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Laura-Leena Kiiskinen

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

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Laura-Leena Kiiskinen

VTT Biotechnology

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VTT, Bergsmansvägen 5, PB 2000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 5, P.O.Box 2000, FIN–02044 VTT, Finland
phone internat. +358 20 722 111, fax +358 20 722 4374

VTT Biotekniikka, Tietotie 2, PL 1500, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 2103

VTT Bioteknik, Datavägen 2, PB 1501, 02044 VTT
tel. växel 020 722 111, fax 020 722 2103

VTT Biotechnology, Tietotie 2, P.O.Box 1501, FIN–02044 VTT, Finland
phone internat. +358 20 722 111, fax +358 020 722 2103

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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 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.

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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).

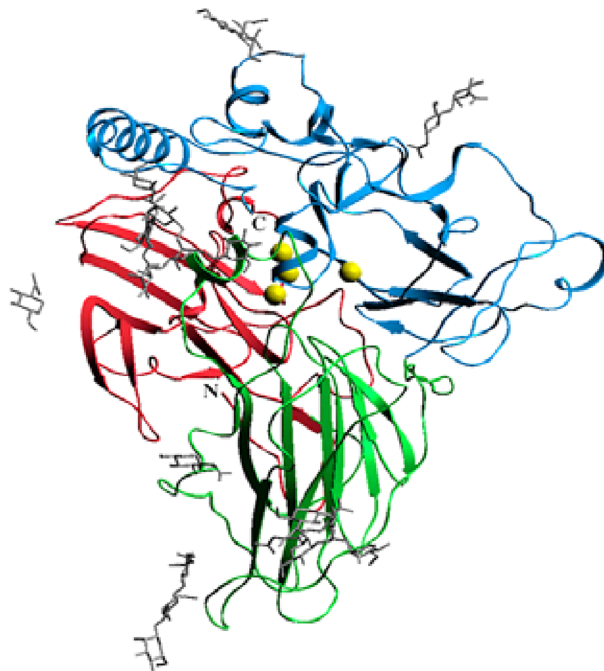
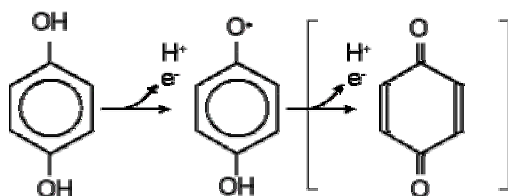


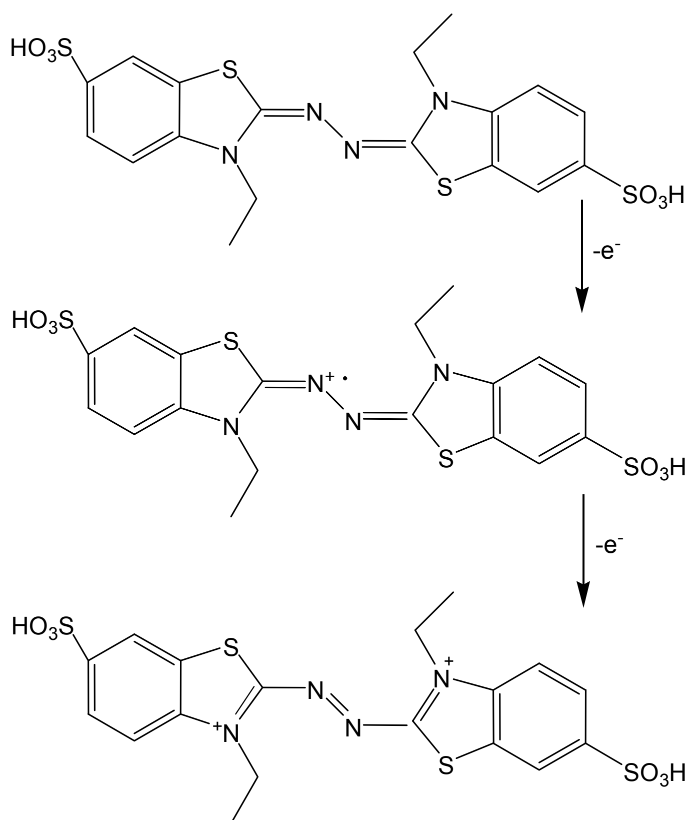
Figure 1. Three-dimensional structure of M. albomyces *laccase (III, reprinted with permission from Nature Publishing Group). Domains A, B, and C are colored red, green and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks.*

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.

