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Characterization and heterologous production of a novel laccase from *Melanocarpus albomyces*

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Abstract

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze oxidation of various substituted phenolic compounds, aromatic amines and even certain inorganic compounds by using molecular oxygen as the electron acceptor. Their substrate versatility makes laccases highly interesting for various applications, including textile dye bleaching, pulp bleaching and bioremediation, where enzymatic catalysis could serve as a more environmentally benign alternative than the currently used chemical processes. However, most laccases studied thus far are not well-suited for the applications because of their low stability at high temperatures or pH values. This work focused on identifying and characterizing novel fungal laccases having potential for the applications as well as on development of efficient production methods for laccases.

Laccase-producing fungi were screened from various environmental samples by plate tests using the indicator compounds guaiacol, tannic acid and the polymeric dyes Remazol brilliant blue R and Poly R-478. A total of 26 positive fungal strains were isolated, and their ability to produce laccase was studied in liquid media. Four fungal strains produced significant amounts of laccase, and these enzymes were preliminarily characterized. The novel laccases were found to be rather typical basidiomycete laccases, although they had notably high thermostabilities as compared to other fungal laccases.

A novel laccase from the ascomycete *Melanocarpus albomyces* was purified and biochemically characterized. The substrate specificity and susceptibility towards inhibitors were shown to be typical for laccases. Spectral data measured for the purified laccase indicated that the characteristic three types of copper were present. Interestingly, *M. albomyces* laccase showed good thermostability and it had a pH optimum at neutral pH with phenolic substrates. Both of these are unusual properties for fungal laccases. The crystal structure of *M. albomyces*

laccase containing all four copper atoms was resolved at 2.4 Å resolution. The overall structure was shown to consist of three cupredoxin-like domains, similarly to other blue copper oxidases. Surprisingly, elongated electron density was observed in the trinuclear center, indicating binding of a dioxygen molecule with a novel geometry. In addition, an exceptional C-terminal end, which protrudes into the active site of the enzyme, was detected.

The gene encoding *M. albomyces* laccase was isolated and it was shown to encode a protein of 623 amino acids. The level of homology of the laccase was about 60-70% with laccases from other ascomycetes and about 30% with basidiomycete laccases. Maturation of *M. albomyces* laccase was shown to consist of the removal of a putative signal sequence, a propeptide and a C-terminal extension. *M. albomyces* laccase cDNA was expressed in *Saccharomyces cerevisiae* under the inducible *GAL1* promoter. Very low laccase production was detected with the expression construct containing laccase cDNA with its own signal and propeptide sequences. The production was significantly improved by replacing these with the prepro-sequence of the *S. cerevisiae* α-factor gene. Further six-fold improvement in the production level was obtained by introducing a stop codon into the cDNA after the native C-terminal processing site. These results suggested that correct post-translational processing was essential for efficient production of *M. albomyces* laccase in *S. cerevisiae*.

M. albomyces laccase was also expressed in the filamentous fungus *Trichoderma reesei*. The laccase was expressed as a non-fused laccase and as a fusion protein that contained the *T. reesei* hydrophobin I protein at the N-terminus. About five times higher activity levels were obtained with the non-fused laccase than with the fusion protein in shake flask cultures. Analyses of transformants from both expression constructs indicated that production of the fusion protein was limited at the post-transcriptional level by proteolytic degradation and inefficient secretion. No induction of the unfolded response pathway by laccase production was detected in the transformants. The unmodified recombinant *M. albomyces* laccase was produced in batch and fed-batch fermentations and the production level of 920 mg l^{-1} in the fed-batch cultivation was the highest heterologous laccase production level hitherto reported. Recombinant *M. albomyces* laccase was purified and biochemically characterized and it was shown to be very similar to the native laccase.

This work also showed for the first time that a laccase can adsorb on cellulose, as *M. albomyces* laccase was shown to bind to lignocellulose and purified cellulose. The binding isotherm obtained with bacterial microcrystalline cellulose fitted well the Langmuir type one-site binding model. The adsorption parameters obtained from the model indicated that *M. albomyces* laccase binds to cellulose very efficiently but with a relatively low binding capacity. The binding was shown to be reversible and not influenced by non-specific protein or the presence of salt. No binding was detected with laccases from *Trametes hirsuta* or *Mauginiella* sp., which suggests that binding to cellulose is not a common feature among laccases.

Preface

The work described in this thesis was carried out at VTT Biotechnology during the years 2000–2004. I wish to thank Professor Juha Ahvenainen, Professor Liisa Viikari and Research Manager Richard Fagerström for offering me the opportunity to work at the excellent facilities of VTT Biotechnology. Professor Matti Leisola at Helsinki University of Technology is thanked for his support and advices during my work. Dr. Taina Lundell and Dr. Leif Jönsson are thanked for reviewing my thesis and for providing valuable comments on how to improve it. Financial support from Neste Oy Foundation is gratefully acknowledged.

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My colleagues and all the personnel in the laboratory are acknowledged for creating a pleasant working atmosphere. I had the chance to work in two different laboratories at VTT Biotechnology during this work and I really enjoyed working in both of them. My special thanks are due to Outi Liehunen, Riitta Nurmi and Seija Nordberg for their skillful and patient technical assistance. M.Sc. Hanna Kontkanen, Dr. Martina Andberg and M.Sc. Pasi Halonen are thanked for all their help in scientific issues and, above all, for their friendship.

My warmest gratitude I wish to express to Sami for his love and support. Our son Konsta is thanked for always helping me to remember that the most important things in life are found outside the laboratory.

List of publications

This thesis consists of an overview and of the following 6 publications which are referred to in the text by Roman numerals I–VI:

I. Kiiskinen, L.-L., Rättö, M. and Kruus, K. (2004) Screening for novel laccase-producing microbes. Journal of Applied Microbiology 97:640–646.

II. Kiiskinen, L.-L., Viikari, L. and Kruus, K. (2002) Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. Applied Microbiology and Biotechnology 59:198–204.

III. Hakulinen, N., Kiiskinen, L.-L., 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.

IV. Kiiskinen, L.-L. 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.

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

VI. Kiiskinen, L.-L., Palonen, H., Linder, M., Viikari, L. and Kruus, K. (2004) Laccase from *Melanocarpus albomyces* binds effectively to cellulose. FEBS Letters 576:251–255.

Contents

Appendices of this publication are not included in the PDF version.
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List of symbols

ABTS	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
BMCC	bacterial microcrystalline cellulose
BSA	bovine serum albumin
CBD	cellulose-binding domain
CBH	cellobiohydrolase
CDH	cellobiose dehydrogenase
2,6-DMP	2,6-dimethoxyphenol
EPR	electron paramagnetic resonance
ER	endoplasmic reticulumn
HFB	hydrophobin
MW	molecular weight
MALDI-TOF	matrix assisted laser desorption/ionization –time of flight
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
RBBR	Remazol brilliant blue R
SPS	steam-pretreated softwood
UPR	unfolded protein response

1. Introduction

Proteins that contain copper atoms as cofactors are crucial for numerous reactions in cellular metabolism. They are involved in photosynthesis, oxidative phosphorylation, metal ion homeostasis and catabolism of various nutrients and toxic chemical compounds. The incorporation of copper atoms in protein structures allows the proteins to perform electron transfer reactions involved in the above-mentioned processes, because copper atoms are able to switch their oxidation states between Cu^{I} and Cu^{II}. The protein structure functions as a complex polymeric ligand for the catalytically active coppers, providing them with a coordination environment where switches between the reduction states are thermodynamically feasible. The structurally simplest copper-containing proteins, such as plant plastocyanins and bacterial azurins, are typically electron-carriers involved in electron-transfer reaction chains. More complex copper proteins are generally oxidoreductases, i.e. enzymes that catalyze oxidation/reduction reactions. Examples of these are microbial galactose oxidase, laccase and nitrite reductase, mammalian ceruloplasmin and plant ascorbate oxidase.

Several copper-containing enzymes have more than one copper atom in the active center. The combination of various copper sites in one protein molecule allows the enzyme to catalyze reactions that involve the transfer of several electrons at a time. This is especially important when molecular oxygen is used as an electron acceptor in the catalytic cycle, since oxygen derivatives generated by single electron transfers are highly detrimental to the cell. Multicopper oxidases typically contain two or four copper atoms per protein molecule and they catalyze oxidation reactions in which electrons are removed from the reducing substrate molecules and transferred to oxygen to form water or hydrogen peroxide. Examples of multicopper oxidases are ceruloplasmin, ascorbate oxidase, ferredoxin, phenoxazinone synthase, bilirubin oxidase and laccase. Ceruloplasmin (EC 1.16.3.1) is a ferredoxin which is essential for iron homeostasis in plasma. Its corresponding representative in yeast has been shown to be yeast ferredoxin Fet3p (Hasset et al. 1998). Plant ascorbate oxidase (EC 1.10.3.3) is apparently involved in balancing the reduction potential in growing cells, but its actual function is not yet known (Pignocchi et al. 2003). Laccases (*p*-benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are multicopper oxidases present mainly in plants and fungi. They are structurally homologous to

ceruloplasmin and ascorbate oxidase and are interesting as model enzymes for multicopper oxidases. Laccases are also of particular interest with regard to potential industrial applications, because of their capability to oxidize a wide range of industrially relevant substrates.

1.1 Distribution of laccases and their physiological roles

Laccases are common enzymes in nature, and they are found widely in plants and fungi as well as in some bacteria and insects. The first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree (review: Reinhammar 1984), from which the designation laccase was derived. Laccases have subsequently been discovered from numerous other plants, for example sycamore (Bligny and Douce 1983), poplar (Ranocha et al. 1999), tobacco (De Marco and Roubelakis-Angelakis 1997) and peach (Lehman et al. 1974). Plant laccases are found in the xylem, where they presumably oxidize monolignols in the early stages of lignification (Bao et al. 1993; O'Malley et al. 1993; Mayer and Staples 2002; Gavnholt and Larsen 2002). In addition, laccases have been shown to be involved in the first steps of healing in wounded leaves (De Marco and Roubelakis-Angelakis 1997). Detection and purification of plant laccases is often difficult because crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha et al. 1999), which is probably the reason why detailed information about the biochemical properties of plant laccases is limited. However, *Rhus vernicifera* laccase is an exception which has been extensively studied, especially with regard to its spectroscopic properties (e.g. Malmström et al. 1970; Woolery et al. 1984). *R. vernicifera* laccase has also widely been used in investigations of the general reaction mechanism of laccases (Lee et al. 2002; Battistuzzi et al. 2003; Johnson et al. 2003).

The majority of laccases characterized so far have been derived from fungi, especially from white-rot basidiomycetes that are efficient lignin degraders. Well-known laccase-producers include fungi such as *Agaricus bisporus* (Wood 1980), *Botrytis cinerea* (Marbach et al. 1984), *Chaetomium thermophilum* (Chefetz et al. 1998), *Coprinus cinereus* (Schneider et al. 1999), *Neurospora crassa* (Froehner and Eriksson 1974), *Phlebia radiata* (Niku-Paavola et al. 1988), *Pleurotus ostreatus* (Sannia et al. 1986), *Pycnoporus cinnabarinus* (Eggert et al. 1996b) and *Trametes (Coriolus, Polyporus) versicolor* (Rogalski et

al. 1991). The physiological roles of fungal laccases are various. Laccases from white-rot fungi, such as *Trametes versicolor* and *Pycnoporus cinnabarinus*, participate in lignin biodegradation, where they mainly oxidize the phenolic subunits of lignin (Bourbonnais and Paice 1990; Eggert et al. 1996a; Eggert et al. 1996b; Thurston 1994; Hatakka 2001). In plant-pathogenic fungi, laccases are important virulence factors. The grapevine grey mould, *Botrytis cinerea*, produces a laccase that is necessary for pathogenesis, and the role of the laccase is presumably related to detoxification of toxic defence metabolites produced by the plant (Bar-Nun et al. 1988). Laccases have also been shown to be important for pathogenesis in the chestnut blight fungus *Cryphonectria parasitica* (Rigling and van Alfen 1991; Choi et al. 1992; Mayer and Staples 2002) and in the human pathogen *Cryptococcus neoformans* (Williamson 1994). In *Aspergillus nidulans*, laccase activity is related to pigment production, and deletion of the laccase gene *yA* abolishes the green color of conidial spores (Clutterbuck 1972; Aramayo and Timberlake 1993; Adams et al. 1998). Laccases have also been proposed to participate in fungal morphogenesis in *Armillaria* spp. (Worral et al. 1986), *Lentinus edodes* (Leatham and Stahmann 1981) and *Volvariella volvacea* (Chen et al. 2004).

Only a few bacterial laccases have been described hitherto. The first bacterial laccase was detected in the plant root-associated bacterium *Azospirillum lipoferum* (Givaudan et al. 1993), where it was shown to be involved in melanin formation (Faure et al. 1994). An atypical laccase containing six putative copper-binding sites was discovered from *Marinomonas mediterranea*, but no functional role has been assigned to this enzyme (Solano et al. 1997; Sanchez-Amat et al. 2001). *Bacillus subtilis* produces a thermostable CotA laccase which participates in pigment production in the endospore coat (Martins et al. 2002). Laccases have recently also been found from *Streptomyces cyaneus* (Arias et al. 2003) and *Streptomyces lavendulae* (Suzuki et al. 2003). In addition to plants, fungi and bacteria, laccases or laccase-like activities have been found in some insects, where they have been suggested to be active in cuticle sclerotization (Sugumaran et al. 1992; Dittmer et al. 2004).

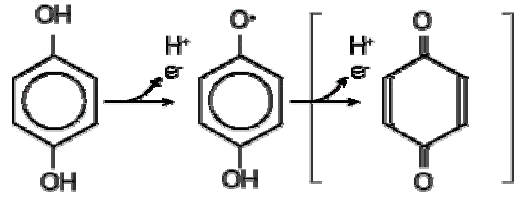
1.2 Structure and catalytic mechanism of laccases

The overall fold of laccases comprises three cupredoxin-like domains A, B and C, that are about equal in size (Figure 1; Ducros et al. 1998; Bertrand et al. 2002; Piontek et al. 2002; Enguita et al. 2003). The cupredoxin fold is common among copper-containing proteins, and it has also been found in the simple copper proteins plant plastocyanin (Guss and Freeman 1983; Inoue et al. 1999) and bacterial azurin (Norris et al. 1983), as well as in the more complex multicopper oxidases ascorbate oxidase (Messerschmidt et al. 1992) and ceruloplasmin (Zaitseva et al. 1996; Murphy et al. 1997). All three domains are important for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains B and C, a mononuclear copper center is located in domain C, and a trinuclear copper center is located at the interface between domains A and C (Figure 1). The mononuclear copper center contains one type-1 (T1) copper atom that is trigonally coordinated to two histidines and a cysteine. The coordination bond between T1 and S_{Cys} is highly covalent, which causes a strong absorption around 600 nm and gives laccases their typical blue color (Solomon et al. 1996). T1 also has an distant axial ligand which is a leucine or phenylalanine residue in fungal laccases (Ducros et al. 1998; Bertrand et al. 2002; III; Piontek et al. 2002) and a methionine residue in the bacterial *Bacillus subtilis* CotA laccase and in other multicopper oxidases (Enguita et al. 2003; Messerschmidt 1997). The trinuclear cluster contains one type-2 (T2) copper atom and a pair of type-3 (T3) coppers (Messerschmidt 1997). The T2 copper is coordinated by two and the T3 copper atoms by six conserved histidines (Bertrand et al. 2002; III; Piontek et al. 2002). The T1 and T2 coppers are paramagnetic and can be identified in electron paramagnetic resonance (EPR) spectrum. The T3 copper pair is antiferromagnetically coupled by a bridging hydroxide, which makes the T3 coppers EPR-silent (Solomon et al. 1996). However, they can be detected by their characteristic absorbance at 330 nm (Solomon et al. 1996).

