethanol by applying error-prone PCR and site-directed mutagenesis. By in vitro randomised mutagenesis of a plasmid containing a *Fome lignosus* laccase gene, Hu et al.

(2007) obtained a mutant laccase which showed a change in two amino acid residues that resulted in a more hydrophilic water channel of the laccase and, thus, in the higher catalytical efficiency determined for the mutant laccase.

This shows that randomised mutagenesis (e.g. by chemical compounds) followed by adequate screening methods, such as for higher stability in organic solvents, can lead to improved laccase activities. Additionally, also site-directed mutations to replace amino acids were applied e.g. to enhance the redox potential of the T1 site (Xu et al. 1998, Durão et al. 2008), to study the trinuclear copper cluster (Ueki et al. 2006) or to increase stability of the enzyme (Bornscheuer et al. 2002). Whether randomised mutagenesis or site directed mutation is the best choice to change the laccase characteristics has to be decided for each individual case.

III. General conclusions

Further studies to reveal characteristics of growth and protein secretion in SmF and SSF are of highly interest for comparison of both cultivation systems. *C. cinerea* and *P. ostreatus* are adequate organisms for such studies since they can be cultivated in both systems (chapter 2 and 3), their genomes are available (*C. cinerea* at http://www.broad.mit.edu and *P. ostreatus* at http://www.jgi.doe.gov) and molecular techniques for genetic modifications exist (Granado et al. 1997, Irie et al. 2001a and 2001b). Additionally, they possess a ligninolytic system, which makes them capable to grow on lignocellulosic waste materials. Especially in the case of *P. ostreatus*, where standard cultivation techniques for SSF in the industrial range are available, transformation of genes coding for interesting fungal enzymes would be of interest for the biotechnological industry (chapter 2).

For an example, laccase is a good candidate for further studies, since both of the organisms are able to secrete relatively high amounts of this enzyme, which was enhanced in the case of *C. cinerea* by using specific strains and recombinant production (chapter 4). With the characterisation of four laccases coming from the same organism we showed differences in the functionality and stability of the *C. cinerea* laccases (chapter 5). Ongoing studies to produce and characterise the remaining *C. cinerea* laccases will give a closer look into relations between laccase gene sequences and their biochemical characteristics. With this knowledge, enhamcement of the laccase using site directed mutation should be possible.

IV. References

Alves AMCR, Record E, Lomascolo A, Scholtmeijer K, Asther M, Wessels JGH and Wosten HAB (2004) Highly efficient production of laccase by the basidiomycete *Pycnoporus cinnabarinus*. Applied and Environmental Microbiology 70:6379-6384

Ardon O, Kerem Z and Hadar Y (1998) Enhancement of lignin degradation and laccase activity in *Pleurotus ostreatus* by cotton stalk extract. Canadian Journal of Microbiology 44:676-680

Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiology Reviews 30:215-242

Baldrian P and Gabriel J (2002) Variability of laccase activity in the white-rot basidiomycete *Pleurotus ostreatus*. Folia Microbiologica (Praha) 47:385-390

Bernauer T, Herberg FW and Langer E (2009) Analyse des extrazellulären, für die Cellulose- und Ligninhydrolyse verantwortlichen Proteinkomplexes von *Phanerochaete chrysosporium*. Proceedings of the conference "3. Mykologisches Kooloquim: Molekularbiologische Pilzdiagnostik", 7+8.05.2009, Dresden, Germany

Bocking SP, Wiebe MG, Robson GD, Hansen K, Christiansen LH and Trinci APJ (1999) Effect of branch frequency in *Aspergillus oryzae* on protein secretion and culture viscosity. Biotechnology and Bioengineering 65:638-648

Böhmer U, Suhardi SH and Bley T (2006) Decolorizing reactive textile dyes with white-rot fungi by temporary immersion cultivation. Engineering in Life Sciences 6:417-420

Bornscheuer UT, Bessler C, Srinivas R and Krishna SH (2002) Optimizing lipases and related enzymes for efficient application. Trends in Biotechnology 20:433-437

Conesa A, Punt PJ, van Luijk N and van den Hondel CAMJ (2001) The secretion pathway in filamentous fungi: A biotechnological view. Fungal Genetics and Biology 33:155-171

Couto SR and Toca-Herrera JL (2007) Laccase production at reactor scale by filamentous fungi. Biotechnology Advances 25:558-569

Deshpande N, Wilkins MR, Packer N and Nevalainen H (2008) Protein glycosylation pathways in filamentous fungi. Glycobiology 18:626-637