Organism	Gene		Protein encoded by the gene			Reference
	Name	EMBL Acc. No.	Length (aa)	MW† (kDa)	pI	
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 $MF\alpha$ 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 x 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.

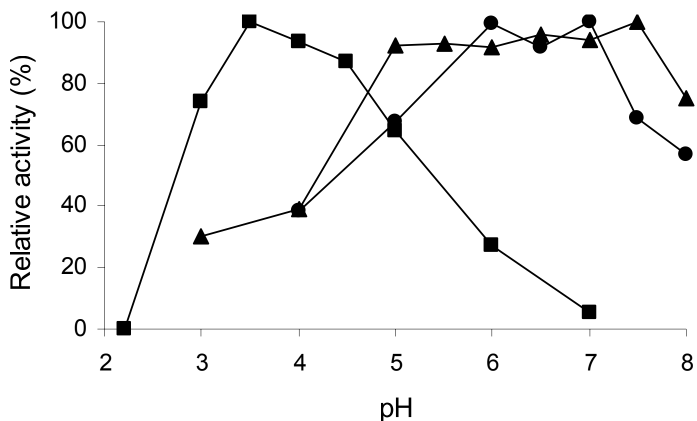
Plate-test screening with laccase indicator compounds has been reported by many groups (Nishida et al. 1988; de Jong et al. 1992; Barbosa et al. 1996; Goncalves and Steiner 1996; Chefetz et al. 1998; Raghukumar et al. 1999), but most of the laccases found in these screening studies have not been purified 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)

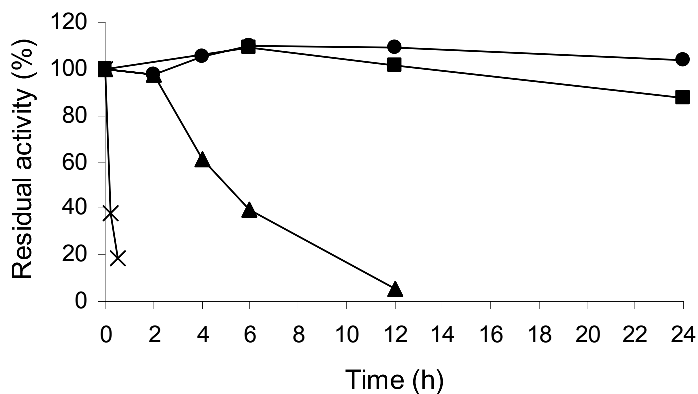
Earlier studies conducted at VTT Biotechnology had indicated that the thermophilic ascomycete *Melanocarpus albomyces* produced laccases (Ravanko 1996). Because *M. albomyces* has previously been reported to produce thermostable and neutral cellulases and xylanases (Vehmaanperä et al. 1996; Prabhu and 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.