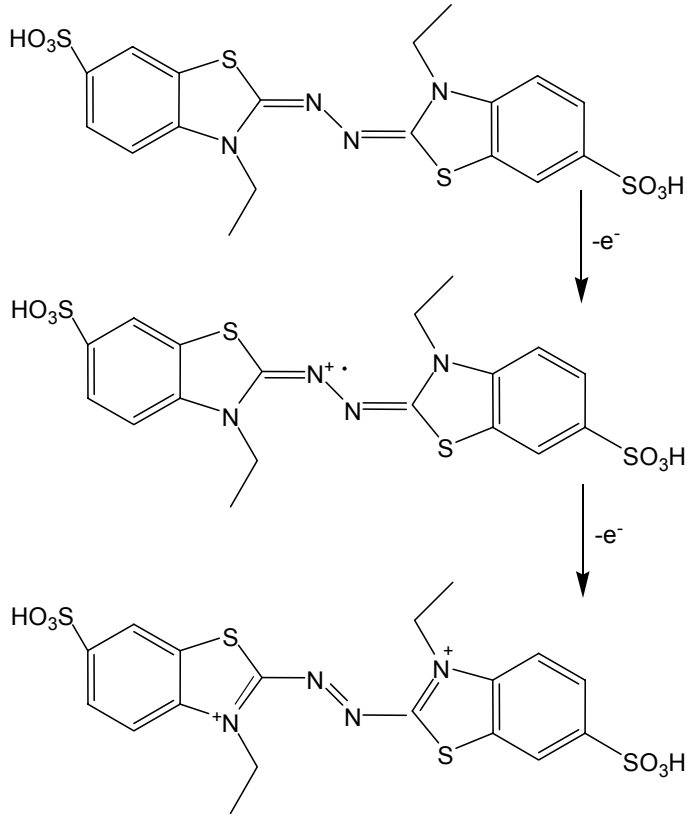
Figure 1. Three-dimensional structure of M. albomyces *laccase (III, reprinted with permission from Nature Publishing Group). Domains A, B, and C are colored red, green and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks.*

Laccases are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize diphenols and related substances and use molecular oxygen as an electron acceptor. In contrast to most enzymes, which are generally very substrate specific, laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols, and even some inorganic compounds such as iodine (Xu 1996). When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical (Kersten et al. 1990; Thurston 1994). The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerisation (Thurston 1994). Figure 2 shows the schematic laccase-catalyzed oxidation of a *p*-diphenol (A) and the commonly used nonphenolic laccase substrate ABTS [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] (B). Other well-known substrates for laccases include syringaldazine (3,5-

dimethoxy-4-hydroxybenzaldehyde azine), 1-naphthol, *p*-cresol (1-hydroxy-4-methylbenzene), 2,6-dimethoxyphenol and guaiacol (2-methoxyphenol).



A



B

Figure 2. (A) Laccase-catalyzed oxidation of a diphenol (modified from Thurston 1994). (B) Oxidation of ABTS by laccase (modified from Potthast et al. 2001).

When oxidized by laccase, the substrate donates an electron to the T1 copper. The reduction of oxygen takes place in the trinuclear copper center which is located about 12 Å away from T1 (Bertrand et al. 2002; Piontek et al. 2002). One catalytic cycle involves the transfer of altogether four electrons, which are carried from T1 to the T2/T3 cluster presumably through a conserved His-Cys-His tripeptide (Messerschmidt et al. 1992; Bertrand et al. 2002; Piontek et al. 2002). The reaction mechanism of laccases has been studied intensively by monitoring the coordination states of the coppers during the reaction cycle by spectroscopical methods, such as EPR, magnetic circular dichroism (MCD) and X-ray absorption spectroscopy (XAS); however, particularly the mechanism of oxygen reduction in the trinuclear center is still unclear (Cole et al. 1990; Shin et al. 1996; Solomon et al. 1996; Lee et al. 2002). The first step of the catalytic cycle of laccases involves the formation of a fully reduced laccase in which all four coppers are in a reduced state (Shin et al. 1996; Solomon et al. 1996; Lee et al. 2002). Molecular oxygen then oxidizes the fully reduced laccase, presumably via a peroxy intermediate, and is reduced to water (Shin et al. 1996; Solomon et al. 1996; Lee et al. 2002). According to Lee et al. (2002), the oxidation of the peroxy intermediate generates an oxygen-activated native intermediate laccase, in which all four coppers are in oxidized form and the three trinuclear copper atoms are all bridged by hydroxide or oxo groups. This bridging makes the native intermediate prone to reduction and it can quickly enter another catalytic cycle (Lee et al. 2002). In contrast to the native intermediate, the resting oxidized laccase is proposed to have a T2 copper that is electronically isolated from the two T3 coppers, and the resting oxidized form is reduced by substrates at a much slower rate than the native intermediate (Lee et al. 2002). The native intermediate slowly transforms into the resting oxidized form in the absence of reducing substrates.

The suitability of a chemical compound as a laccase substrate depends on two factors. Firstly, the substrate must dock at the T1 copper site, which is mainly determined by the nature and position of substituents on the phenolic ring of the substrate, especially those with bulky side chains (Xu 1996; Bertrand et al. 2002). Secondly, the redox-potential (E^0) of the substrate must be low enough, because the rate of a laccase-catalyzed reaction has been shown to depend on the difference between the redox-potentials of the enzyme and the substrate, ΔE^0[laccase-substrate] (Xu 1996; Xu et al. 1996; Xu et al. 2000; Xu et al. 2001). The redox-potential of the substrate is determined by its chemical structure, and

different substituents have different impacts on the E^0[substrate] depending on their propensity to withdraw or donate electrons (Xu 1996). Methoxy substituents, for example, are electron-donating and increase the electron density at the phenoxy group, thus making it more readily oxidized (Xu 1996; Garzillo et al. 1998).

The redox-potentials of laccases vary from 0.4 to 0.8 V, and the most critical factor determining the E^0[laccase] is the coordination sphere of the T1 copper (Xu et al. 1996; Palmer et al. 1999; Xu et al. 1999). The axial ligand has been proposed to be especially important for the redox-potential, because other multicopper oxidases that have a coordinating Met in this position have lower redox-potentials than laccases, in which the axial ligand is usually a non-coordinating phenylalanine or leucine residue (Palmer et al. 1999; Xu et al. 1999). This was studied by mutating the corresponding Phe to Met in *Trametes villosa* laccase, and the results showed that the E^0 of the mutated laccase was indeed lowered by 0.1 V (Xu et al. 1999). The nature of the non-coordinating axial ligand has also been suggested to be important, because high E^0 laccases generally have a Phe at this position whereas a Leu is usually found in the low E^0 laccases (Eggert et al. 1998). This hypothesis has been studied by mutating the corresponding Leu to Phe in the low redox-potential *Myceliophthora thermophila* and *Rhizoctonia solani* laccases (Xu et al. 1998), and vice versa in the high E^0 *Trametes villosa* laccase (Xu et al. 1999). However, these mutations did not have any effect on the redox-potentials of the enzymes (Xu et al. 1998; Xu et al. 1999). On the basis of these results and the three-dimensional structure of *Trametes versicolor* laccase, Piontek et al. (2002) recently suggested that the E^0[laccase] is actually determined by a relatively large network of interactions, such as hydrogen bonds around the T1 site, which affect the bond lengths between the coordinating N_{His} and the T1 copper atom. According to Piontek et al. (2002), hydrogen bonds that stretch a coordinating histidine residue away from the T1 may decrease the electron density of the copper atom, thus making it more electron deficient. In addition, it must be taken into account that other factors in the vicinity of the T1 site, such as solvent accessibility and charge distribution, may contribute to the oxidation potential of laccases (Xu et al. 1996; Garzillo et al. 2001). Recently it was also reported that the expression host affected the E^0 of *Pycnoporus cinnabarinus* laccase, and it was suggested that this resulted from differences introduced during protein folding or glycosylation (Sigoillot et al. 2004).

Coordination chemistry of the coppers is also related to the impact mechanism of many laccase inhibitors. The most effective laccase inhibitors are small anions, especially azide, cyanide, and fluoride ions, which bind to the trinuclear copper center and interfere with the electron flow (Solomon et al. 1996; Xu 1996, Battistuzzi et al. 2003; Johnson et al. 2003). Other laccase inhibitors include EDTA, fatty acids, tropolone, kojic acid and coumaric acid, but their inhibitory concentrations are generally higher than those of the small anions (Wood 1980, Bollag and Leonowicz 1984; Faure et al. 1995; Eggert et al. 1996b; Chefetz et al. 1998; Sethuraman et al. 1999; Xu 1999; Jung et al. 2002). Many sulfhydryl-containing compounds, such as L-cysteine, dithiothreitol and thioglycolic acid, have also often been considered as laccase inhibitors. However, when the effect of inhibitors has been further studied by the oxygen consumption method instead of absorbance measurements, it has been found that the observed inhibition was actually caused by reduction of the oxidized substrate by the sulfhydryl compounds and not by inhibition of the enzyme (Johannes and Majcherczyk 2000).

1.3 Biochemical properties of laccases

The catalytic action of an enzyme is quantitatively described by the Michaelis constant K_m and the catalytic efficiency constant k_{cat}. These constants have been measured for a large number of laccases, and rather great variance can be observed among them (Table 1). The K_m values of laccases are generally in the range of 2–500 μM depending on the enzyme source and the reducing substrate (Table 1). The lowest K_m values have been measured with syringaldazine, which is a dimer of two molecules of 2,6-dimethoxyphenol linked by an azide bridge. Either the azide bridge or the dimer form is apparently beneficial for the affinity of syringaldazine to laccases, because the K_m values measured for monomeric 2,6-dimethoxyphenol are generally higher than those obtained with syringaldazine (Table 1). The comparison of K_m values also shows that laccases from different source organisms have different substrate preferences (Xu et al. 1996). The specificity for oxygen is less dependent on the enzyme, and K_m values of 20–50 μM for O_2 have been reported for several laccases (Yaver et al. 1999; Xu 2001).

Very significant variance has also been observed in the catalytic efficiencies (k_{cat}) of various laccases. Differences as high as 3500-fold can be seen in the k_{cat} values between different laccases with the same substrates (Table 1). On the other hand, the k_{cat} values for a single laccase do not generally differ more than 2–10-fold between different substrates, which reflects the fact that k_{cat} describes the rate of the electron-transfer reactions taking place inside the enzyme after substrate binding (Xu 2001). This can be seen, for example, for laccases from *Pleurotus sajor-caju*, *Trametes pubescens* and *Trametes trogii* in Table 1. However, the variance in assay conditions must always be taken into account when the catalytic constants measured in different laboratories are compared. The constants in Table 1 have been measured in varying pH, ionic strength and temperature conditions and using different protein concentrations, all of which have a great effect on the results. In addition, different molar extinction coefficients for oxidation products have sometimes been used in spectrophotometric assays, because the nature of the actual oxidation products is often complex or poorly understood. This affects particularly the numerical values of k_{cat}.

Table 1. Kinetic constants of laccases. The pH-values at which the constants have been measured are also included.

Substrate	K_m (μM)	k_{cat} (min^{-1})	pH	Laccase	Reference
ABTS	14	41400	3	*Trametes pubescens* LAP2	Galhaup et al. 2002a
	23	1090	5.5	*Coprinus cinereus* Lcc1	Schneider et al. 1999
	30	198	3.4	*Trametes trogii* POXL3	Garzillo et al. 1998
	32	n.r.*	3	*Panaeolus sphinctrinus*	Heinzkill et al. 1998
	41	n.r.	5	*Coprinus friesii*	Heinzkill et al.1998
	45	620	5.5	*Trichophyton rubrum*	Jung et al. 2002
	50.6	n.r.	3	*Panaeolus papilionaceus*	Heinzkill et al. 1998
	52	n.r.	5.3	*Rhizoctonia solani* Lcc4	Xu et al. 1996
	55	n.r.	4	*Pycnoporus cinnabarinus* Lac1	Record et al. 2002
	58	2700	5.3	*Trametes villosa* Lcc1	Xu et al. 1996
	90	350000	3	*Pleurotus ostreatus* POXA1	Palmieri et al. 1997
	106	1000	4	*Bacillus subtilis* CotA	Martins et al. 2002
	120	n.r.	3	*Pleurotus ostreatus* POXA2	Palmieri et al. 1997

	190	n.r.	6	*Chaetomium thermophilum*	Chefetz et al. 1998
	280	57000	3	*Pleurotus ostreatus* POXC	Palmieri et al. 1997
	290	790	6	*Myceliophthora thermophila* Lcc1	Bulter et al. 2003
	380	n.r.	4.5	*Streptomyces cyaneus*	Arias et al. 2003
	2500	74000	3.3	*Pleurotus sajor-caju* Lac4	Soden et al. 2002
2,6-DMP	26	n.r.	4.5	*Gaeumannomyces graminis* LAC2	Edens et al. 1999
	72	24000	3	*Trametes pubescens* LAP2	Galhaup et al. 2002a
	96	n.r.	6	*Chaetomium thermophilum*	Chefetz et al. 1998
	100	n.r.	3.5	*Botrytis cinerea*	Slomczynski et al. 1995
	120	58000	6	*Pleurotus sajor-caju* Lac4	Soden et al. 2002
	410	109	3.4	*Trametes trogii* POXL3	Garzillo et al. 1998
	230	430	5	*Pleurotus ostreatus* POXC	Palmieri et al. 1997
	740	n.r.	6.5	*Pleurotus ostreatus* POXA2	Palmieri et al. 1997
	2100	21000	5	*Pleurotus ostreatus* POXA1	Palmieri et al. 1997
Guaiacol	66	6800	6.5	*Pleurotus sajor-caju* Lac4	Soden et al. 2002
	36	10800	3	*Trametes pubescens* LAP2	Galhaup et al. 2002a
	400	n.r.	6	*Chaetomium thermophilum*	Chefetz et al. 1998
	510	n.r.	4.5	*Gaeumannomyces graminis* LAC2	Edens et al. 1999
	1200	150	6	*Pleurotus ostreatus* POXC	Palmieri et al. 1997
	3100	n.r.	6	*Pleurotus ostreatus* POXA2	Palmieri et al. 1997
	5120	115	3.4	*Trametes trogii* POXL3	Garzillo et al. 1998
Syringald-azine	1.6	2100	6	*Myceliophthora thermophila* Lcc1	Bulter et al. 2003
	3.9	3000	5.3	*Trametes villosa* Lcc1	Xu et al. 1996
	6	16800	4.5	*Trametes pubescens* LAP2	Galhaup et al. 2002a
	26	180	5.5	*Coprinus cinereus* Lcc1	Schneider et al. 1999
	26	200	6	*Bacillus subtilis* CotA	Martins et al. 2002
	28	n.r.	5.3	*Rhizoctonia solani* Lcc4	Xu et al. 1996

34	n.r.	6	*Chaetomium thermophilum*	Chefetz et al. 1998
20	23000	6	*Pleurotus ostreatus* POXC	Palmieri et al. 1997
130	28000	6	*Pleurotus ostreatus* POXA1	Palmieri et al. 1997
140	n.r.	6	*Pleurotus ostreatus* POXA2	Palmieri et al. 1997
280	35000	6.5	*Pleurotus sajor-caju* Lac4	Soden et al. 2002

*n.r., not reported

In addition to the kinetic constants, the catalytic performance of laccases is described by their activity and stability in different pH and temperature conditions. The pH activity profiles of laccases are often bell-shaped, with optima around 4–6, when measured with phenolic substrates (Hoffmann and Esser 1977; Palmieri et al. 1993; Eggert et al. 1996b; Xu 1997; Chefetz et al. 1998; Schneider et al. 1999; Garzillo et al. 2001). The decrease in laccase activity in neutral or alkaline pH values is affected by increasing hydroxide anion inhibition, because as a small anion, hydroxide ion is also a laccase inhibitor (Xu 1997). On the other hand, the increasing pH decreases the redox potential of the phenolic substrate, which makes the substrate more susceptible to oxidation by laccase (Xu 1997). The bell-shaped pH profile is thus the result of two opposite effects: increasing ΔE^0[laccase-substrate] and inhibition by hydroxide anion (Xu 1997). Oxidation of non-phenolic substrates, such as ABTS, does not involve proton exchange, and therefore nearly monotonic pH activity profiles with highest activities at pH values of 2–3 are obtained (Hoffmann and Esser 1977; Xu 1997; Garzillo et al. 2001). In contrast to their activity, the stability of laccases is generally highest at pH values around 8–9 (Nishizawa et al. 1995; Xu et al. 1996; Chefetz et al. 1998).