Durão P, Chen ZJ, Silva CS, Soares CM, Pereira MM, Todorovic S, Hildebrandt P, Bento I, Lindley PF and Martins LO (2008) Proximal mutations at the type 1 copper site of CotA laccase: spectroscopic, redox, kinetic and structural characterization of I494A and L386A mutants. Biochemical Journal 412:339-346

Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Kharziani T and Kvesitadze G (2008) *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresource Technology 99:457-462

Fenice M, Giovannozzi SG, Federici F and D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. Journal of Biotechnology 100:77-85

Festa G, Autore F, Fraternali F, Giardina P and Sannia G (2008) Development of new laccases by directed evolution: Functional and computational analyses. Proteins-Structure Function and Bioinformatics 72:25-34

Gordon CL, Khalaj V, Ram AFJ, Archer DB, Brookman JL, Trinci APJ, Jeenes DJ, Doonan JH, Wells B, Punt PJ, van den Hondel CAMJ and Robson GD (2000) Glucoamylase::green fluorescent protein fusions to monitor protein secretion in *Aspergillus niger*. Microbiology-SGM 146:415-426

Granado JD, Kertesz-Chaloupková K, Aebi M, and Kües U (1997) Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Molecular & General Genetics 256:28-36

Grimrath A (2007) Heterologous expression of laccase gene *lcc5* from different strains of *Trametes versicolor* in the basidiomycete *Coprinopsis cinerea*. Master thesis, Georg-August Universität Göttingen, Germany

Hölker U, Hofer M and Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Applied Microbiology and Biotechnology 64:175-186

Hu MR, Chao YP, Zhang GQ, Yang XQ, Xue ZQ and Qian SJ (2007) Molecular evolution of *Fome lignosus* laccase by ethyl methane sulfonate-based random mutagenesis in vitro. Biomolecular Engineering 24:619-624

Irie T, Honda Y, Hirano T, Sato T, Enei H, Watanabe T and Kuwahara M (2001a) Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using *Lentinus edodes* GPD expression signals. Applied Microbiology and Biotechnology 56:707-709

Irie T, Honda Y, Watanabe T and Kuwahara M (2001b) Homologous expression of recombinant manganese peroxidase genes in ligninolytic fungus *Pleurotus ostreatus*. Applied Microbiology and Biotechnology 55:566-570

Iwashita K (2002) Recent studies of protein secretion by filamentous fungi. Journal of Bioscience and Bioengineering 94:530-535

Jiménez-Tobon G, Kurzatkowski W, Rozbicka B, Solecka J, Pocsi I and Penninckx MJ (2003) In situ localization of manganese peroxidase production in mycelial pellets of *Phanerochaete chrysosporium*. Microbiology-SGM 149:3121-3127

Kajita S, Sugawara S, Miyazaki Y, Nakamura M, Katayama Y, Shishido K and Iimura Y (2004) Overproduction of recombinant laccase using a homologous expression system in *Coriolus versicolor*. Applied Microbiology and Biotechnology 66:194-199

Kellner H, Jehmlich N, Benndorf D, Hoffmann R, Rühl M, Hoegger PJ, Majcherczyk A, Kües U, von Bergen M and Buscot F (2007) Detection, quantification and identification of fungal extracellular laccases using polyclonal antibody and mass spectrometry. Enzyme and Microbial Technology 41:694-701

Kilaru S (2006) Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology. Cuvillier Verlag, Göttingen, Germany

Kilaru S, Hoegger PJ, Majcherczyk A, Burns C, Shishido K, Bailey A, Foster GD and Kües U (2006) Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Applied Microbiology and Biotechnology 71:200-210

Koroleva OV, Stepanova EV, Gavrilova VP, Yakovleva NS, Landesman EO, Yavmetdinov IS and Yaropolov AI (2002) Laccase and Mn-peroxidase production by *Coriolus hirsutus* strain 075 in a jar fermentor. Journal of Bioscience and Bioengineering 93:449-455

Lilly WW, Stajich JE, Pukkila PJ, Wilke SK, Inoguchi N and Gathman AC (2008) An expanded family of fungalysin extracellular metallopeptidases of *Coprinopsis cinerea*. Mycological Research 112:389-398

Mazumder S, Basu SK and Mukherjee M (2009) Laccase production in solid-state and submerged fermentation by *Pleurotus ostreatus*. Engineering in Life Sciences 9:45-52

Meyer V, Arentshorst M, van den Hondel CAMJ and Ram AFJ (2008) The polarisome component SpaA localises to hyphal tips of *Aspergillus niger* and is important for polar growth. Fungal Genetics and Biology 45:152-164