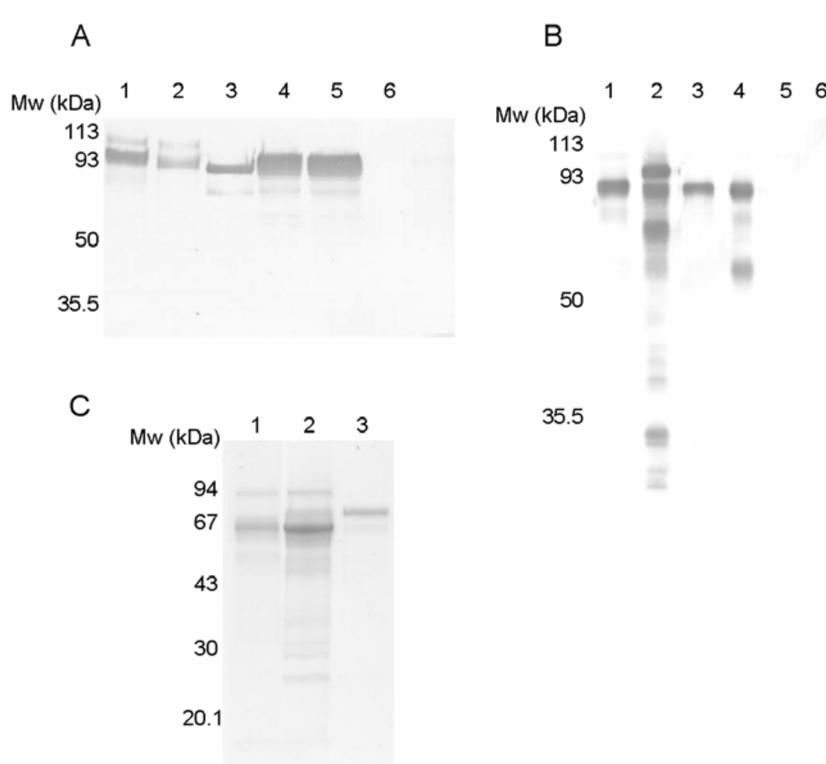


Figure 4. (A) Western blot analysis of culture supernatants of HFBI-laccase fusion strains (lanes 1–2) and transformants producing non-fused laccase (lanes 4–5). Lane 3 contained 0.2 μg of native M. albomyces *laccase. Lane 6 is the control strain* Trichoderma reesei *RutC-30. (B) Western blot analysis 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.

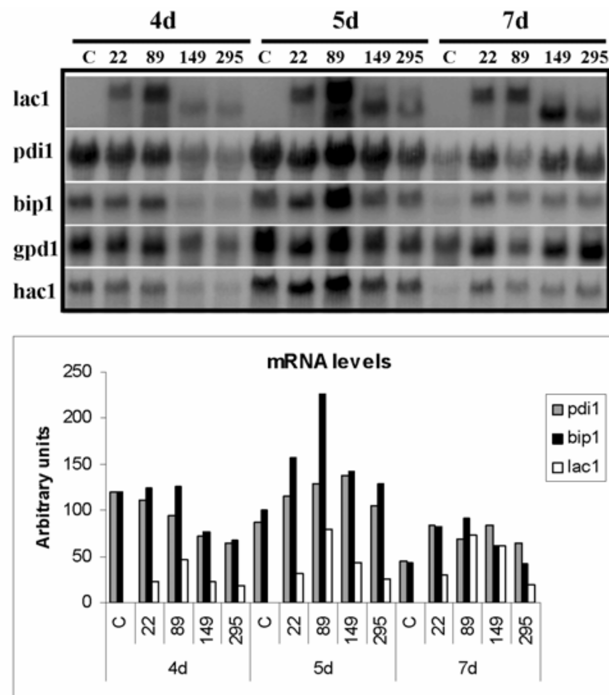


Figure 5. Gene expression analysis of the control strain and of the two best-producing transformants from the HFBI-laccase fusion construct (transformants 22 and 89) and from the non-fusion construct (149 and 295) performed by hybridizing T. reesei *total RNA with* M. albomyces lac1 *and the UPR-related 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°C (A) or alkali lignin at 4°C (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).

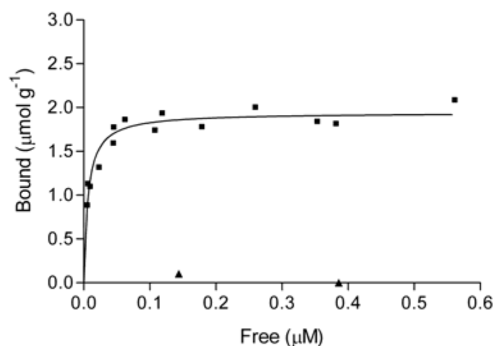


Figure 6. The adsorption isotherm of M. albomyces *(■) laccase obtained with BMCC at 22 ℃, pH 5. (▲) binding of* Trametes hirsuta *laccase.*

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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Screening for novel laccase-producing microbes

L.-L. Kiiskinen, M. Rättö and K. Kruus
VTT Biotechnology, Espoo, Finland

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ABSTRACT

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Aims: To discover novel laccases potential for industrial applications.

Methods and Results: Fungi were cultivated on solid media containing indicator compounds that enabled the detection of laccases as specific colour reactions. The indicators used were Remazol Brilliant Blue R (RBBR), Poly R-478, guaiacol and tannic acid. The screening work resulted in isolation of 26 positive fungal strains. Liquid cultivations of positive strains confirmed that four efficient laccase producers were found in the screening. Biochemical characteristics of the four novel laccases were typical for fungal laccases in terms of molecular weight, pH optima and pI. The laccases showed good thermal stability at 60°C.