Temperature stabilities of laccases vary considerably, depending on the source organism. In general, laccases are stable at 30–50°C and rapidly lose activity at temperatures above 60°C (Wood 1980; Xu et al. 1996; Chefetz et al. 1998; Heinzkill et al. 1998; Schneider et al. 1999; Galhaup et al. 2002a; Jung et al. 2002; Palonen et al. 2003). The most thermostable laccases have been isolated from bacteria; the half-life of *Streptomyces lavendulae* laccase was 100 minutes at 70°C (Suzuki et al. 2003) and that of *Bacillus subtilis* CotA was 112 minutes at 80°C (Martins et al. 2002). The typical half-lives of fungal laccases are clearly

below one hour at 70°C and below 10 minutes at 80°C (Wood 1980; Nishizawa et al. 1995; Xu et al. 1996; Chefetz et al. 1998; Schneider et al. 1999; Galhaup et al. 2002a; Jung et al. 2002; Palonen et al. 2003).

1.4 Molecular biology of laccases

The first laccase genes were isolated and sequenced about 15 years ago from the fungi *Neurospora crassa* (Germann et al. 1988), *Aspergillus nidulans* (Aramayo and Timberlake 1990), *Coriolus hirsutus* (Kojima et al. 1990) and *Phlebia radiata* (Saloheimo et al. 1991). Since then, the number of laccase genes sequenced has increased considerably, and searches from protein and gene sequence databases currently yield several hundreds of laccase gene sequences. However, a significant number of these are only partial stretches of putative laccase genes that have been found in genome-wide sequencing projects and have been annotated on the basis of sequence homology with known laccases. The number of laccase genes of which the corresponding protein products have been experimentally characterized is significantly lower. To date, there are about 20 such enzymes, most of which are fungal laccases (Table 2). In addition to the genes shown in Table 2, several laccase genes have been characterized in detail at the nucleotide level but have not been specified to code for a known laccase protein.

Table 2. Examples of laccase genes that have been shown to encode a biochemically characterized laccase protein.

	Gene		Protein encoded by the gene			
Organism	**Name**	**EMBL Acc. No.**	**Length (aa)**	**MW[†] (kDa)**	**pI**	**Reference**
Bacillus subtilis	*cotA*	U51115	513	65	7.7	Martins et al. 2002
Ceriporiopsis subvermispora	*lcs-1*	AY219235	519	79	3.6	Salas et al. 1995; Karahanian et al. 1998
Coprinus cinereus	*lcc1*	AF118267	539	63	3.7–4.0	Yaver et al. 1999; Schneider et al. 1999
Cryptococcus neoformans	*CNLAC1*	L22866	624	75	n.d.*	Williamson 1994
Gaeumannomyces graminis var. *tritici*	*LAC2*	AJ417686	577	70	5.6	Edens et al. 1999; Litvintseva and Henson 2002
Marasmius quercophilus (Basidiomycete C30)	*lac1*	AF162785	517	62	3.6	Dedeyan et al. 2000
Myceliophthora thermophila	*lcc1*	AR023901	619	80	4.2	Berka et al. 1997a & b
Neurospora crassa	2 alleles	M18333-4	619	64	6.8	Germann et al. 1988
Phlebia radiata	*lac1*	X52134	548	64	3.5	Niku-Paavola et al. 1990; Saloheimo et al. 1991
Pleurotus ostreatus	*poxa1b*	AJ005017	533	62	6.9	Giardina et al. 1999
Pleurotus ostreatus	*poxc (=pox2)*	Z49075	533	67	4.7	Palmieri et al. 1993; Giardina et al. 1996
Basidiomycete PM1 (CECT 2971)	*lac1*	Z12156	517	64	3.6	Coll et al. 1993a; Coll et al. 1993b
Podospora anserina	*lac2*	Y08827	621	70	7–10	Fernández-Larrea and Stahl 1996
Populus euramericana	*lac90*	Y13772	574	90	9.2	Ranocha et al. 1999
Rhizoctonia solani	*lcc4*	Z54277	530	66	7.5	Wahleithner et al. 1996
Streptomyces lavendulae	-	AB092576	631	73	n.d.*	Suzuki et al. 2003
Trametes pubescens	*lap2*	AF414807	523	65	2.6	Galhaup et al. 2002a
Trametes trogii	*lcc1*	Y18012	496	70	3.3–3.6	Garzillo et al. 1998; Colao et al. 2003
Trametes versicolor	*lccI*	L49376	519	67	n.d.*	Bourbonnais et al. 1995; Ong et al. 1997
Trametes versicolor	*lcc2*	U44430	520	64	3.1–3.3	Cassland and Jönsson 1999
Trametes villosa	*lcc1*	L49377	520	63	3.5	Yaver et al. 1996
Trametes villosa	*lcc2*	AY249052	519	63	6.2–6.8	Yaver et al. 1996

* n.d., not determined.

[†] Molecular weights determined by SDS-PAGE.

A typical laccase gene codes for a protein of 500–600 amino acids (Table 2). The coding regions of fungal laccase genes are usually intervened by 8–13 introns of about 50–90 basepairs in length, and the splicing junctions generally adhere to the GT-AG rule (Padgett et al. 1984; Kojima et al. 1990; Saloheimo et al. 1991; Choi et al. 1992; Yaver et al. 1996; Yaver et al. 1999; Zhao and Kwan 1999; Galhaup et al. 2002a). There are, however, also some laccase genes that have only one intron (*Neurospora crassa* laccase gene; Germann et al. 1988), as well as genes with even up to 19 introns (*Pleurotus ostreatus pox1*; Giardina et al. 1995). Conserved intron positions have been found in laccase genes only from the same fungal phylum, and this has been considered as an indicator of the evolutionary distance between basidiomycetous and ascomycetous fungi (Saloheimo et al. 1991; Fernández-Larrea and Stahl 1996; Berka et al. 1997b). All the fungal laccases listed in Table 2 are secreted proteins, and typical eukaryotic signal peptide sequences of about 20 amino acids are found at the N-termini of the protein sequences. In addition to the secretion signal sequence, laccase genes from *Neurospora crassa*, *Podospora anserina*, *Myceliophthora thermophila* and *Coprinus cinereus* contain regions that code for N-terminal cleavable propeptides (Germann et al. 1988; Fernández-Larrea and Stahl 1996; Berka et al. 1997b; Yaver et al. 1999). These laccases also have C-terminal extensions, i.e. the last amino acids from the predicted amino acid sequence are not present in the mature protein (Germann et al. 1988; Berka et al. 1997b; Yaver et al. 1999).

The molecular weights of laccases are usually in the range of 60 to 90 kDa when determined by SDS-PAGE (Table 2). The difference between the molecular weight (MW) predicted from the peptide sequence and the experimentally obtained MW is caused by glycosylation, which typically accounts for about 10–20% of the total MW (Froehner and Eriksson, 1974; Coll et al. 1993a; Giardina et al. 1996; Wahleithner et al. 1996; Dedeyan et al. 2000; Galhaup et al. 2002a). The isoelectric points of microbial laccases are generally around 3–6 (Table 2). However, many laccase-producing fungi produce several laccase isoforms, and laccases with pIs at neutral or slightly alkaline pH values have also been detected in several fungi, such as *Podospora anserina* (Fernández-Larrea and Stahl 1996), *Rhizoctonia solani* (Wahleithner et al. 1996), *Trametes villosa* (Yaver et al. 1996) and *Pleurotus ostreatus* (Palmieri et al. 1997).

Many fungal genomes contain more than one laccase gene. *Trametes villosa*, for example, contains at least five laccase genes (Yaver et al. 1996; Yaver and Golightly 1996), *Coprinus cinereus* at least eight (Hoegger et al. 2004), and *Rhizoctonia solani* (Wahleithner et al. 1996), *Pleurotus sajor-caju* (Soden and Dobson 2001) and *Pleurotus ostreatus* (Palmieri et al. 2003) at least four laccase genes. The precise quantification of laccase genes is complicated by the existence of different laccase gene alleles in the chromosomes, because most of the studied laccase-producing fungi are diploid (Yaver et al. 1996; Eggert et al. 1998). Laccase proteins and thereby also laccase genes are identified by the presence of four highly conserved copper binding motifs, all involving the sequence HXH and containing altogether 10 conserved histidines and one conserved cysteine (Fernández-Larrea and Stahl 1996; Yaver et al. 1999; Kumar et al. 2003). These copper binding regions can also be found in other multicopper oxidases, which complicates the identification of laccase genes without knowledge of the properties of the corresponding protein. For example, fungal ferroxidase from *Phanerochaete chrysosporium* has been shown to be about 30% identical to fungal laccases, and it contains the same conserved copper binding residues as laccases (Larrondo et al. 2003b).

The expression levels of different laccase genes typically depend on cultivation conditions. For example, high nitrogen content of the medium has been shown to induce transcription of laccase genes in the Basidiomycete I-62 (CECT 20197) (Mansur et al. 1998) and in *Pleurotus sajor-caju* (Soden and Dobson 2001). Copper is also often a strong inducer of laccase gene transcription, and this has been suggested to be related to a defence mechanism against oxidative stress caused by free copper ions (Fernández-Larrea and Stahl 1996; Collins and Dobson 1997; Palmieri et al. 2000; Soden and Dobson 2001; Galhaup et al. 2002a; Litvintseva and Henson 2002). In addition to copper, other metal ions such as Mg^{2+}, Cd^{2+} or Hg^{2+} can stimulate laccase expression (Scheel et al. 2000; Soden and Dobson 2001; Galhaup et al. 2002a). Certain aromatic compounds that are structurally related to lignin precursors, such as 2,5-xylidine or ferulic acid, have also been shown to increase laccase gene transcription in *Trametes villosa*, *Trametes versicolor* and *Pleurotus sajor-caju* (Yaver et al. 1996; Collins and Dobson 1997; Soden and Dobson 2001). On the other hand, *Trametes villosa* and *Pleurotus sajor-caju* have also been shown to contain constitutively expressed laccase genes, and this may be related to different physiological roles of the various laccases in the fungi (Yaver et al. 1996; Soden and Dobson 2001).

The transcriptional induction of laccase genes by metal ions and phenolic compounds has been suggested to result from the presence of specific regulatory sites in the promoter regions of the genes. The upstream regulatory regions of several laccase genes have been shown to contain putative metal-responsive elements (MRE) that have also been found in promoter regions of metallothionein proteins involved in metal homeostasis and detoxification (Karahanian et al. 1998; Mansur et al. 1998; Giardina et al. 1999; Galhaup et al. 2002a; Faraco et al. 2003). Furthermore, putative heat-shock elements (HSE), xenobiotic response elements (XRE) and antioxidant response elements (ARE) have been discovered from the promoter regions of laccase genes (Saloheimo et al. 1991; Fernández-Larrea and Stahl 1996; Giardina et al. 1999; Soden and Dobson 2001; Galhaup et al. 2002a), although the roles of these regulatory regions have not yet been experimentally demonstrated.

Comparison of laccase gene nucleotide sequences indicates that laccases can be divided into at least three different groups: basidiomycete, ascomycete and plant laccases (Eggert et al. 1998; Cassland and Jönsson 1999; Valderrama et al. 2003). The level of amino acid identity between laccases from the same group is generally above 50%, whereas identity between laccases from different groups is below 40%. The translated laccase genes *yA* and *tilA* from the ascomycete *Aspergillus nidulans* differ significantly from other laccase protein sequences (Aramayo and Timberlake 1990; Scherer and Fischer 2001); the level of amino acid identity between the predicted *Aspergillus* laccases and other laccases is only about 30% based on BLAST similarity searches. Unfortunately, the substrate specificities of these laccases have not been characterized. The bacterial laccase proteins from *Bacillus subtilis* (Martins et al. 2002) and *Streptomyces lavendulae* (Suzuki et al. 2003) are 47% similar to each other but differ very much from other laccases. Pairwise similarity between the bacterial and fungal laccase proteins is less than 30%. The similarity of bacterial laccases is actually higher with other bacterial multicopper proteins, such as *Streptomyces antibioticus* phenoxazinone synthase (Hsieh and Jones 1995) and *Escherichia coli* copper homeostasis protein CueO (Roberts et al. 2002), than with other laccases.

1.5 Heterologous production of laccases

Laccase genes are often expressed at very low levels in the native hosts. In order to improve laccase production, fungal laccases have been expressed heterologously in *Saccharomyces cerevisiae* (Kojima et al. 1990), *Trichoderma reesei* (Saloheimo and Niku-Paavola 1991), *Aspergillus oryzae* (Yaver et al. 1996; Wahleithner et al. 1996; Berka et al. 1997b; Yaver et al. 1999; Sigoillot et al. 2004), *Pichia pastoris* (Jönsson et al. 1997; Otterbein et al. 2000; Brown et al. 2002; Soden et al. 2002; Liu et al. 2003), *Aspergillus sojae* (Hatamoto et al. 1999), *Aspergillus niger* (Record et al. 2002; Larrondo et al. 2003a), *Aspergillus nidulans* (Larrondo et al. 2003a), tobacco (LaFayette et al. 1999) and maize (Bailey et al. 2004). In addition, heterologous yeast expression systems have been developed to facilitate protein engineering of laccases (Gelo-Pujic et al. 1999; Bulter et al. 2003) or to improve the resistance of yeast to phenolic growth inhibitors (Cassland and Jönsson 1999). Bacterial laccases from *Bacillus subtilis* and *Streptomyces lavendulae* have been expressed in *Escherichia coli* (Martins et al. 2002; Suzuki et al. 2003) but successful expression of fungal laccases in *E. coli* has not been reported.

Laccases have been expressed in *Aspergillus* spp. under the control of the strong constitutive TAKA-amylase (*amyA*) (Yaver et al. 1996; Wahleithner et al. 1996; Berka et al. 1997b; Yaver et al. 1999; Larrondo et al. 2003a) or glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoters (Record et al. 2002). In *Trichoderma reesei*, the promoter region of the major cellulase gene *cbh1* has been used (Saloheimo and Niku-Paavola 1991). Secretion of heterologous laccases has generally been directed by using native laccase signal sequences in the expression constructs. However, it may be possible to increase the production levels by using signal sequences derived from host genes. For example, *Pycnoporus cinnabarinus* Lac1 was produced 80 times more efficiently in *Aspergillus niger* when the laccase signal sequence was replaced by the prepro sequence of the *A. niger* glucoamylase gene *glaA* (Record et al. 2002). The effect of signal sequences on heterologous laccase production has also been studied in *Pichia pastoris* strains expressing various laccases, but in these experiments the native laccase signal sequences have performed better than the commonly used N-terminal signal peptide from the *S. cerevisiae* mating-type factor *MFα* gene (Jönsson et al. 1997; Brown et al. 2002; Soden et al. 2002; Liu et al. 2003).