Meza JC, Auria R, Lomascolo A, Sigoillot JC and Casalat L (2007) Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. Enzyme and Microbial Technology 41:162-168

Moukha SM, Wosten HAB, Asther M and Wessels JGH (1993) Insitu localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. Journal of General Microbiology 139:969-978

Oda K, Kakizono D, Yamada O, Iefuji H, Akita O and Iwashita K (2006) Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. Applied and Environmental Microbiology 72:3448-3457

Peberdy JF (1994) Protein secretion in filamentous fungi - trying to understand a highly productive black box. Trends in Biotechnology 12:50-57

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J and van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends in Biotechnology 20:200-206

Revankar MS, Desai KM and Lele SS (2007) Solid-state fermentation for enhanced production of laccase using indigenously isolated *Ganoderma* sp. Applied Biochemistry and Biotechnology 143:16-26

Rühl M, Fischer C and Kües U (2008) Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate. Current Trends in Biotechnology and Pharmacy 2: 478-492

Schmidt FR (2004) Recombinant expression systems in the pharmaceutical industry. Applied Microbiology and Biotechnology 65:363-372

Schneider P, Caspersen MB, Mondorf K, Halkier T, Skov LK, Ostergaard PR, Brown KM, Brown SH and Xu F (1999) Characterization of a *Coprinus cinereus* laccase. Enzyme and Microbial Technology 25:502-508

te Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, van Driel KGA, Schutyser MAI, Dijksterhuis J, Zhu Y, Weber FJ, de Vos WM, van den Hondel KAMJ, Rinzema A and Punt PJ (2002) Aspergillus oryzae in solid-state and submerged fermentations - Progress report on a multi-disciplinary project. FEMS Yeast Research 2:245-248

te Biesebeke R, Levin A, Sagt C, Bartels J, Goosen T, Ram A, van den Hondel C and Punt P (2005a) Identification of growth phenotype-related genes in *Aspergillus oryzae* by heterologous macroarray and suppression subtractive hybridization. Molecular Genetics and Genomics 273:33-42

te Biesebeke R, van Biezen N, de Vos WM, van den Hondel CAMJ and Punt PJ (2005b) Different control mechanisms regulate glucoamylase and protease gene transcription in *Aspergillus oryzae* in solid-state and submerged fermentation. Applied Microbiology and Biotechnology 67:75-82

Téllez-Téllez M, Fernandez FJ, Montiel-Gonzalez AM, Sanchez C and Díaz-Godinez G (2008) Growth and laccase production by *Pleurotus ostreatus* in submerged and solid-state fermentation. Applied Microbiology and Biotechnology 81:675-679

Tsukihara T, Honda Y, Watanabe T and Watanabe T (2006) Molecular breeding of white rot fungus *Pleurotus ostreatus* by homologous expression of its versatile peroxidase MnP2. Applied Microbiology and Biotechnology 71:114-120

Ueki Y, Inoue M, Kurose S, Kataoka K and Sakurai T (2006) Mutations at Asp112 adjacent to the trinuclear Cu center in CueO as the proton donor in the four-electron reduction of dioxygen. FEBS Letters 580:4069-4072

Uldschmid A, Engel M, Dombi R and Marbach K (2002) Identification and functional expression of *tahA*, a filamentous fungal gene involved in copper trafficking to the secretory pathway in *Trametes versicolor*. Microbiology-Sgm 148:4049-4058

Uldschmid A, Dombi R and Marbach K (2003) Identification and functional expression of *ctaA*, a P-type ATPase gene involved in copper trafficking in *Trametes versicolor*. Microbiology-Sgm 149:2039-2048

Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SD, Díaz-Godinez G and Augur C (2003) Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal 13:157-167

Wösten HAB, Moukha SM, Sietsma JH and Wessels JGH (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. Journal of General Microbiology 137:2017-2023

Xu JF, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE and Solomon EI (1998) Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. Biochemical Journal 334:63-70

Xu JF, Wang LP, Ridgway D, Gu TY and Moo-Young M (2000) Increased heterologous protein production in *Aspergillus niger* fermentation through extracellular proteases inhibition by pelleted growth. Biotechnology Progress 16:222-227

Yaver DS, Overjero MD, Xu F, Nelson BA, Brown KM, Halkier T, Bernauer S, Brown SH and Kauppinen S (1999) Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase Lcc1. Applied and Environmental Microbiology 65:4943-4948