Conclusions: Plate-test screening based on polymeric dye compounds, guaiacol and tannic acid is an efficient way to discover novel laccase producers. The results indicated that screening for laccase activity can be performed with guaiacol and RBBR or Poly R-478.

Significance and Impact of the Study: Laccases have many potential industrial applications including textile dye decolourization, delignification of pulp and effluent detoxification. It is essential to find novel, efficient enzymes to further develop these applications. This study showed that relatively simple plate test screening method can be used for discovery of novel laccases.

Keywords: guaiacol, laccase, Poly R-478, RBBR, screening, tannic acid.

INTRODUCTION

Laccases (benzenediol : oxygen oxidoreductases; EC 1.10.3.2) exist widely in nature. They are predominantly found in higher plants and fungi (Thurston 1994; Mayer and Staples 2002), and recently some bacterial laccases have also been characterized from *Azospirillum lipoferum* (Givaudan *et al.* 1993), *Bacillus subtilis* (Martins *et al.* 2002), *Streptomyces lavendulae* (Suzuki *et al.* 2003) and *S. cyaneus* (Arias *et al.* 2003). Most of the laccases studied thus far are of fungal origin, especially from white-rot fungi, such as *Phlebia radiata* (Niku-Paavola *et al.* 1988), *Pleurotus ostreatus* (Palmieri *et al.* 2000) and *Trametes versicolor* (Bourbonnais *et al.* 1995). The physiological roles of fungal laccases include degradation of lignocellulosic materials, pigment production and plant pathogenesis (Thurston 1994). Laccases oxidize a surprisingly wide variety of organic and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines and aromatic amines, with concomitant reduction of molecular oxygen to water (Thurston 1994). Large variety of potential substrates has raised interest in the use of laccases in several industrial applications, such as pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation (Gianfreda *et al.* 1999; Xu 1999).

Discovery of novel laccases with different substrate specificities and improved stabilities is important for industrial applications. Microbes that produce laccases have been screened for either on solid media containing coloured indicator compounds that enable the visual detection of laccase production (Nishida *et al.* 1988; De Jong *et al.* 1992; Barbosa *et al.* 1996) or with liquid cultivations monitored

Present address: Laura-Leena Kiiskinen, Finnzymes Oy, Keilaranta 16 A, Fin-02150 Espoo, Finland.

Correspondence to: Kristiina Kruus, VTT Biotechnology, PO Box 1500, Fin-02044 VTT, Espoo, Finland (e-mail: kristiina.kruus@vtt.fi).

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with enzyme activity measurements (Szklarz *et al.* 1989; Peláez *et al.* 1995; Luterek *et al.* 1997). The use of coloured indicators is generally simpler as no sample handling and measurement is required. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. The traditional screening reagents tannic and gallic acid (Harkin and Obst 1973) have nowadays mostly been replaced with synthetic phenolic reagents, such as guaiacol and syringaldazine (Nishida *et al.* 1988; De Jong *et al.* 1992) or with the polymeric dyes Remazol Brilliant Blue R (RBBR) and Poly R-478 (Barbosa *et al.* 1996; D'Souza *et al.* 1999; Raghukumar *et al.* 1999). RBBR and Poly R-478 are decolourized by lignin-degrading fungi (Gold *et al.* 1988; Barbosa *et al.* 1996), and the production of ligninolytic enzymes is observed as a colourless halo around microbial growth. With guaiacol a positive reaction is indicated by the formation of a reddish-brown halo (Nishida *et al.* 1988), while with tannic and gallic acid the positive reaction is a dark-brown coloured zone (Harkin and Obst 1973).

In this study, laccase-producing fungi were isolated from various environmental samples using four different indicator compounds in agar plates. The screening results with different indicators were also compared in order to reduce the number of indicator compounds needed in future screening procedures. In addition, the production of laccase by the positive strains was monitored in liquid cultures. The laccases that were produced in significant amounts were preliminarily characterized.