Laccase production levels have often been improved significantly by expression in heterologous hosts, but the reported levels have still been rather low for industrial applications (Table 3). The highest yields have been obtained in filamentous fungi, especially in *Aspergillus* spp. that are widely used in the production of industrial enzymes. Improved laccase production levels have also been achieved by expression in *Pichia pastoris*, whereas expression in *S. cerevisiae* has generally resulted in very low activity levels (Larsson et al. 2001; Bulter et al. 2003). The highest reported laccase production levels thus far have been obtained in homologous production systems in a shake flask cultivation of *Pycnoporus cinnabarinus*, which yielded 1000–1500 mg l^{-1} laccase (Lomascolo et al. 2003), and a fermentor cultivation of *Trametes pubescens*, which yielded 700 mg l^{-1} laccase (Galhaup et al. 2002b). The production of two bacterial laccases from *Bacillus subtilis* and *Streptomyces lavendulae* in *E. coli* resulted in extensive intracellular aggregation of laccases (Martins et al. 2002; Suzuki et al. 2003). *Bacillus subtilis* laccase could not be recovered from the inclusion bodies, and only the soluble fraction representing about 10% of the heterologous laccase was purified (Martins et al. 2002). *Streptomyces lavendulae* laccase was refolded to an active form after treatment with urea and 2-mercaptoethanol (Suzuki et al. 2003).

Table 3. Laccase production in heterologous hosts.

Laccase gene	**Production host**	**Laccase production (mg l^{-1})***	**Reference**
Ceriporiopsis subvermispora lcs-1	*Aspergillus nidulans*	1.5	Larrondo et al. 2003a
	Aspergillus niger	1.5	Larrondo et al. 2003a
Coprinus cinereus lcc1	*Aspergillus oryzae*	135	Yaver et al. 1999
Myceliophthora thermophila lcc1	*Aspergillus oryzae*	19	Berka et al. 1997b
	Saccharomyces cerevisiae	18	Bulter et al. 2003
Phlebia radiata lac1	*Trichoderma reesei*	20	Saloheimo and Niku-Paavola 1991
Pleurotus sajor-caju lac4	*Pichia pastoris*	4.9	Soden et al. 2002
Pycnoporus cinnabarinus lac1	*Pichia pastoris*	8	Otterbein et al. 2000
	Aspergillus niger	70	Record et al. 2002
	Aspergillus oryzae	80	Sigoillot et al. 2004

* The reported production levels have been obtained in shake flask cultivations, except in the case of *Phlebia radiata* laccase which was produced in a laboratory fermentor.

Production of heterologous laccase has often been improved by varying the cultivation conditions. For example, better production of heterologous laccase has been achieved in yeast systems by controlling the pH of the culture medium and by lowering cultivation temperatures (Jönsson et al. 1997; Cassland and Jönsson 1999; Larsson et al. 2001; O'Callaghan et al. 2002; Soden et al. 2002; Liu et al. 2003). Buffering of the culture medium to maintain the pH above 4 has been proposed to be important for stability of secreted laccases and inactivation of acidic proteases (Jönsson et al. 1997; Larsson et al. 2001; Soden et al. 2002), whereas lowered cultivation temperatures may result in better production due to improved folding of heterologous proteins (Cassland and Jönsson 1999). In addition, overexpression of Sso2p, a membrane protein involved in the protein secretion machinery (Aalto et al. 1993), has been shown to improve heterologous laccase production in *S. cerevisiae* (Larsson et al. 2001). The addition of copper into the culture medium has also proved to be important for heterologous laccase production in *Pichia pastoris* and *Aspergillus* spp. (O'Callaghan et al. 2002; Larrondo et al. 2003a; Liu et al. 2003). In contrast to homologous laccase production, in which copper addition often affects laccase gene expression, the increased laccase production by copper addition is probably related to improved folding of the active laccase in heterologous production (Larrondo et al. 2003a). The importance of adequate copper concentration for proper laccase folding was further corroborated by studies in which two genes related to copper-trafficking in *Trametes versicolor* were overexpressed in *S. cerevisiae* expressing *Tr. versicolor lacIII* gene; the heterologous laccase production by *S. cerevisiae* was improved up to 20-fold (Uldschmid et al. 2003). The effect was suggested to result from more efficient transport of copper to the Golgi compartment (Uldschmid et al. 2003). Directed evolution has also been used for improving heterologous laccase production. Mutations in the *Myceliophthora thermophila* laccase gene resulted in the highest reported laccase production level in *S. cerevisiae*, 18 mg l^{-1} (Bulter et al. 2003).

1.6 Laccase applications

Oxidation reactions are widely used in industrial processes, for example in the textile, food, wood processing, pharmaceutical and chemical industries. Many of the currently used oxidation methods are not economically or environmentally satisfactory, because they produce unwanted side reactions and the oxidants or

reaction catalysts are often toxic. Enzymatic oxidation is a potential alternative to chemical methods, because enzymes are very specific and efficient catalysts, and are ecologically sustainable. Laccases are currently seen as very interesting enzymes for industrial oxidation reactions, because they are capable of oxidizing a wide variety of substrates. In addition, they use readily available molecular oxygen as an electron acceptor instead of expensive cofactors such as $NAD(P)^+$.

Laccases are currently studied intensively for many applications and they are already used in large scale in the textile industry. Together with low molecular weight redox-mediator compounds, laccases can generate a desired worn appearance on denim by bleaching indigo dye (Pedersen and Kierulff 1996; Campos et al. 2001). They could also be used for decolorizing dye house effluents, that are hardly decolorized by conventional sewage treatment plants (Abadulla et al. 2000; Wesenberg et al. 2003). In addition to dye house effluents, laccases can decolorize waste waters from olive oil mills (D'Annibale et al. 2000; Dias et al. 2004) and pulp mills (Manzanares et al. 1995) by removing colored phenolic compounds. Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases are able to oxidize toxic organic pollutants, such as polycyclic aromatic hydrocarbons (Collins et al. 1996) and chlorophenols (Gianfreda et al. 1999; Ahn et al. 2002). The most useful method for this application would probably be inoculating the soil with fungi that are efficient laccase-producers, because the use of isolated enzymes is not economically feasible for soil remediation in large scale.

The involvement of fungal laccases in lignin biodegradation has raised interest in the use of laccases in lignocellulose processing. The proposed applications include pulp bleaching (Bourbonnais and Paice 1992; Call and Mücke 1997) and fiber modification (Felby et al. 1997; Chandra and Ragauskas 2002). Laccases are able to delignify pulp when they are used together with mediators (Bourbonnais and Paice 1992; Call and Mücke 1997). The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates (Bourbonnais and Paice 1990; Bourbonnais and Paice 1992; Call and Mücke 1997). Although the laccase-mediator system has been studied extensively, there are still unresolved problems concerned with mediator recycling, cost and toxicity. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be used in the enzymatic

adhesion of fibers in the manufacturing of lignocellulose-based composite materials, such as fiber boards. Laccase has been proposed to activate the fiber-bound lignin during manufacturing of the composites, and boards with good mechanical properties have been obtained without toxic synthetic adhesives by using laccases (Felby et al. 1997; Hüttermann et al. 2001). Another possibility is to functionalize lignocellulosic fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolic acid derivatives onto kraft pulp fibers (Lund and Ragauskas 2001; Chandra and Ragauskas 2002). This ability could be used in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties, such as hydrophobicity or charge.

Because laccases are able to catalyze electron-transfer reactions via a direct mechanism, i.e. without additional cofactors, their use has also been studied in biosensors that detect various phenolic compounds (Ghindilis et al. 1992; Lisdat et al. 1997; Kulys and Vidziunaite 2003), oxygen (Gardiol et al. 1996) or azides (Leech and Daigle 1998). In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems (Chen et al. 2001; Calabrese Barton et al. 2002). In the food industry laccases have potential in wine, fruit juice and beer stabilization by removing the polyphenols that cause haze formation and discoloration (Cantarelli et al. 1989; Giovanelli and Ravasini 1993; Minussi et al. 2002 and references therein). In addition, laccases can be used in baking to improve the mixing properties of the dough and the structure of the baking product (Si 1993; Labat et al. 2001). A novel application field for laccases is in cosmetics. For example, laccase-based hair dyes could be less irritant and easier to handle than current hair dyes (Roure et al. 1992; Aaslyng et al. 1996; Xu 1999). In the future laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative deprotection (Semenov et al. 1993) and production of complex polymers and medical agents (Xu 1999 and references therein; Mai et al. 2000; Uyama and Kobayashi 2002; Kurisawa et al. 2003; Nicotra et al. 2004).

1.7 Aims of the study

Laccases are important and promising enzymes for various applications. Many known fungal laccases are active only in the acidic pH range and they are not sufficiently thermostable for industrial applications. The aim of the present work was to discover a novel alkaline and thermostable laccase. The work also aimed at developing an efficient production system for the laccase, because relatively low production levels have hitherto hindered the efficient exploitation of laccases. More specifically, the aims were:

1. Testing of simple screening methods to isolate novel fungal laccases from environmental samples.

2. Purification, biochemical characterization and structure determination of the novel laccase from *Melanocarpus albomyces*.

3. Cloning of the gene encoding *M. albomyces* laccase and its expression in *Saccharomyces cerevisiae* and *Trichoderma reesei*.

4. Characterization of the recombinant *M. albomyces* laccase produced in *T. reesei*.

2. Materials and methods

The materials and methods used in this study are described in detail in the original publications I–VI. A general outline of the methodology is presented below.

2.1 Laccase activity measurements

Laccase activity was measured by monitoring the oxidation of 5 mM ABTS in 25 mM succinate buffer (pH 4.5) at 436 nm at 25ºC. The activities were calculated using an extinction coefficient of 29 300 M^{-1} cm^{-1} (Niku-Paavola et al. 1988) and expressed as katals (mol s^{-1}). In addition, specific activities for purified laccases were measured with three other substrates: syringaldazine (525 nm; ε 65 000 M^{-1} cm^{-1}; Leonowicz and Grzywnowicz 1981), 2,6-dimethoxyphenol (469 nm; ε 49 600 M^{-1} cm^{-1}; Wariishi et al. 1992), and guaiacol (465 nm; ε 12 100 M^{-1} cm^{-1}; Paszczynski et al. 1985) in 25 mM succinate buffer (pH 4.5) (II) or in 40 mM MES-NaOH buffer (pH 6) (V).

2.2 Screening for laccase-positive fungi (I)

Laccase-producing fungi were screened from several samples taken in Finland: decomposing tree stump, oak leaf compost, birch log, spruce chip pile and VTT test compost of municipal biowaste at different composting stages. In addition, crude cork material and process waters of a Portuguese cork factory (A. Silva, Porto, Portugal) and soiled pulp from a pulp mill in Svetogorsk, Russia, were used as sources of laccase-producing fungi. Fungi were cultivated by placing pieces of samples on malt extract agar and potato dextrose agar plates containing the following indicator compounds: 0.04% (w/v) Remazol Brilliant Blue R (RBBR), 0.04% Poly R-478, 0.01% guaiacol or 0.5% tannic acid. In addition, 0.01% (w/v) chloramphenicol and chlorotetracycline were added to the media in order to inhibit the growth of bacteria and 1% Benomyl in order to select for wood decay fungi (Maloy 1974). The plates were incubated at 30ºC and positive strains were subcultured when clear positive color reactions were detected. Fungal strains indicating laccase production in the plate test were grown in different types of liquid media and the production of laccase was monitored with activity measurements.

2.3 Production and purification of native *Melanocarpus albomyces* laccase (II, III)

Melanocarpus albomyces (VTT D-96490) was cultivated in shake flasks at 37ºC on a rotary shaker (160 rpm). Extracellular laccase activity was assayed daily from the culture filtrate and the enzyme was collected when laccase activity reached its maximum.

The mycelium was removed by filtration through Whatman n:o 1 filter paper and the buffer was changed to 10 mM acetate buffer (pH 5) by ultrafiltration. *M. albomyces* laccase was purified with three chromatographic steps at room temperature (Table 4).

Table 4. Purification of M. albomyces *laccase.*

	Step	Resin	Equilibration buffer	Elution protocol
1.	Anion exchange chromatography	DEAE Sepharose Fast Flow	10 mM Na-acetate, pH 5	Increasing linear gradient of 0–200 mM Na_2SO_4
2.	Hydrophobic interaction chromatography	Phenyl Sepharose Fast Flow	20 mM Na-citrate, pH 5	1) Decreasing linear gradient of 400-0 mM Na_2SO_4 2) 2 mM Na-citrate buffer, pH 5 3) Distilled H_2O
3.	Gel filtration	Sephacryl S-100 HR	100 mM Na-phosphate, pH 7	

Crystallisation of the purified *M. albomyces* laccase, collection of x-ray diffraction data and determination of the crystal structure were performed at the Department of Chemistry, University of Joensuu, Finland, by the group of professor Juha Rouvinen (see more details in the publication III).

2.4 Biochemical characterization of laccases (I, II, V)

The molecular weights of purified laccases were determined with SDS-PAGE (I, II) and MALDI-TOF (V). The isoelectric points were determined by isoelectric focusing using active staining with ABTS (I, II, V). Enzyme stabilities were determined at different pH-values and temperatures (I, II, V) and pH optima were determined in McIlvaine universal buffer over a pH range of 2.2–8.0 with ABTS (I, II, V), guaiacol (I, II, V), 2,6-DMP (II) or syringaldazine (II) as substrates. The effects of various inhibitors on laccase activity were determined by measuring oxygen consumption during the enzyme reaction with ABTS in the presence of the inhibitor compounds (II). The UV-visible spectrum of purified native *M. albomyces* laccase was measured with a Hitachi U-2000 spectrophotometer and the EPR spectrum with a Bruker ESP 300 X-band spectrometer (II). The redox-potentials of the T1 coppers of laccases were determined by photometric copper titration in 0.1 M KH_2PO_4 (pH 6.0) as described by Xu et al. (1996) using the redox titrant couple $K_3Fe(CN)_6/K_4Fe(CN)_6$.

2.5 Cloning of the *M. albomyces lac1* gene (IV)

DNA and RNA manipulations were performed according to standard methods (Sambrook et al. 1989). A genomic library of *M. albomyces* was constructed into SuperCos I cosmid, and approximately 5×10^5 clones from the library were hybridized with the *Podospora anserina lac2* gene encoding laccase II (Fernández-Larrea and Stahl 1996). Six strongly hybridising cosmid clones were isolated, and a 4.5-kb EcoRI fragment that hybridised with *Podospora anserina lac2* was subcloned from the cosmids. *M. albomyces lac1* gene was sequenced from the insert by using the primer walking technique. The cDNA encoding the laccase was cloned using RACE-PCR (rapid amplification of cDNA ends). Similarities between *M. albomyces* laccase protein sequence and other laccase proteins were searched with BLAST program at http://www.ch.embnet.org/software/BottomBLAST.html.

2.6 Heterologous expression of *M. albomyces* laccase in *Saccharomyces cerevisiae* (IV)

Saccharomyces cerevisiae strain INVSc1 was transformed with four *M. albomyces lac1* expression vectors (Table 8) and the corresponding empty control vectors using the lithium acetate procedure (Gietz et al. 1992). Production of laccase by the yeast transformants was first assayed on plates by soaking the well-grown colonies with ABTS and monitoring the formation of green color around the colonies. To study laccase production in liquid cultures, the transformants were grown in SC-Ura medium (Sherman 1991), supplemented with 2% glucose (transformants with pLLK7) or 2% raffinose (transformants carrying pLLK10, pMS174 or pMS175) and 0.5 mM $CuSO_4$. After two days of cultivation at 30ºC (250 rpm), cells from transformants carrying pLLK10, pMS174 or pMS175 were transferred into induction medium (containing galactose instead of raffinose). Extracellular laccase activity was monitored daily using ABTS as substrate. In addition, the presence of extra- and intracellular laccase was studied with Western blotting using polyclonal rabbit antibodies raised against purified *M. albomyces* laccase.