Zumárraga M, Bulter T, Shleev S, Polaina J, Martinez-Arias A, Plow FJ, Ballesteros A and Alcalde M (2007) In vitro evolution of a fungal laccase in high concentrations of organic cosolvents. Chemistry & Biology 14:1052-1064

Zumárraga M, Camarero S, Shleev S, Martinez-Arias A, Ballesteros A, Plou FJ and Alcalde M (2008) Altering the laccase functionality by in vivo assembly of mutant libraries with different mutational spectra. Proteins-Structure Function and Bioinformatics 71:250-260

Curriculum vitae

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Education

since 11.2004	Scientific research assistant at the section Molecular Wood Biotechnology and Technical Mycology of the Georg-August University of Göttingen, Lower Saxony, Germany Prof. Dr. Ursula Kües
09.2003-08.2004	Diploma thesis at the Max-Planck Institute of Biochemistry in Martinsried (Munich), Bavaria, Germany group of PD Dr. Hermann Heumann Title: „Automatisierung und Optimierung einer autotrophen Fermentationsanlage zur Anzucht des Bakteriums *Ralstonia eutropha*" (Automation and optimisation of an autotrophic fermentation installation for the cultivation of *Ralstonia eutropha*)
10.1999-08.2004	Study of Biotechnology and Bioinformatics at the University of Applied Sciences Weihenstephan, Freising, Bavaria, Germany
07.1998-08.1999	Civil service at the youth rectory of the church district Braunfels, Hesse, Germany
06.1998	Abitur (higher education entrance qualification) at the Gymnasium Nidda, Nidda, Hesse, Germany
1991-1998	Gymnasium Nidda, Nidda, Hesse, Germany
1985-1991	Kurt-Moosdorfschule, Echzell, Hesse, Germany

Internships

09.2002-02.2003: University of Massachusetts Medical School in Worcester, MA

- Analysis on variances in gene expression in schizophrenian tissue and contribution to other neuroscientific projects.
- Scholarship holder of the Carl-Duisberg-Gesellschaft (now Inwent)

03.2001-07.2001: Aventis Pharma Deutschland GmbH in Frankfurt (Main), Germany

- Working on different molecular biology projects and techniques in the departments Core Operations and Gene Expression

08.1999-09.1999: Veterinarian Institute Südhessen in Frankfurt (Main), Germany

- Processing of different microbiological and chemical processes for determination of food quality.

Publications

2009 **Rühl M** and Kües U (2009) Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete *Coprinopsis cinerea*. Current Trends in Biotechnology and Pharmacy 3:241-253

2008 **Rühl M**, Fischer C and Kües U (2008) Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate. Current Trends in Biotechnology and Pharmacy 2:478-492

2007 **Rühl M**, Kilaru S, Navarro-Gonzaléz M, Hoegger PJ, Kharazipour A and Kües U (2007) Production of laccase and other enzymes for the wood industry. In: Kües U (Ed.), *Wood Production, Wood Technology, and Biotechnological Impacts.* Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, 469-507

Rühl M and Kües U (2007) Mushroom production. In: Kües U (Ed.), *Wood Production, Wood Technology, and Biotechnological Impacts.* Universitätsverlag Göttingen, Göttingen, Germany, http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf, 555-586

Kellner H, Jehmlich N, Benndorf D, Hoffmann R, **Rühl M**, Hoegger PJ, Majcherczyk A, Kües U, von Bergen M and Buscot F (2007) Detection, quantification and identification of fungal extracellular laccases using polyclonal antibody and mass spectrometry. Enzyme and Microbial Technology 41:694-701

Rühl M, Kilaru S, Majcherczyk A, Hoegger PJ, Navarro-Gonzaléz M, Chaisaena W, Naumann A, Peddireddi S, Malik I and Kües U (2007) Biotechnological research on basidiomycete fungi. In: Kharazipour A, Müller C and Schöpper C (Eds.), *Review of forests, wood products and wood biotechnology of Iran and Germany*. Implemented within the scope of the special program "German-Arabian/Iranian Education Dialogue" of the German Academic Exchange Service (DAAD), 58-69

2006 Kilaru S, **Rühl M**, Saathoff A, Dwivedi RC, Zomorrodi M, Lange K, Majcherczyk A, Hoegger PJ and Kües U (2006) Overexpression of laccases in *Coprinopsis cinerea*. In: Pisabarro AG and Ramírez L (Eds.) *IV Genetics and Cellular Biology of Basidiomycetes*, Univ. Pública de Navarra, Pamplona, Spain, 123-138