MATERIALS AND METHODS

Solid media used in the screening

Malt extract agar (MEA) and potato dextrose agar (PDA) from Difco Laboratories (Detroit, MI, USA) were used to isolate fungi. 0·01% (w/v) of chloramphenicol and chlorotetracycline (Sigma, St Louis, MO, USA) were added to the media in order to inhibit the growth of bacteria. In addition, 1% (w/v) of Benomyl, a benzimidazole fungicide (Du Pont de Nemours & Co., Wilmington, DE, USA), was added in order to select for wood decay fungi (Maloy 1974). Benomyl was autoclaved together with the media, whereas chloramphenicol and chlorotetracycline were added to the media after autoclaving.

Indicator compounds used in the screening

Different indicator compounds were added to the solid media in order to detect microbes that produced ligninolytic enzymes: 0·04% (w/v) RBBR (Sigma), 0·04% Poly R-478 (Sigma), 0·01% guaiacol (Sigma) or 0·5% tannic acid (Merck, Whitehouse Station, NJ, USA). Guaiacol was added to the media before autoclaving, and RBBR and Poly R-478 after autoclaving as sterile-filtered water solutions. Tannic acid was autoclaved separately before addition to the media. The correlation between positive reactions with different indicators was investigated by cross-cultivating positive strains on all different indicator plates. The white-rot fungus *Trametes hirsuta* (VTT D-95443) that produces laccase (Rittstieg *et al.* 2002), manganese peroxidase and lignin peroxidase (unpublished data), was used as a positive control for indicator plates.

Isolation of micro-organisms

Laccase-producing microbes were screened from various environmental samples taken in Finland: decomposing tree stump, oak leaf compost, birch log, spruce chip pile and test compost of municipal biowaste at different composting stages. In addition, microbes were isolated from crude cork material and process waters of a Portuguese cork factory (A. Silva, Porto, Portugal) and from a soiled pulp sample from a pulp mill in Svetogorsk, Russia. Fungi were isolated by placing pieces of samples on MEA and PDA plates containing different indicator compounds. The plates were incubated at 30°C for at least a week. Positive strains were subcultured when clear positive reactions were visible.

Cultivations in liquid media

Fungal strains showing positive reactions in the plate-test screening were grown in different types of liquid media. At first, the positive fungi were cultivated in malt extract, potato dextrose and yeast mold (YM) broth (Difco) to test for the production of laccase. Subsequently, laccase-positive fungal strains were cultivated in two types of rich media to improve laccase production: soya meal medium (10 g l^{-1} soya meal, 20 g l^{-1} glucose and minerals; Kiiskinen *et al.* 2002) and yeast extract medium (27·5 g l^{-1} yeast extract, 25 g l^{-1} glucose and minerals). In order to optimize enzyme production, the ratio of carbon and nitrogen was varied in the latter medium. The effect of different inducers on laccase production was also studied in liquid cultures: 0·05% Tween 80 (Sigma), 0·2 mmol l^{-1} veratryl alcohol (Sigma), 0·05% Indulin AT (Sigma), 1 mmol l^{-1} guaiacol or 20 g l^{-1} spruce chips were added to malt extract, potato dextrose, YM, soya meal or yeast extract medium. The microbes were cultivated in 50 ml of medium in Erlenmeyer flasks at 30°C on a rotary shaker (100–150 rev min^{-1}). Extracellular laccase production was measured by monitoring the oxidation of 5 mmol l^{-1} ABTS in 25 mmol l^{-1} succinate buffer (pH 4·5) at 436 nm and using an extinction coefficient of 29 300 l mol^{-1} cm^{-1} (Niku-Paavola *et al.* 1988). The activities were expressed as katals (mol s^{-1}).

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Purification of laccases

Laccases from strains LLP13 and AH2 were partially purified by two chromatographic steps at room temperature. Clear culture supernatants were first applied to a DEAE Sepharose Fast Flow column (1·6 × 13 cm; Pharmacia, Uppsala, Sweden), which was equilibrated with 20 mmol l^{-1} Tris-HCl buffer (pH 7·1) for LLP13 laccase or with 20 mmol l^{-1} acetate buffer (pH 5·5) for AH2 laccase. Proteins were eluted with a linear increasing Na_2SO_4 gradient of 0–300 mM for LLP13 or 0–500 mM for AH2, and laccase-positive fractions were pooled. LLP13 laccase was further purified with a Phenyl Sepharose Fast Flow column (1·6 × 9 cm; Pharmacia) equilibrated with 20 mmol l^{-1} citrate buffer (pH 5·0), containing 700 mmol l^{-1} Na_2SO_4. Proteins were eluted with a linear decreasing 700–0 mmol l^{-1} Na_2SO_4 gradient. AH2 laccase was further purified with gel filtration on a Sephacryl S-100 HR column (1·6 × 90 cm; Pharmacia) equilibrated with 100 mmol l^{-1} sodium phosphate buffer (pH 7·0) containing 100 mmol l^{-1} NaCl. Laccase purification was monitored with SDS-PAGE (12% Tris-HCl Ready Gel; Bio-Rad; Hercules, CA, USA) (Laemmli 1970). Purification of the major laccase from the strain LLP12 is published elsewhere (Niku-Paavola *et al.* 2004).