2.7 Transformation of *Trichoderma reesei* and characterization of the transformants (V)

M. albomyces laccase was produced in *Trichoderma reesei* from two expression constructs, both containing the *cbh1* promoter and terminator sequences. pLLK13 contained the full-length laccase cDNA, whereas pLLK12 contained the *T. reesei* hydrophobin gene *hfb1* (Nakari-Setälä et al. 1996) fused to the 5'-end of the *lac1* cDNA region encoding the mature laccase. *T. reesei* RutC-30 was transformed with pLLK13, which also contained the *E. coli* hygromycin resistance gene, using the procedure described in Penttilä et al. (1987). As pLLK12 did not contain a hygromycin resistance gene, its laccase expression cassette was cotransformed with pBluekan7-1.NotI, carrying a hygromycin resistance cassette. The transformants were plated on minimal medium (Penttilä et al. 1987) containing glucose and hygromycin, and well-growing transformants were purified to uninuclear clones.

Laccase production was tested on plates containing minimal medium, lactose and hygromycin by pipetting ABTS onto fungal colonies and monitoring the formation of green color for two hours. Selected laccase-positive transformants were cultivated in shake flasks in minimal medium (Penttilä et al. 1987) supplemented with 40 g l^{-1} lactose, 20 g l^{-1} spent grain, 0.1 mM $CuSO_4$, and 10 g l^{-1} potassium hydrogen phthalate for buffering to pH 6. For monitoring the growth properties of the fungi, selected transformants were also grown in a soluble medium in which the spent grain was replaced by 2 g l^{-1} peptone. All the cultivations were performed at 28°C and 200 rpm and production of extracellular laccase was monitored with activity measurements. The intra- and extracellular laccase levels were compared by Western blotting.

Hybridization of the electrophoresed *T. reesei* total RNA with *M. albomyces lac1* cDNA was used to study the *M. albomyces lac1* expression levels from different constructs. In addition, the possible induction of the unfolded protein response (UPR) pathway was studied by hybridizing the *T. reesei* total RNA with the specific nucleotide probes generated for the transcripts of the following *T. reesei* genes: *pdi1* encoding protein disulphide isomerase (Saloheimo et al. 1999), *bip1* encoding the major ER chaperone (Pakula et al. 2003) and *hac1* encoding the transcription factor of unfolded protein response (Saloheimo et al. 2003).

2.8 Production and purification of recombinant *M. albomyces* laccase (V)

The *T. reesei* transformant which produced the highest laccase activities in shake flasks was cultivated in a fermentor in batch and fed-batch modes. The working volume was 20 liters, pH was adjusted to 5.5–6, agitation was 400–500 rpm, aeration was 2–10 liters min^{-1} and the cultivation temperature was 28°C. The dry weight, lactose and total protein concentration, as well as laccase, cellobiohydrolase I (CBHI) (Bailey and Tähtiharju 2003) and β-1,4-endoglucanase activities (IUPAC 1987) were measured daily. In the fed-batch cultivation, lactose feed was controlled by an algorithm which calculated the rate of base addition required for pH control (Bailey and Tähtiharju 2003). Decrease in the rate of base addition indicated slower growth and resulted in increased lactose feeding.

Recombinant *M. albomyces* laccase was purified from the culture supernatant of the batch fermentation. The supernatant was first clarified by bentonite treatment and the clear solution was treated with papain in order to facilitate the separation of recombinant *M. albomyces* laccase from the major extracellular protein CBHI. After papain digestion the laccase was purified from the solution with three chromatographic steps at room temperature (Table 5).

Table 5. Chromatographic purification steps used to purify recombinant M. albomyces *laccase.*

Step	Resin	Equilibration buffer	Elution protocol
1. Hydrophobic interaction chromatography	Phenyl Sepharose Fast Flow	5 mM Na-citrate, pH 5	Decreasing linear gradient of 600-0 mM Na_2SO_4
2. Anion exchange chromatography	DEAE Sepharose Fast Flow	20 mM Na-acetate, pH 5	Increasing linear gradient of 0–400 mM Na_2SO_4
3. Gel filtration	Sephacryl S-100 HR	50 mM Na-phosphate, pH 7, 150 mM NaCl	

2.9 Cellulose binding studies (VI)

The following enzymes were used in experiments studying binding of laccases to cellulose: native and recombinant *M. albomyces* laccases, *Trametes hirsuta* laccase (purified according to Rittstieg et al. 2002) and *Mauginiella* sp. laccase (purified according to Palonen et al. 2003). Various amounts of the laccases were incubated in 50 mM citrate buffer (pH 5) in suspensions of steam-pretreated softwood (SPS) (Palonen and Viikari 2004), Avicel (plant-derived microcrystalline cellulose), bacterial microcrystalline cellulose (BMCC) (Gilkes et al. 1992) or alkali lignin (Indulin AT, Sigma). With BMCC suspensions, 0.5% of bovine serum albumin (BSA) was added to the buffer in order to reduce non-specific adsorption of the laccase at low protein concentrations. After gentle mixing in an end-over-end rotary shaker for one hour (with lignin at 4°C and with the other matrices at 22°C), the samples were centrifuged and the residual laccase activity in the supernatant was measured spectrophotometrically using ABTS as substrate. The amount of bound laccase was calculated from the

difference between initial and free enzyme concentrations. The activity of cellulose-bound laccase was measured with an end-point activity assay after binding to Avicel.

The reversibility of binding was determined by dilution experiments. *M. albomyces* laccase was first allowed to adsorb on BMCC. Subsequently, the mixture was diluted five-fold with the sample buffer, and the formation of a new equilibrium was monitored by removing small samples from the mixture at different time points and measuring the free laccase activity. The effect of non-specific adsorption on binding to BMCC was studied by omitting BSA from the BMCC suspension. The role of ionic interactions in adsorption was analyzed by adding 0.1 or 0.5 M Na_2SO_4 to the reaction mixture.

3. Results and discussion

3.1 Screening for laccases (I)

In order to find novel laccases with potential for industrial applications and for structure-function studies, laccase-producing fungi were isolated from various environmental samples taken in Finland, as well as from samples taken in a Portuguese cork factory and a Russian pulp mill. Production of ligninolytic enzymes was detected on solid media containing indicator compounds. The screening resulted in isolation of a total of 26 positive fungal strains, of which 15 were identified by the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands; Table 6).

Table 6. Identification of the ligninolytic fungal strains isolated in the screening.

Strain	Identification
LLP2	*Mucor circinelloides* v. Tieghem (Zygomycete)
LLP4	most likely *Bjerkandera* (Basidiomycete)
LLP5	*Chrysosporium queenslandicum* (Ascomycete)
LLP6	*Sporotrichum pruinosum* (Basidiomycete)
LLP7	*Rhizopus microsporus* var. *rhizopodiformis* (Zygomycete)
LLP8	*Mucor hiemalis* f. *corticolus* (Zygomycete)
LLP9	*Mucor circinelloides* v. Tieghem (Zygomycete)
LLP12	most likely *Peniophora* species (Basidiomycete)
LLP14	most likely *Phlebia* cf. *spongipellis* (Basidiomycete)
LLP16	*Trichoderma atroviride* (Ascomycete)
LLP17	*Peniophora* sp. or *Lopharia* sp. (Basidiomycete)
LLP19	*Trichoderma harzianum* (Ascomycete)
LLP20	*Mucor circinelloides* v. Tieghem (Zygomycete)
LLP21	*Trichoderma atroviride* (Ascomycete)
AH2	*Pholiota* sp. (Basidiomycete)

The isolated fungal strains represented nine different genera. Interestingly, four strains from the genera *Peniophora*, *Rhizopus* or *Mucor*, which have not hitherto been reported to produce ligninolytic enzymes, were found in the screening. In addition, three clearly positive isolates were identified as *Trichoderma atroviride* or *Trichoderma harzianum*. Indications of the presence of laccases in *Trichoderma* spp. have previously been reported by Flegel et al. (1982) and Assavanig et al. (1992). In addition, Hölker et al. (2002) recently discovered cell-wall associated laccases in conidia of *T. atroviride* and *T. harzianum*. However, none of these *Trichoderma* laccases have been characterized with respect to their substrates, copper content or amino acid sequence. The production of native laccases by *Trichoderma* species is very interesting, because *T. reesei* secretes very high amounts of both native and recombinant cellulases and is currently used for production of industrial enzymes.

The isolated fungal strains that were positive in the plate-test screening were cultivated in liquid media. Various combinations of different nitrogen and carbon sources were used, as well as compounds that have been reported to be inducers of laccase production. Extracellular laccase production was detected in shake flask cultures with nine of the 26 strains tested. Most strains produced very low laccase activity levels (below 1 nkat ml^{-1}), but four fungal strains, LLP12, LLP13, LLP17 and AH2, produced significant amounts of laccase (22–40 nkat ml^{-1}). No extracellular laccase production was observed with the isolated *Trichoderma* strains. The identification results of LLP12, LLP17 and AH2 are shown in Table 6, whereas LLP13 was an unidentifiable haploid basidiomycete. Laccase production with these strains was very dependent on culture medium, and the highest laccase production was only detected in very rich media containing soya meal. Laccase production by fungi has previously been shown to depend markedly on the composition of the cultivation medium; for example carbon source, nitrogen content and phenolic inducer compounds have been reported to have significant effects on laccase production (Niku-Paavola et al. 1990; Rogalski et al. 1991; Schlosser et al. 1997). In this study, nitrogen-containing compounds derived from the soya meal might have been the crucial factor for efficient laccase production. Induction of laccase production by high medium nitrogen content has recently been detected in the Basidiomycete I-62 (CECT 20197) (Mansur et al. 1998), in *Pleurotus sajor-caju* (Soden and Dobson 2001) and in *Trametes trogii* (Colao et al. 2003). It is also possible that the soya meal contained plant-derived phenolic compounds that might have induced laccase production.

The laccases produced by LLP12, LLP13, and AH2 were characterized with regard to molecular weight, isoelectric point (pI), pH optimum and thermostability. The laccases from LLP13 and AH2 were purified and characterized in this study and LLP12 laccase was characterized by Niku-Paavola et al. (2004). In addition, the properties of LLP17 laccase were studied from the culture filtrate. The results showed that the sizes, pI values and pH optima of these novel laccases were typical for basidiomycete laccases (Table 2, Table 7). In addition, the peptide sequences of the N-terminus and two internal peptides of AH2 (*Pholiota* sp.) laccase were determined. AH2 laccase was shown to be highly homologous to other basidiomycete laccases, such as *Trametes trogii* Lcc1 (Colao et al. 2003), *Trametes villosa* Lcc4 (Yaver and Golightly 1996), *Trametes versicolor* Lcc1 (Jönsson et al. 1995), *Pycnoporus cinnabarinus* LAC1 (Eggert et al. 1998) and *Phlebia radiata* laccase (Saloheimo et al. 1991). The exceptional feature of the novel laccases found in this screening was their relatively high thermostability in citrate buffer at pH 6. The half-lives at 60°C were 3–6 h (Table 7), whereas many fungal laccases have clearly shorter half-lives at this temperature (Heinzkill et al. 1998; Schneider et al. 1999; Tagger et al. 1998; Palonen et al. 2003). These results were very promising for future studies on the novel laccases, because thermostability is one of the key factors determining the applicability of an enzyme for industrial use.

Table 7. Selected biochemical properties of fungal laccases found in the screening. Molecular weights were determined by SDS-PAGE. The pI of the main isoform is shown in boldface type.

Fungal strain	Mw (kDa)	pI	pH optimum (substrate)	t½ 60°C	Reference
LLP12	63	3.7; 3.8; **4.1**	4.0 (guaiacol)	5 h	Niku-Paavola et al. 2004
LLP13	70	3.5; 4.1; **4.2**	3.0 (ABTS)	3 h	I
LLP17*	70	3.5–4.0	< 2 (ABTS); 3.0 (guaiacol)	n.d.	I
AH2	60	**4.1**; 5.1	3 (ABTS); 4.0 (guaiacol)	6 h	I

*LLP17 laccase was characterized from the culture supernatant.

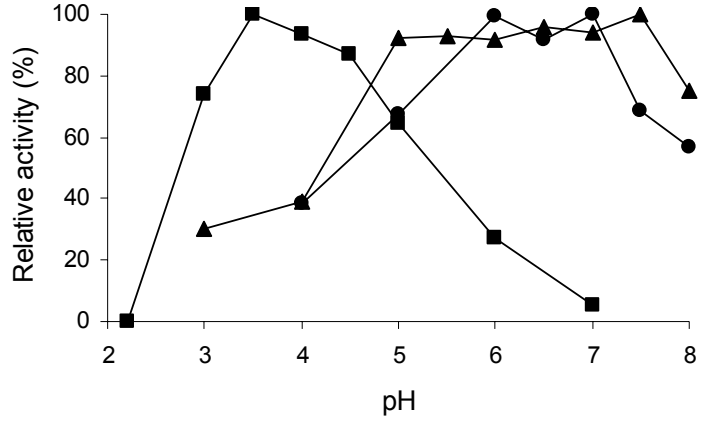
Plate-test screening with laccase indicator compounds has been reported by many groups (Nishida et al. 1988; de Jong et al. 1992; Barbosa et al. 1996; Goncalves and Steiner 1996; Chefetz et al. 1998; Raghukumar et al. 1999), but most of the laccases found in these screening studies have not been purified or characterized. However, Goncalves and Steiner (1996) and Chefetz et al. (1998) reported the enzymatic characteristics of the isolated novel laccases from *Polyporus* sp. and *Chaetomium thermophilum*, respectively. The *Polyporus* sp. laccase was a typical basidiomycete laccase with respect to its catalytical properties (Goncalves and Steiner 1996), whereas laccase from the thermophilic ascomycete *Chaetomium thermophilum* was shown to be exceptionally thermostable and to have a pH optimum for syringaldazine oxidation at pH 6 (Chefetz et al. 1998). In addition, the use of indicator plates facilitated isolation of the basidiomycete fungus *Flavodon flavus* strain 312, which has potential in bioremediation (Raghukumar et al. 1999). These results support our conclusion that indicator plates are an efficient and simple method for discovering novel laccases for both research and industrial application purposes.

3.2 Purification and biochemical characterization of *Melanocarpus albomyces* laccase (II)

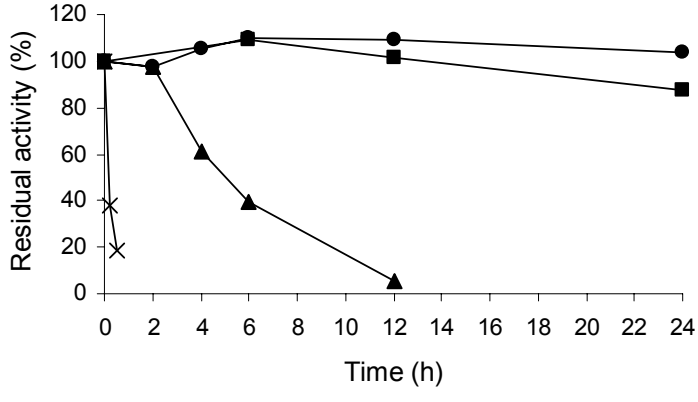
Earlier studies conducted at VTT Biotechnology had indicated that the thermophilic ascomycete *Melanocarpus albomyces* produced laccases (Ravanko 1996). Because *M. albomyces* has previously been reported to produce thermostable and neutral cellulases and xylanases (Vehmaanperä et al. 1996; Prabhu and Maheshwari 1999; Jain et al. 1998), laccase production by *M. albomyces* was studied in more detail in this work. *M. albomyces* laccase was purified by three chromatographic steps: anion exchange chromatography, hydrophobic interaction chromatography and gel filtration and the purified enzyme was biochemically characterized. It showed a molecular mass of 80 kDa on SDS-PAGE, which is slightly higher than the values determined for most fungal laccases (Table 2). Unlike many fungi that often produce several laccase isoforms (Table 7; Bourbonnais et al. 1995; Yaver et al. 1996; Palmieri et al. 2000; Palonen et al. 2003), *M. albomyces* produced only one laccase isoform with a pI of 4.0 in the selected culture conditions. The UV-VIS and EPR spectra of *M. albomyces* laccase were typical for blue copper proteins, indicating the presence of the type-1, -2 and -3 copper atoms.