2005 Akbarian S, **Ruehl MG**, Bliven E, Luiz LA, Peranelli AC, Baker SP, Roberts RC, Burnicy WE, Conley RC, Jones EG, Tamminga CA and Guo Y (2005) Chromatin alterations associated with down-regulated metabolic gene expression in the prefrontal cortex of subjects with schizophrenia. Archives of General Psychiatry 62:829-840

Selective posters and oral presentations

2009 Poster „ Laccase and other multicopper oxidase genes in Agaricomycotina" Kües U, **Rühl M**, Hoegger PJ, Navarro-González M, Fragner D and Majcherczyk A; 25th Fungal Genetics Conference; Asilomar; CA; 17. – 22.03.2009

2008 Poster „Studies on the morphology of the filamentous basidiomycete *Coprinopsis cinerea* grown in liquid cultures" **Rühl M**, Richter M, Kilaru S, Lange K and Kües U; 9th European Conference on Fungal Genetics; Edinburgh; 5. – 8.04.2008

Presentation "Cultivation of *Coprinopsis cinerea*: laccase production and growth morphology" **Rühl M**; TU Dresden, Institute of Food Technology and Bioprocess Engineering, Dresden; 13.03.2008

Poster "Optimisation of culture conditions for recombinant laccase production with the basidiomycete *Coprinopsis cinerea*" **Rühl M**, Richter M, Kilaru S, Rittershaus PC, Lange K and Kües U; Annual Conference of the Association for General and Applied Microbiology (VAAM); Frankfurt; 9. – 11.03.2008

2007 Presentation "Submerged cultivation of *Coprinopsis cinerea*: laccase production and growth morphology" **Rühl M**; BRAIN AG, Zwingenberg; 24.10.2007.

Poster "Morphological studies on submerged cultures of the basidiomycete *Coprinopsis cinerea* and secretion of recombinant laccases"; Rühl M, Kilaru S, Rittershaus PC, Hoegger PJ, Lange K and Kües U; Molecular Biology of Fungi; Hamburg; 23. – 26.09.2007

Poster „Abbau von Holzwerkstoffen auf Küstentannenbasis durch Pilze"; Cherdchim B, Navarro-González M, Malik I, Majcherczyk A, Vos H, Kharazipour A, **Rühl M** and Kües U; 10. Symposium Nachwachsende Rohstoffe für die Chemie, Fachagentur für Nachwachsende Rohstoffe; Oldenburg, 27. – 29.03.2007

2006 Poster „Submerged cultivation of *Coprinopsis cinerea* transformants for laccase production"; **Rühl M**, Kilaru S, Lange K, Zomorrodi M, Hoegger PJ, Majcherczyk A and Kües U; Biology of Yeasts and Filamentous Fungi; Bochum; 12. – 14.10.2006

Presentation „Ligninolytic enzymes of *Pleurotus ostreatus*" **Rühl M**, Schöpper C, Kharazipour A and Kües U; Annual Conference of the Association for General and Applied Microbiology (VAAM); Jena; 19. – 22.03.2006

2005 Poster "Laccases from white-rot fungi in the production of wood composites"; **Rühl M**, Schöpper C, Kilaru S, Saathoff A, Majcherczyk A, Hoegger PJ, Müller C, Zomorrodi M, Lange K, Kharazipour A and Kües U; Molecular Biology of Fungi Conference; Bochum; 4. – 7.09.2005

Poster "Overexpression of basidiomycetous enzymes in Coprinopsis cinerea"; Kilaru S, **Rühl M**, Saathoff A, Dwivedi RC, Zomorrodi M, Lange K, Majcherczyk A, Hoegger PJ and Kües U; 23rd Fungal Genetics Conference; Asilomar; CA; 15. – 20.03.2008

2004 Presentation "Cultivation, distribution and markets of edible mushrooms in Germany"; **Rühl M**; 1st International Workshop on Non-Timber Forest Products; Georg-August University, Göttingen, 11. & 12.12.2004

Poster "Niedersächsisches Kompetenznetz für Nachhaltige Holznutzung (NHN) Forschungsprojekte"; **Rühl M** et al.; Zukunftsweisende Umweltforschung - Stiftungslehrstühle der DBU stellen sich vor; ZUK in Osnabrück; 6. & 7.12.2004

Poster "Computer controlled autotrophic fermentation for in vivo enrichment of stabile isotopes (2H, 15N, $13CO_2$) using *Ralstonia eutropha*"; **Rühl M**, Fuß H, Maier C and Heumann H; Poster 2.71; Bioperspectives2004; Wiesbaden; 4. - 6.05.2004