Characterization of novel laccases

The isoelectric points of LLP13, LLP17 and AH2 laccases were determined with isoelectric focusing as previously described (Kiiskinen *et al.* 2002). The pH optima for laccases were determined in the universal McIlvaine buffer (Dawson *et al.* 1959) within a pH range of 2·2–8·0 using ABTS and guaiacol as substrates (Kiiskinen *et al.* 2002). The temperature stabilities were determined by incubating the enzyme solutions in 60 mmol l^{-1} citrate buffer (pH 6) at different temperatures and determining the residual enzyme activity at room temperature with ABTS as a substrate. The N-terminus of AH2 laccase as well as the internal peptides were sequenced according to Edman degradation chemistry using PE Biosystems Procise Sequencer (PE Biosystems, Foster City, CA, USA) (Kiiskinen *et al.* 2002).

RESULTS

Isolation of microbes

Altogether 26 fungal strains showing positive reactions on indicator plates were isolated from the collected samples. Positive strains were found from all the sources explored. The crude cork material from Portugal proved to be the best source, as altogether 10 positive strains were isolated from it. As for the test compost of municipal biowaste, three positive strains were isolated only at the late maturation phase. Of the 26 positive fungal strains, 16 were isolated from plates containing the polymeric indicators RBBR or Poly R-478, and eight strains from the guaiacol plates. Tannic acid gave relatively weak positive reactions with many samples. However, the ability of most positive microbes to form brown colour on tannic acid weakened during subculturing, whereas with other indicators the same effect was not observed. Only two fungal strains (LLP11 and LLP16) were so strongly positive on tannic acid plates that they were considered as laccase positives. Malt extract agar proved to be the best media for isolating laccase-producing fungi. The most promising positive fungal strains were identified by the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands; Table 1). Fungi from nine different genera were discovered from the environmental samples. Most of the isolated fungi were separate species, but *Mucor circinelloides* van Tieghem was found from three different environmental samples.

Specificity of the indicators

Reactions with the four different indicators, RBBR, Poly R-478, guaiacol and tannic acid, were tested with the fungal strains LLP1-LLP21 on malt extract agar plates. The positive reactions with RBBR and Poly R-478 correlated well with each other: only one strain, LLP20, of 20 tested showed a positive reaction only with Poly R-478 and not with RBBR (Table 2). The correlation between the polymeric dyes and guaiacol was also good, as only one strain, LLP9, was positive on guaiacol without being positive on polymeric dye indicators. Tannic acid seemed to be less specific, as it failed to give positive results with 10 strains that were positive on other indicators. In addition, there was one strain, LLP11, that was positive only on tannic acid.

Table 1 Identification of the ligninolytic fungal strains isolated in the screening

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

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Table 2 Comparison of reactions of the isolated fungal strains LLP1–LLP21 with different indicators on malt extract agar plates

Strain	RBBR	Poly R-478	Guaiacol	Tannic acid
LLP1	+	+	+	+
LLP2	+	+	+	−
LLP3	+	+	+	+
LLP4	+	+	+	+
LLP5	+	+	+	+
LLP6	+	+	+	−
LLP7	+	+	+	−
LLP8	+	+	+	−
LLP9	−	−	+	+
LLP11	−	−	−	+
LLP12	+	+	+	+
LLP13	+	+	+	+
LLP14	+	+	+	−
LLP15	+	+	+	−
LLP16	+	+	+	+
LLP17	+	+	+	+
LLP18	+	+	+	−
LLP19	+	+	+	−
LLP20	−	+	+	−
LLP21	+	+	+	−
Trametes hirsuta	+	+	+	+

The control strain *T. hirsuta* gave strong positive reactions with all the tested indicators.