In addition to the spectral data, the purified enzyme was verified to be a laccase by substrate and inhibitor studies. *M. albomyces* laccase was able to oxidize the typical laccase substrates ABTS, syringaldazine, 2,6-DMP and guaiacol, and it did not oxidize tyrosine. The known laccase inhibitors KCN, NaN_3 and NaF efficiently inhibited *M. albomyces* laccase activity. The N-terminal and two internal peptide sequences of *M. albomyces* laccase were also determined and compared with those of other fungal laccases. The closest homology was found with other ascomycete laccases from *Myceliophthora thermophila* (Berka et al. 1997a), *Podospora anserina* (Fernández-Larrea and Stahl 1996) and *Neurospora crassa* (Germann et al. 1988).

The activity and stability of purified *M. albomyces* laccase were determined at different pH values and temperatures. The pH optimum was measured with four different substrates, and the lowest pH optimum, 3.5, was determined in oxidizing ABTS, similarly to other fungal laccases (Figure 3A; Robles et al. 2000; Xu 1997). However, oxidation of guaiacol and syringaldazine by *M. albomyces* laccase showed a pH optimum in the neutral pH range (Figure 3A). Dependence of pH optima on the substrate has also been observed with other laccases and it has been proposed to reflect the difference in oxidation mechanisms between phenolic and non-phenolic substrates (see Introduction; Hoffmann and Esser 1977; Palmieri et al. 1993; Eggert et al. 1996b; Xu 1997; Chefetz et al. 1998; Schneider et al. 1999). Compared to other laccases, the exceptional property of *M. albomyces* laccase was its activity at alkaline pH values; the enzyme still showed 75% of its maximum activity at pH 8 with guaiacol. Most fungal laccases have pH optima around 4–6 when measured with phenolic substrates and they rapidly lose activity when the pH is increased above 6 (Hoffmann and Esser 1977; Palmieri et al. 1993; Eggert et al. 1996b; Xu 1997; Chefetz et al. 1998; Schneider et al. 1999). The stability of *M. albomyces* laccase was also good at neutral or slightly alkaline pH values, which is common for other laccases as well (Nishizawa et al. 1995; Xu et al. 1996; Chefetz et al. 1998).



A



B

Figure 3. (A) pH activity profiles of purified M. albomyces *laccase with ABTS (■), guaiacol (▲) and syringaldazine (•) as substrates. (B) Thermostability of purified* M. albomyces *laccase measured with guaiacol at pH 6 at 40 (•), 50 (■), 60 (▲), and 70ºC (×).*

In addition to high activity and stability at elevated pH values, *M. albomyces* laccase was shown to have good thermostability at 40, 50 and 60°C (Figure 3B). Its activity remained virtually unaltered for 24 hours at 40 and 50°C and for two hours at 60°C. In addition, thermal activation at 50°C was observed, which has

previously been detected with laccases from *Myceliophthora thermophila* and *Scytalidium thermophilum* (Xu et al. 1996). Combination of thermostability and activity at neutral to alkaline pH values is very rare among fungal laccases, as many other laccases with a pH optimum around 6–7 are not thermostable. For example, unusually high pH optima (7–8) have been measured with 2,6-dimethoxyphenol for laccases from *Coprinus friesii*, *Panaeolus papilionaceus* and *Panaeolus sphinctrinus*, but the enzymes rapidly lose activity at 60°C (Heinzkill et al. 1998). However, an exceptionally thermostable bacterial laccase (CotA) was recently isolated from *Bacillus subtilis*, and this laccase also has an optimum at pH 7 when measured with syringaldazine (Martins et al. 2002). The crystal structure of CotA has been solved at 2.4 Å resolution, and the authors suggested that the high thermostability of CotA is related to its function as an endospore coat component (Enguita et al. 2003). Interestingly, the structure indicated tight internal packing of the protein, which is required for its assembly into the spore coat, and this packing might confer improved thermostablity to the quaternary structure (Enguita et al. 2003). Since *M. albomyces* also produces thermostable cellulases and xylanases, the role of the laccase may be related to degradation of lignocellulosic material in the naturally warm habitat of the fungus, tropical soil. Laccase could also be related to spore formation or pigmentation, because *M. albomyces* forms melanized ascomata after prolonged cultivation, as indicated by the name *Melanocarpus*. Several laccases have been shown to be involved in melanization reactions (Eggert et al. 1995; Williamson et al. 1998; Edens et al. 1999; Martins et al. 2002), but the exact role of laccase in *M. albomyces* is not known.

3.3 Crystal structure of *M. albomyces* laccase (III)

Determination of crystal structures of laccases is complicated by their high degree of glycosylation, difficulties in achieving efficient expression, and the presence of multiple laccase isoforms in several laccase-producing fungi. For several years, only one laccase structure was available, for Lcc1 of *Coprinus cinereus* (Ducros et al. 1998). Unfortunately, it was not complete because it lacked the T2 copper. The three-dimensional structure of *M. albomyces* laccase containing all four copper atoms was solved by x-ray crystallography at 2.4 Å resolution at the University of Joensuu. The overall structure was shown to consist of three cupredoxin-like domains (Figure 1), in accordance with the

structures of *Coprinus cinereus* Lcc1 (Ducros et al. 1998) and other blue copper oxidases (Messerschmidt et al. 1992; Zaitseva et al. 1996). A similar fold has also recently been published for laccases from *Trametes versicolor* (Bertrand et al. 2002; Piontek et al. 2002) and *Bacillus subtilis* (Enguita et al. 2003).

All the characteristic four copper atoms were found in the *M. albomyces* laccase structure. The T1 copper was trigonally coordinated to two histidines and a cysteine residue, and the distant axial ligand was a non-coordinating leucine residue. Both of the T3 coppers were coordinated to three histidines and the T2 copper was shown to be coordinated to two histidines and a chloride ion. The coordination bonds between the coppers and the amino acids are well conserved in multicopper oxidases, and they have also been observed in *Trametes versicolor* (Bertrand et al. 2002; Piontek et al. 2002) and *Bacillus subtilis* laccases (Enguita et al. 2003), as well as in ascorbate oxidase from zucchini (Messerschmidt et al. 1992). The only exception is the axial ligand of T1, which is a non-coordinating leucine in *M. albomyces* laccase and *Coprinus cinereus* Lcc1 (Ducros et al. 1998) and a phenylalanine in *Trametes versicolor* laccases (Bertrand et al. 2002; Piontek et al. 2002), but a coordinating methionine in ascorbate oxidase (Messerschmidt et al. 1992) and *Bacillus subtilis* CotA (Enguita et al. 2003). The coordinating propensity of the axial ligand has been suggested to be important in determining the redox-potential of multicopper oxidases, but recent results have also emphasized the role of other bonds around the T1 copper (see Introduction).

The crystal structure of *M. albomyces* laccase showed two exceptional features as compared to other multicopper oxidase structures. First, elongated electron density was detected in the trinuclear center, indicating the presence of a molecular oxygen molecule in the middle of the T3 copper pair. This kind of binding geometry has not previously been observed at a trinuclear copper site. However, a dioxygen molecule was recently detected in the solvent channel directed towards the trinuclear center in the crystal structure of CotA adducted with ABTS, and the authors suggested that the dioxygen is caught in a holding position prior to binding to the copper atoms (Enguita et al. 2004). The second exceptional feature in the *M. albomyces* laccase structure was its C-terminus, which packed inside a tunnel leading to the trinuclear center. The C-terminal carboxylate group was also shown to form a hydrogen bond to a side chain of His140, which coordinates one of the T3 coppers. In the other reported laccase

structures, the tunnel is not blocked, and it has been proposed to provide access for the solvent molecules to the trinuclear site (Messerschmidt et al. 1992; Bertrand et al. 2002; Piontek et al. 2002). If the tunnel actually forms the access route for oxygen or the exit route for water molecules, the C-terminal plug might be one reason for the presence of the oxygen molecule in the active site of the crystallized *M. albomyces* laccase.

3.4 Cloning of the *M. albomyces lac1* gene (IV)

The N-terminal and two internal peptide sequences determined from purified *M. albomyces* laccase showed high homology to *Podospora anserina* laccase 2 (II). Therefore, *Podospora anserina lac2* gene (Fernández-Larrea and Stahl 1996) was used as a heterologous hybridization probe in isolation of the gene encoding *M. albomyces* laccase. Screening of an *M. albomyces* genomic DNA library with *Podospora anserina lac2* resulted in isolation of a 4.5-kb DNA fragment, and the laccase-encoding region of the fragment was sequenced by the primer walking method. The amino acid sequence deduced from the isolated gene was shown to contain the conserved copper-binding motifs typical for laccases. All the three peptide sequences obtained previously from purified *M. albomyces* laccase could also be identified in the amino acid sequence, which verified that the gene encoded the biochemically characterized *M. albomyces* laccase enzyme.

M. albomyces lac1 gene codes for a protein of 623 amino acids corresponding to one of the largest fungal laccases described so far (Table 2). The corresponding *lac1* cDNA was cloned by RACE-PCR and its sequence revealed the presence of five introns in the genomic laccase gene. When compared to *Myceliophthora thermophila lcc1* gene (Berka et al. 1997a), all the five introns in *M. albomyces lac1* were located in conserved positions. The single intron in the *Neurospora crassa* laccase gene (Germann et al. 1988) and introns I and II in *Podospora anserina lac2* (Fernández-Larrea and Stahl 1996) also aligned with *M. albomyces lac1* introns. These results are in accordance with previous results showing conserved intron architectures among laccase genes from the same fungal phylum (Saloheimo et al. 1991; Fernández-Larrea and Stahl 1996; Berka et al. 1997b). The overall amino acid identity of *M. albomyces* laccase was also high with other ascomycete laccases. The level of amino acid identity was 73% with *Myceliophthora thermophila* laccase (Berka et al. 1997a), 68% with

Podospora anserina laccase 2 (Fernández-Larrea and Stahl 1996), and 63% with *Neurospora crassa* laccase (Germann et al. 1988). On the other hand, the similarity with basidiomycete laccases was only about 30%, which complies with the hypothesis of the separation of fungal laccases into two divergent groups.

The first 22 N-terminal amino acids of *M. albomyces* laccase consisted of a predicted signal sequence typical for eukaryotic proteins (Nielsen et al. 1997). Comparison of the deduced amino acid sequence with the N-terminal peptide sequence of the purified protein showed that the signal sequence was followed by a cleavable propeptide of 28 amino acids. Furthermore, *M. albomyces* laccase was shown to be processed at its C-terminus: C-terminal sequencing of the purified laccase identified Ser-Gly-Leu as the last three amino acids, which indicated that the last 14 predicted amino acid residues were absent from the mature protein. Similar processing has also been suggested for other ascomycete laccases, namely *Neurospora crassa* (Germann et al. 1988), *Podospora anserina* (Fernández-Larrea and Stahl 1996) and *Myceliophthora thermophila* (Berka et al. 1997b) laccases. The last four amino acids of these mature proteins are also Asp-Ser-Gly-Leu. Interestingly, the sequence Asp-Ser-Gly-(Leu/Ile/Val) also exists in the C-termini of laccases from the ascomycetes *Cryphonectria parasitica* (Choi et al. 1992), *Botrytis cinerea* (Cantone and Staples 1993), *Glomerella (Colletotrichum) lagenarium* (Tsuji et al. 2001) and *Gaeumannomyces graminis* var. *tritici* (Litvintseva and Henson 2002), although in these laccases the conserved tetrapeptides are the last amino acid residues of the open reading frames and are not followed by cleavable extensions. This kind of conservation of C-terminal ends, along with the presence of the C-terminal plug in the crystal structure of *M. albomyces* laccase, strongly suggests that the conserved C-terminus has an important role in the function of ascomycete laccases. No similar conservation has been found among basidiomycete laccases.

3.5 Heterologous expression of *M. albomyces* laccase in *Saccharomyces cerevisiae* (IV)

Saccharomyces cerevisiae is often used as a host organism for production of heterologous eukaryotic proteins because of its easy handling in the laboratory, the broad knowledge of its molecular biology as well as the availability of versatile tools for its genetic engineering. Another benefit of *S. cerevisiae* is that

it does not secrete high amounts of contaminating native proteins into the culture medium. In this study, *S. cerevisiae* was used as a heterologous production host for *M. albomyces* laccase in order to create a relatively simple laccase expression system for directed evolution studies to be conducted in the future. Four different expression vectors with different types of promoters, signal sequences and propeptides as well as modified laccase cDNAs were constructed (Table 8).

Table 8. Plasmids for expression of M. albomyces *laccase in* S. cerevisiae.

Plasmid	**Promoter**	**Origin of signal sequence and propeptide**	***M. albomyces lac1* cDNA**	**Maximum laccase activity in the supernatant (nkat ml^{-1})**
pLLK7	*PGK1*	*M. albomyces lac1*	Unmodified	0
pLLK10	*GAL1*	*M. albomyces lac1*	Unmodified	around 0.002
pMS174	*GAL1*	*S. cerevisiae MFα1*	Signal sequence and propeptide removed	0.45
pMS175	*GAL1*	*S. cerevisiae MFα1*	As in pMS174 but has a stop codon after the C-terminal cleavage site	2.8

Laccase production was not detected from pLLK7, which contained the constitutive *PGK1* promoter, and very low laccase activity was detected from pLLK10, which contained the galactose-inducible *GAL1* promoter and the native laccase prepro sequence (Table 8). The most prominent improvement in laccase production was achieved by replacing the signal and propeptide sequences of *lac1* with the yeast α-factor signal sequence and propeptide (plasmid pMS174). This substitution improved extracellular laccase activity levels about 200-fold (Table 8). When the cleavable C-terminal extension of the laccase was removed by introducing a stop codon after the native cleavage site (i.e. pMS175), laccase production was further improved about six-fold (Table 8). If the enzyme produced in yeast has the same specific activity as the native *M. albomyces*

laccase, the highest laccase production level in yeast should correspond to about 3 mg l^{-1}. This is well adequate for high throughput screening of directed evolution experiments.

The prepro sequence of the *MFα1* gene of *S. cerevisiae* has been used as a secretion signal in different yeast expression systems, including *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris* and *S. cerevisiae* as host organisms (Weydemann et al. 1995; Jönsson et al. 1997; Hsieh and Da Silva 1998; Otterbein et al. 2000; Brown et al. 2002; Soden et al. 2002; Liu et al. 2003). It has also been used in basidiomycete laccase production in *Pichia pastoris*, but contrary to our results, these studies did not show higher laccase production levels with α-factor prepro sequence compared to native laccase signal sequences (Jönsson et al. 1997; Otterbein et al. 2000; Brown et al. 2002; Soden et al. 2002; Liu et al. 2003). However, it must be taken into account that our results were obtained with *S. cerevisiae*, which may recognize and process its own native prepro sequence more efficiently than *P. pastoris*. In addition, none of the laccases expressed in *P. pastoris* contained cleavable propeptide regions similar to that of *M. albomyces* laccase. It is possible that the propeptide cleavage of *M. albomyces* laccase was a major problematic step for the production of this enzyme in *S. cerevisiae*, and the significant improvement in laccase production brought about by the α-factor prepro sequence resulted from improved processing and secretion of the mature laccase protein. The importance of correct proteolytic processing for laccase expression was also verified by further improved laccase activity levels when the region encoding the cleavable C-terminal extension was removed. Improper folding of the product of the intact *lac1* cDNA was also suggested by Western blotting of yeast cell lysate samples, as the laccase with the C-terminal extension (pMS174) appeared to be more degraded intracellularly than the product of the truncated laccase gene (pMS175).