Production of laccases in liquid media

Laccase production by the positive strains found in the screening was studied in liquid cultures. Detectable laccase production was observed with nine isolated fungal strains: LLP1–5, LLP12–13, LLP15, LLP17–18 and AH2 (Table 3). Considerable laccase activities were measured with strains LLP12, LLP13, LLP17 and AH2, whereas other positive strains produced very low activity levels (Table 3). Laccase production proved to be very much dependent on the medium, and the highest laccase activities were detected on soya meal medium. With the strains LLP13 and LLP17, laccase production was enhanced by the addition of wood chips, whereas the strain AH2 produced highest laccase activities when glucose concentration was decreased to 10 g l^{-1}, and the medium was supplemented with 5 g l^{-1} Bacto peptone.

Table 3 Laccase activity produced in liquid cultivations by the laccase-positive strains

Strain	Maximal laccase activity (nkat ml^{-1})
LLP1	0·3
LLP2	0·1
LLP3	0·1
LLP4	0·2
LLP5	0·4
LLP12	22
LLP13	40
LLP15	<0·1
LLP17	28
LLP18	0·5
AH2	35

Characterization of novel laccases

The highest laccase production was detected with the strains LLP12, LLP13, LLP17 and AH2, and the laccases from these strains were further analysed. All the strains showed multiple laccase isoforms in isoelectric focusing, and the major isoforms from LLP13 and AH2 were purified in this study. The pH optima were determined with ABTS or guaiacol as substrates. All novel laccases discovered in this screening had their pH optima at acidic pH values (Table 4). The thermal stabilities were relatively high for these laccases as the half-lives at 60°C ranged from 3 to 5 h (Table 4).

The AH2 laccase was considered the most interesting because of its high thermal stability. Therefore, it was further characterized. Altogether 15 amino acid residues from the N-terminus as well as two internal peptide sequences of 14 and 15 amino acids, respectively, were determined for the purified protein and compared with other known laccase sequences (Table 5). The level of amino acid identity at the N-terminus was 86% with *Trametes trogii* laccase (Colao *et al.* 2003), 86% with *Trametes villosa* Laccase 4 (Yaver and Golightly 1996), 80% with *Trametes versicolor* Laccase 4 (Jönsson *et al.* 1995), 73% with *Pycnoporus cinnabarinus* laccase (Eggert *et al.* 1998) and 73% with *Phlebia radiata* laccase (Saloheimo *et al.* 1991).

CONCLUSIONS

Screening for laccase-producing microbes on plates containing coloured indicators resulted in isolation of 26 fungal strains. Crude cork material and soiled pulp proved to be highly interesting sources for laccase producers. This is presumably because of the presence of relatively high concentration of laccase substrates in these environments. Besides the well known ligninolytic fungi, some positive strains isolated in this work were identified to belong to fungal species not previously reported to produce laccases. Two different isolates were identified as *Peniophora* species, one as *Rhizopus microsporus* var. *rhizopodiformis* and four as *Mucor* species. Fungi from the genera *Peniophora*, *Rhizopus* or *Mucor* have not thus far been reported to produce ligninolytic enzymes. An interesting finding was also that three clearly positive isolates were identified as *Trichoderma*

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Table 4 Biochemical characteristics of the novel laccases

Fungal strain	Mw (kDa)	pI	pH optimum (substrate)	*t*½ 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	This article
LLP17*	70	3·5–4·0	<2 (ABTS); 3·0 (guaiacol)	n.d.	This article
AH2	60	**4·1**; 5·1	3 (ABTS); 4·0 (guaiacol)	6 h	This article
Melanocarpus albomyces	80	**4·0**	3·5 (ABTS); 5·0–7·5 (guaiacol)	5 h	Kiiskinen *et al.* (2002)
Mauginiella sp.	63	4·8; 5·0; 5·2; 5·6; 6·0; 6·4	<2 (ABTS); 4·0 (guaiacol)	40 min	Palonen *et al.* (2003)

The pI of the main isoform is shown in boldface type.
*LLP17 laccase characterized from the culture supernatant.
Molecular weights as determined by SDS-PAGE. Previously determined properties of *Melanocarpus albomyces* and *Mauginiella* sp. laccases are included for comparison.