Interestingly, expression of the closely homologous *Myceliophthora thermophila* laccase in *S. cerevisiae* was also recently reported (Bulter et al. 2003). The initial production level was very low, but the production was considerably improved by directed evolution of the laccase gene, and a rather high laccase production level, 18 mg l^{-1}, was eventually reached (Bulter et al. 2003). The results of Bulter et al. (2003) also highlighted the importance of correct proteolytic processing of the preproenzyme, because three of the 13 amino acid substitutions found in the

best-produced mutant laccase were located at the three processing sites of the preproenzyme. Bulter et al. (2003) also studied the role of the C-terminal extension in laccase production by inserting a stop codon after the C-terminal processing site. In contrast to our results, removal of the C-terminal extension led to decreased laccase production (Bulter et al. 2003). However, the point mutation was made in *Myceliophthora thermophila lcc1* altered by a series of random mutations, and these random mutations may have affected the observed production levels. Despite this discrepancy, the results for *Myceliophthora thermophila* laccase mutants supported our conclusion that correct processing and folding is essential for production of these ascomycete laccases in yeast.

3.6 Transformation of *Trichoderma reesei* with *M. albomyces lac1* and characterization of the transformants (V)

M. albomyces produced laccase in rather low amounts (max 20 mg l^{-1}) and the cultivations were difficult to reproduce. The yields from expression of *M. albomyces lac1* cDNA in *S. cerevisiae* were adequate for directed evolution purposes but not high enough for efficient laccase production. Therefore *M. albomyces* laccase was expressed in *Trichoderma reesei*, a well-known filamentous fungus capable of producing high amounts of extracellular hydrolytic enzymes (Mäntylä et al. 1998). *M. albomyces lac1* cDNA was placed under the strongly inducible promoter of the major cellulase gene *cbh1*, which has previously been used for production of e.g. *Phlebia radiata* laccase (Saloheimo and Niku-Paavola 1991).

M. albomyces laccase was expressed in *T. reesei* in two forms: as a non-fused laccase and as a fusion protein containing the *T. reesei* hydrophobin I (HFBI) protein at the N-terminus. HFBI was chosen as a fusion partner because fusions with secreted native proteins have been reported to enhance the production of foreign proteins in *T. reesei* (Nyyssönen et al. 1993; Paloheimo et al. 2003), and also because HFBI can facilitate purification of the recombinant protein in aqueous two-phase systems (Linder et al. 2001; Selber et al. 2001). Several laccase-positive *T. reesei* transformants were obtained from both expression constructs. Approximately fivefold activity levels were obtained with the non-fused laccase than with the HFBI-laccase fusion protein. The highest laccase

activity produced by the non-fusion transformants was 193 nkat ml^{-1}, which corresponds to about 230 mg l^{-1}. Coomassie-stained SDS-PAGE of the culture supernatant samples showed that the recombinant laccase was one of the major secreted proteins of the transformants (Figure 4C).

Fusion to a secreted host protein had no effect on the production of the heterologous *M. albomyces* laccase. On the other hand, HFBI has not previously been used as a production carrier protein. To examine the secretion of heterologous laccase, transformants from both expression constructs were studied by Western blotting. The cell lysate and culture supernatant samples showed that most of the HFBI-laccase fusion protein was cleaved between the fusion partners (Figures 4A and 4B, lanes 1–2). Cell lysate samples also showed intracellular proteolytic degradation of both the non-fused laccase and the fusion protein (Figure 4B, lanes 2 and 4), but for the fusion protein the intracellular proportion was much higher. This suggests that secretion of the fusion protein was a problematic step in the production. This was further indicated by RNA hybridization analyses, which showed that the laccase mRNA levels obtained from the fusion construct were at the same level or even higher than those derived from the non-fusion construct (Figure 5). The difference between the production level of the two expression constructs could thus not be explained by transcription efficiency or mRNA stability.

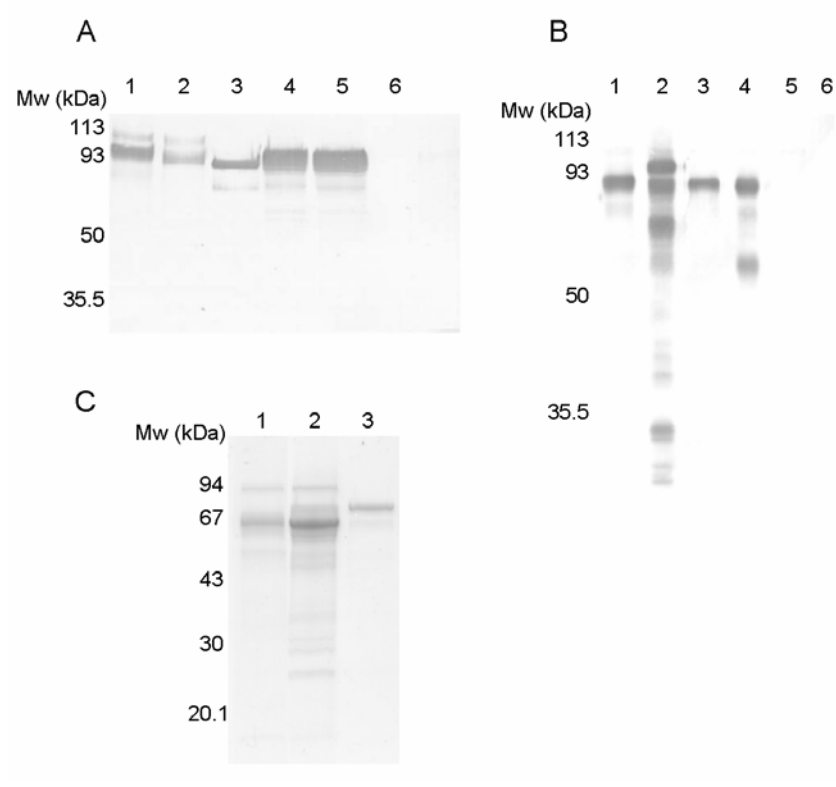


Figure 4. (A) Western blot analysis of culture supernatants of HFBI-laccase fusion strains (lanes 1–2) and transformants producing non-fused laccase (lanes 4–5). Lane 3 contained 0.2 μg of native M. albomyces *laccase. Lane 6 is the control strain* Trichoderma reesei *RutC-30. (B) Western blot analysis of culture supernatants (lanes 1, 3, 5) and cell lysates (lanes 2, 4, 6) of HFBI-laccase fusion strains (lanes 1–2), non-fusion laccase strains (lanes 3–4), and the control strain (lanes 5–6). The supernatant and cell lysate samples loaded on the gel corresponded to the same culture volume. (C) Coomassie-stained SDS-PAGE of culture supernatant of* T. reesei *RutC-30 (lane 1), culture supernatant of the best-producing non-fusion laccase transformant pLLK13/295 (lane 2), and purified recombinant* M. albomyces *laccase (lane 3).*

Overexpression of heterologous proteins often causes secretion stress in host cells. This can induce the unfolded protein response (UPR) pathway, which is a signal transduction pathway reacting to accumulation of unfolded proteins in the endoplasmic reticulumn (ER) and inducing genes involved in folding,

degradation or transport of proteins (Mori 2003). The possible induction of UPR by laccase expression in *T. reesei* was studied by hybridizing *T. reesei* total RNA with the cDNA-probes of the UPR-related genes *pdi1*, *bip1* and *hac1*. The results showed that *pdi1* and *bip1* mRNAs were not present at elevated levels as compared to the control, and the truncated form of the *hac1* mRNA was not found in any of the laccase-producing transformants (Figure 5). This indicated that laccase production did not cause severe secretion stress in *T. reesei*. The result was slightly surprising, since rather extensive intracellular accumulation of the fusion protein and its degradation products was detected by the Western blot analyses with the antibody against *M. albomyces* laccase (Figure 4B). This might be explained by trapping of the fusion protein in other parts of the secretory pathway than the ER, e.g. in the vacuole.

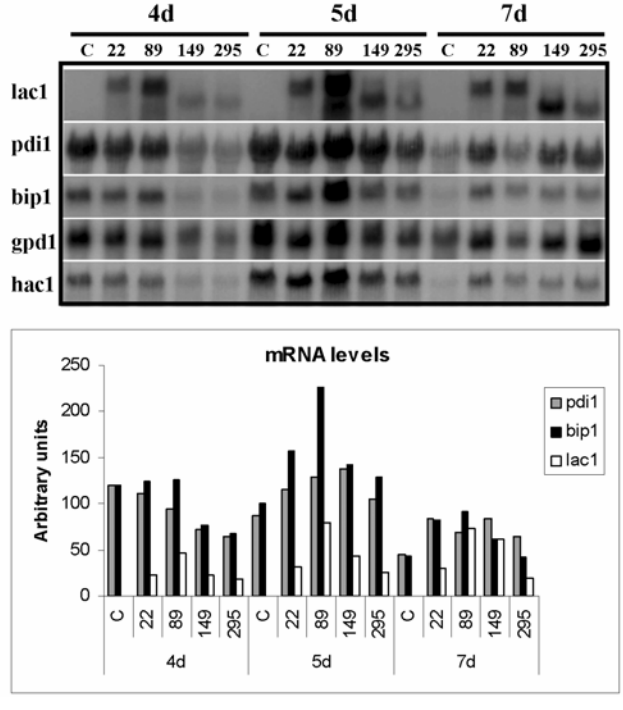


Figure 5. Gene expression analysis of the control strain and of the two best-producing transformants from the HFBI-laccase fusion construct (transformants 22 and 89) and from the non-fusion construct (149 and 295) performed by hybridizing T. reesei *total RNA with* M. albomyces lac1 *and the UPR-related cDNA-probes* pdi1, bip1 *and* hac1 *after 4, 5 and 7 days of cultivation. The signal intensities were quantified and normalized to* gpd1 *(glyceraldehyde-3-phosphate dehydrogenase) signal.*

3.7 Production, purification and biochemical characterization of recombinant *M. albomyces* laccase from *T. reesei* (V)

In order to produce high amounts of *M. albomyces* laccase, the *T. reesei* transformant producing highest laccase activities was cultivated in a laboratory-scale fermentor. Two types of cultivations were performed, a batch and a fed-batch fermentation. In the batch fermentation, the highest laccase activity reached 250 nkat ml^{-1}, which corresponds to about 290 mg l^{-1} of laccase. Significant improvement in the production level was achieved by applying the recently published feeding algorithm for continuous cellulase production by *T. reesei* (Bailey and Tähtiharju 2003). When lactose was slowly added into the cultivation medium after exhaustion of the initial carbon source, the laccase activity increased to 780 nkat ml^{-1}, corresponding to about 920 mg l^{-1}. The achieved production level is comparable to the highest reported laccase production levels of 700–1500 mg l^{-1} obtained in the native hosts *Trametes pubescens* and *Pycnoporus cinnabarinus* (Galhaup et al. 2002b; Lomascolo et al. 2003). *M. albomyces* laccase production in the cultivations was rather interesting, as it occurred at a different growth phase than cellulase production, although the laccase gene was expressed from the *cbh1* promoter. The production curves for cellulases were typical for *T. reesei*, and the most efficient cellulase production occurred at the time when exhaustion of lactose started to limit fungal growth (Bailey and Tähtiharju 2003). However, laccase activity in the culture supernatant increased significantly after cellulase production and fungal growth had already ended. This might be related to activation of an apolaccase by proteolytic processing of the propeptide and/or the C-terminal extension rather than actual protein production. As determined by N- and C-terminal sequencing of purified recombinant laccase, *T. reesei* was able to process both termini of the laccase correctly. Involvement of proteolysis in the activation of laccases has previously been detected with a *Pleurotus ostreatus* laccase (Palmieri et al. 2001). Another possibility for the continuously increasing laccase activity in the supernatant could have been the release of intracellular laccase after cell autolysis.

Recombinant laccase was purified from the fermentation culture filtrate. The purification was significantly disturbed by the presence of high amounts of the major cellulase CBHI, because laccase and intact CBHI could not be separated

by the chromatographic methods available. However, purification of laccase succeeded after treating the culture supernatant with papain, which cleaved CBHI between the cellulose binding domain and the core protein (van Tilbeurgh et al. 1986). The cleavage altered the hydrophobicity of CBHI and thus facilitated the separation of laccase and CBHI by hydrophobic interaction chromatography. Papain digestion did not change the activity, size, N- or C-terminal peptide sequences of *M. albomyces* laccase. After hydrophobic interaction chromatography, the laccase was further purified by anion exchange chromatography and gel filtration. Purified recombinant laccase and wild type *M. albomyces* laccase were compared with regard to molecular weights, isoelectric points, pH optima, thermostabilities, redox-potentials and kinetic constants. The results showed that recombinant laccase produced in *T. reesei* was very similar to the wild type laccase (Table 9)

Table 9. Biochemical characteristics of native (MaL) and recombinant (rMaL) M. albomyces *laccases. Kinetic measurements were performed in 25 mM succinate buffer at pH 4.5 with ABTS and in 40 mM MES/NaOH buffer at pH 6 with other substrates.*

Property	**MaL**	**rMaL**
MW (MALDI-TOF)	72 200	71 300
pI	4.0	4.0
pH optimum on guaiacol oxidation	5–7.5	5–7.5
T½ 60°C (h)	3.5	3.5
T½ 50°C (h)	50	50
T½ 40°C (h)	>70	>70
E^0 (mV)	460	470
C- terminus	DSGL	DSGL
K_m (ABTS) (μM)	280 +/- 20	270 +/- 10
K_m (2,6-DMP) (μM)	5.2 +/- 0.2	5.2 +/- 0.1
K_m (syringaldazine) (μM)	1.3 +/- 0.1	1.8 +/- 0.1
k_{cat} (ABTS) (min^{-1})	4500	4700
k_{cat} (2,6-DMP) (min^{-1})	3400	4000
k_{cat} (syringaldazine) (min^{-1})	4700	5500

When compared to other laccases, *M. albomyces* laccase had a rather high K_m value for the non-phenolic ABTS and very low K_m values for the phenolic 2,6-DMP and syringaldazine (Tables 1 and 9). This indicates that phenolic compounds might be better substrates for *M. albomyces* laccase than the commonly used ABTS. The catalytic constant values of *M. albomyces* laccase are closest to the values of *Myceliophthora thermophila* laccase (Table 1; Bulter et al. 2003), which can be explained by the high similarity between the amino-acid sequences of the enzymes.

3.8 Binding of *M. albomyces* laccase to cellulose (VI)

During the purification of recombinant *M. albomyces* laccase, it was discovered that the laccase adsorbed on cellulosic materials. This phenomenon was studied in more detail using steam-pretreated softwood (SPS), microcrystalline cellulose (Avicel and bacterial microcrystalline cellulose, BMCC) and alkali lignin as adsorbents. In addition, the binding of *Trametes hirsuta* and *Mauginiella* sp. laccases was studied in order to compare the behaviours of different laccases.

Binding to SPS was studied using the three different laccases at two concentrations. The results showed that only *M. albomyces* laccase showed extensive binding, whereas *Trametes hirsuta* and *Mauginiella* sp. laccases did not adsorb on softwood. This suggested that binding to lignocellulosic materials is not a common feature of all laccases. Binding of *M. albomyces* laccase was further studied with purified microcrystalline cellulose. The adsorption studies with Avicel showed that less than 3% of the initial activity remained in the supernatant after one hour of binding, i.e. *M. albomyces* laccase was able to bind very efficiently to pure cellulose (Table 10A). To verify that the disappearance of laccase activity from the supernatant was not caused by inactivation of the laccase by cellulose, the activity of bound laccase was also measured. The results showed that all the enzyme activity removed from the supernatant could be recovered by measuring the activity of enzyme bound to cellulose (Table 10A). This confirmed that the activity measurement data described true binding of *M. albomyces* laccase to cellulose.

Lignin is one of the natural substrates of laccases. Therefore, the adsorption of *M. albomyces* laccase on lignin was also studied. Binding to lignin was studied

at 4°C in order to minimize the possible degradation of lignin by the laccase. No binding to Indulin AT (alkali lignin) occurred, and all of the initial activity was detected in the supernatant fraction after mixing for one hour (Table 10B). This is in contrast with the results obtained with many cellulases, which have been shown to adsorb on both cellulose and lignin (Sutcliffe and Saddler, 1986; Bernardez et al. 1993; Palonen et al. 2004). However, it must be taken into account that the adsorption of cellulases on lignin strongly depends on the nature of the lignin preparation (Palonen et al. 2004), and the extraction and drying of the alkali lignin used in this study may have changed its characteristics significantly.

Table 10. Amount of M. albomyces *laccase in the supernatant and in the solid fractions after mixing with 1% Avicel at 22℃ (A) or alkali lignin at 4℃ (B) for one hour at pH 5.*

A

Initial dosage (nkat)	Supernatant fraction (nkat)	Cellulose fraction (nkat)
10	0.2	10
0.8	0	0.8

B

Initial dosage (nkat)	Supernatant fraction (nkat)
7	6
0.7	0.7

Due to the rather heterogeneous structure of Avicel preparations, more detailed analysis on binding of *M. albomyces* to cellulose was performed using bacterial microcrystalline cellulose as adsorbent. The amount of laccase bound to cellulose was measured using different enzyme concentrations, and the adsorption parameters for *M. albomyces* laccase were determined from the adsorption isotherm obtained (Figure 6). The data points on the isotherm fitted well to the classical Langmuir-type binding model, which facilitated the calculation of the maximum binding capacity B_{max} and the dissociation constant

K_d (Bothwell and Walker 1995). The curve fit yielded values of 1.94 ±0.05 μmol g^{-1} and 0.006 ±0.001 μM, respectively. The relative partition coefficient (K_p) of 320 ± 80 l g^{-1} was also calculated by using the slope of the isotherm at low enzyme concentration (Linder et al. 1996). As a comparison, the adsorption of *Trametes hirsuta* laccase was also studied at two points on the isotherm. The results clearly indicated that *Trametes hirsuta* laccase did not bind to BMCC (Figure 6).

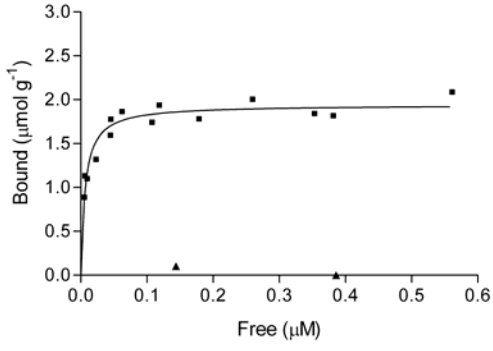


Figure 6. The adsorption isotherm of M. albomyces *(■) laccase obtained with BMCC at 22℃, pH 5. (▲) binding of* Trametes hirsuta *laccase.*

Comparison of the binding parameters of *M. albomyces* laccase with the values obtained for cellulases showed that the maximum binding capacity of *M. albomyces* laccase was relatively low, as binding capacities ranging from 6 to 20 μmol g^{-1} for BMCC have been reported for various cellulases (Reinikainen et al. 1995; Jung et al. 2003). However, very similar binding capacity of 2.1 μmol g^{-1} has previously been observed for a cellobiose dehydrogenase (CDH) from *Phanerochaete chrysosporium* (Henriksson et al. 1997). Despite the low binding capacity, the affinity of *M. albomyces* laccase (represented by K_p) was clearly higher than those reported for cellulases. For example, the partition coefficients of cellobiohydrolases CBHI and CBHII from *Trichoderma reesei* were 18 and 3.4 l g^{-1}, respectively, (Palonen et al. 1999), and those of the cellulases CenA and Cex from *Cellulomonas fimi* were 40.5 and 33.3 l g^{-1}, respectively (Gilkes et al. 1992). The combination of high affinity and low capacity of binding suggests that *M. albomyces* laccase is able to bind very strongly to BMCC, but only at relatively few binding sites. One probable reason for the differences in binding behaviour between cellulases and *M. albomyces* laccase could be the absence of

a cellulose binding domain (CBD) in the laccase. Neither the amino acid sequence nor the crystal structure of *M. albomyces* laccase contain a separate CBD that in most cellulases dominates the binding characteristics. In addition, the *Phanerochaete chrysosporium* CDH, which shows similar binding behaviour to that of *M. albomyces* laccase, does not contain a separate CBD either (Henriksson et al. 1997; Hallberg et al. 2002). The crystal structure of CDH has been analyzed with respect to cellulose binding, but no obvious substructures or surface patches that could be assigned as the binding site have been identified (Hallberg et al. 2002).

One of the assumptions in a Langmuir-type binding model is full reversibility of adsorption, and the reversibility of binding of *M. albomyces* laccase to cellulose was studied by dilution experiments. The desorption data showed that a new equilibrium was established on the same isotherm, which indicated that the binding was fully reversible. In order to elucidate the nature of interactions affecting the adsorption of *M. albomyces* laccase on BMCC, the effect of non-specific protein (BSA) and ionic strength on binding were also studied. The results showed that the binding of *M. albomyces* laccase to cellulose was not affected by BSA. The binding was thus not markedly affected by random protein adsorption on cellulose. The binding was also unaltered in the presence of 0.1–0.5 M Na_2SO_4. As ionic interactions are weakened by increasing ionic strength, it can be concluded that electrostatic forces were not the main cause for the observed binding (Kyriacou et al. 1988). Interestingly, these binding characteristics of *M. albomyces* laccase resemble the binding of CBDs to cellulose, despite the lack of a CBD in the laccase. When CBDs are separated from the core of the cellulase protein, they show many binding characteristics similar to those of *M. albomyces* laccase: the binding is not affected by even high concentrations of a non-specific protein such as BSA, it is fully reversible, and it follows a first order Langmuir isotherm (Linder and Teeri 1996).

Binding of *M. albomyces* laccase to cellulose is very interesting considering the role of fungal laccases in nature. Recently, preliminary results by Paice et al. (2002) showed adsorption of *Myceliophthora thermophila* laccase on Kraft pulp, which suggests that adsorption on cellulose may be a common feature among some laccases. The function of this phenomenon is unknown, but it could be related to the total hydrolysis of lignocellulose, because *M. albomyces* is also known to produce several cellulose- and hemicellulose-degrading enzymes

(Saraswat and Bisaria 2000; Miettinen-Oinonen et al. 2004). Laccase treatment of lignocellulose has been shown to improve the hydrolysis of cellulose by cellulases by about 13% (Palonen and Viikari 2004). Interestingly, the cellulose-binding enzyme CDH uses quinones and phenoxy radicals, i.e. products of laccase-catalyzed reactions, as electron donors when oxidizing cellobiose (Henriksson et al. 2000). It is thus possible that laccase produces electron donors for CDH and that the two enzymes function in close proximity. Total degradation of the chemically resistant lignocellulose most probably requires the concerted action of a wide set of versatile enzymes, and binding to cellulose may assist in increasing the effective concentrations of these enzymes at the reaction site.

4. Conclusions

Laccases are blue copper proteins which catalyze oxidation reactions coupled to the four-electron reduction of molecular oxygen to water. Because of the versatility of potential substrates, laccases are highly interesting as novel biocatalysts in various industrial processes. This work describes screening for laccase-producing fungi from various environmental samples and preliminary analysis of the most interesting isolated enzymes. The work also provides detailed biochemical, structural and genetic information on a novel laccase from the thermophilic fungus *Melanocarpus albomyces*.

Screening for laccase-producing fungi showed that novel ligninolytic micro-organisms can be found by simple plate tests using the indicator compounds guaiacol and the polymeric dyes RBBR or Poly R-478. Four novel fungal laccases were characterized and they were found to have high thermostabilities as compared to other fungal laccases studied thus far.

A novel laccase from *M. albomyces* was purified and biochemically characterized with respect to size, pI, substrate specificity, inhibitors, pH and temperature characteristics and the presence of copper atoms. The data indicated that the purified enzyme was a true laccase. The laccase was shown to be rather thermostable and to have unusually high pH optima, which increases its potential for many industrial applications. The crystal structure of *M. albomyces* laccase was resolved as one of the first laccase structures containing all four copper atoms. This facilitated the determination of copper coordination chemistry at the laccase active site. Copper-containing enzymes are crucial metabolic catalysts throughout all kingdoms of life, and their ability to bind oxygen has been studied widely. Interestingly, the structure of *M. albomyces* laccase revealed elongated electron density between the three coppers in the trinuclear center, suggesting the presence of molecular oxygen bound with a novel binding geometry. The crystal structure of *M. albomyces* laccase also revealed an exceptional C-terminal plug, i.e. the last four amino-acid residues of the protein were packed inside a tunnel leading to the trinuclear copper center. The importance of the C-terminal plug is not yet understood, but it may have a role in the function of the enzyme by affecting the entry of oxygen into the active site.

The gene encoding *M. albomyces* laccase was cloned in order to elucidate the molecular structure of the enzyme and to produce it heterologously. *M. albomyces* laccase was shown to be similar in amino-acid sequence with laccases from other ascomycetous fungi, but significantly less related to basidiomycete laccases. This finding further strengthened the hypothesis of the separation of fungal laccases into two clearly divergent groups. The N- and C-terminal sequences of the purified laccase showed that both ends of the proenzyme were processed after translation. Conservation of the C-terminus in several ascomycete laccases, in combination with the data obtained from the crystal structure of *M. albomyces* laccase, further indicated that the C-terminal processing may be important for laccase activity.

M. albomyces lac1 cDNA was expressed in *S. cerevisiae* using four different expression constructs. Highest laccase production was obtained with a construct that contained the inducible *GAL1* promoter, signal and propeptide sequences from the *S. cerevisiae MFα* gene and *lac1* cDNA encoding mature laccase protein, i.e. without the propeptide and the C-terminal extension. The most significant improvement in laccase production was obtained by replacing the *lac1* signal and propeptide sequences with the α-factor prepro sequence. Because signal sequences are generally interchangeable between eukaryotic species, it is probable that the propeptide cleavage was the problematic step for production of *M. albomyces* laccase in yeast. The efficient yeast expression system created in this study will facilitate the use of high throughput screening methods in directed evolution studies of *M. albomyces* laccase in the future. Directed evolution could be used, for example, to generate laccase mutants with altered substrate specificities, stabilities, pH profiles or redox potentials.

M. albomyces laccase was also expressed in *T. reesei* in order to obtain a reliable and efficient source for the laccase. In addition to unmodified laccase, a hydrophobin-laccase fusion protein was produced. The fusion was made in order to enhance the secretion of the heterologous protein and to simplify its purification. However, about fivefold higher activity levels were obtained with the non-fused laccase. According to the results, factors causing this difference included proteolytic degradation and inefficient secretion of the fusion protein. The non-fused recombinant *M. albomyces* laccase was produced in a fed-batch fermentation and the production level of 920 mg l^{-1} was the highest heterologous laccase production level reported so far. In the future, laccase production may

probably be further improved by optimization of the cultivation parameters and by deleting cellulase genes from the laccase-producing transformant. In addition, the *T. reesei* transformants producing high amounts of laccase can be used to study various factors related to heterologous protein expression in general in this industrially important fungus. Recombinant *M. albomyces* laccase produced in *T. reesei* was purified and compared to the native laccase. The results showed that the molecular weights, pIs, pH optima, temperature stabilities, C-termini, redox-potentials and substrate kinetics were very similar for both enzymes.

Surprisingly, it was discovered that *M. albomyces* laccase binds effectively to cellulose. Binding to cellulose has been demonstrated for many enzymes involved in the modification of cellulose, but this was the first time that a laccase was shown to bind to cellulose. *M. albomyces* laccase was able to bind efficiently to steam-pretreated softwood and to microcrystalline cellulose, but not to alkali lignin. No binding was detected with *Trametes hirsuta* or *Mauginiella* sp. laccases, suggesting that cellulose-binding ability is not a common feature of all laccases. Based on the adsorption parameters of *M. albomyces* laccase, it was concluded that the laccase binds to cellulose with very high affinity but with rather low binding capacity. In addition, the binding was shown to be reversible and specific. In the future, several interesting aspects concerning the binding interactions and the effects of various environmental conditions on binding remain to resolved. The evaluation of the benefits of cellulose binding in various applications, such as textile dye oxidation and modification of lignocellulosics, will be also be important.

This work provided information on novel fungal laccases from different aspects of enzymology, ranging from biochemical and molecular characterization to heterologous expression and binding studies. The thorough analysis of the industrially interesting laccase from *M. albomyces* has significantly increased our knowledge of the mode of action and molecular biology of laccases. These results will be of great help for future studies focusing on the structure-function relatioships and heterologous production of laccases. In addition, deeper understanding of the biochemistry of laccases will facilitate the development of novel and more economical laccase applications.

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Title

Characterization and heterologous production of a novel laccase from *Melanocarpus albomyces*

Abstract

Laccases are copper-containing enzymes that oxidize a variety of industrially relevant substrates. In order to fully exploit laccases in industrial processes, novel laccases with high stability and activity at elevated temperatures and pH values are needed. This work focused on identifying and characterizing novel fungal laccases having potential for the applications as well as on development of efficient production methods for laccases.

Laccase-producing fungi were screened from various environmental samples by plate tests, and a total of 26 positive fungal strains were isolated. Four novel fungal laccases were preliminarily characterized, and these enzymes were found to be rather typical basidiomycete laccases.

A novel laccase from the ascomycete *Melanocarpus albomyces* was purified and biochemically characterized. Interestingly, *M. albomyces* laccase showed good thermostability and activity at neutral to alkaline pH values. The crystal structure of *M. albomyces* laccase was resolved and the overall structure was shown to be similar to other blue copper oxidases. However, novel features were discovered at the active site and at the C-terminal end of the enzyme.

The gene encoding *M. albomyces* laccase was isolated, and the amino acid sequence of the enzyme was shown to be about 60–70% identical with laccases from other ascomycetes. *M. albomyces* laccase cDNA was expressed in *Saccharomyces cerevisiae*. Very low laccase production levels were significantly improved by replacing the native signal and propeptide sequences of laccase cDNA by the prepro-sequence of the *S. cerevisiae* α-factor gene.

M. albomyces laccase was also expressed in the filamentous fungus *Trichoderma reesei*. The laccase was expressed as a non-fused laccase and as a fusion protein with the *T. reesei* hydrophobin I. The unmodified recombinant *M. albomyces* laccase was produced in a laboratory-scale fermentor and the production level reached 920 mg l^{-1}. Recombinant *M. albomyces* laccase was purified and biochemically characterized and it was shown to be very similar to the native laccase.

M. albomyces laccase was shown to bind to lignocellulose and purified cellulose. The adsorption parameters indicated that *M. albomyces* laccase binds to cellulose very efficiently but with a relatively low binding capacity. No binding was detected with other laccases, which suggests that binding to cellulose is not a common feature among laccases.

Keywords
enzyme, laccase, screening, characterization, *Melanocarpus albomyces*, heterologous expression, *Saccharomyces cerevisiae*, *Trichoderma reesei*, cellulose

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