Table 5 Comparison of the N-terminal amino acid sequence and two internal peptide sequences from purified AH2 laccase (*Pholiota* sp.) with *Trametes trogii* laccase (Colao *et al.* 2003), *Trametes villosa* Laccase 4 (Yaver and Golightly 1996), *Trametes versicolor* Laccase 4 (Jönsson *et al.* 1995), *Pycnoporus cinnabarinus* laccase (Eggert *et al.* 1998) and *Phlebia radiata* laccase (Saloheimo *et al.* 1991) sequences

N-terminus																
AH2	**A**	**I**	**G**	**P**	**V**	**T**	**D**	**L**	**T**	**I**	**V**	**N**	**G**	**A**	**V**	
T. trogii	**A**	**I**	**G**	**P**	**V**	A	**D**	**L**	**T**	**I**	S	**N**	**G**	**A**	**V**	
T. villosa Lac4	**A**	**I**	**G**	**P**	**V**	**T**	**D**	**L**	**T**	**I**	S	**N**	**G**	D	**V**	
T. versicolor Lac4	**A**	**I**	**G**	**P**	**V**	**T**	**D**	**L**	**T**	**I**	S	**N**	A	D	**V**	
Pc. cinnabarinus	**A**	**I**	**G**	**P**	**V**	A	**D**	**L**	**T**	L	T	**N**	A	**A**	**V**	
Ph. radiata	S	**I**	**G**	**P**	**V**	**T**	**D**	F	H	**I**	**V**	**N**	A	**A**	**V**	
First internal peptide																
AH2	**Y**	**V**	**G**	**A**	**E**	**D**	**V**	**E**	**P**	**T**	**T**	**T**	**A**	**N**		
T. trogii	**Y**	D	**G**	**A**	A	P	**V**	**E**	**P**	**T**	**T**	S	Q	T		
T. villosa Lac4	**Y**	S	**G**	**A**	S	E	**V**	D	**P**	**T**	**T**	**T**	E	T		
T. versicolor Lac4	**Y**	S	**G**	**A**	S	E	**V**	D	**P**	**T**	**T**	**T**	E	T		
Pc. cinnabarinus	**Y**	D	**G**	**A**	P	E	**V**	**E**	**P**	**T**	**T**	**T**	Q	T		
Ph. radiata	**Y**	D	**G**	**A**	D	V	**V**	**E**	**P**	**T**	**T**	**T**	Q	A		
Second internal peptide																
AH2	**L**	**G**	**P**	**A**	**G**	**G**	**T**	**D**	**S**	–	**E**	**L**	**A**	**V**	**I**	**T**
T. trogii	**L**	**G**	R	S	I	D	**T**	L	N	A	D	**L**	**A**	**V**	**I**	**T**
T. villosa Lac4	G	R	F	**A**	**G**	**G**	–	**D**	**S**	T	D	**L**	**A**	**V**	**I**	**T**
T. versicolor Lac4	G	R	F	**A**	**G**	**G**	–	**D**	**S**	T	D	**L**	**A**	**V**	**I**	**T**
Pc. cinnabarinus	**L**	**G**	R	S	P	**G**	**T**	T	T	A	D	**L**	**A**	**V**	**I**	K
Ph. radiata	C	**G**	E	**A**	**G**	C	P	V	**S**	–	D	**L**	**A**	**V**	**I**	S

atroviride or *Trichoderma harzianum*. *Trichoderma* strains have been reported to produce polyphenoloxidases (Assavanig *et al.* 1992; Score *et al.* 1997), and recently the presence of cell-wall associated laccase in *T. atroviride* and *T. harzianum* was demonstrated by Hölker *et al.* (2002). The rest of the fungal strains isolated in this study belonged to genera that are known to produce ligninolytic enzyme activities: *Sporotrichum* (Rohrmann and Molitoris 1992; Luterek *et al.* 1997), *Bjerkandera* (Rohrmann and Molitoris 1992; Kaal *et al.* 1993), *Chrysosporium* (Buckley and Dobson 1998), *Pholiota* (Bollag and Leonowicz 1984) and *Phlebia* (Niku-Paavola *et al.* 1990).

Comparison of the reactions with different indicators showed that the polymeric dyes RBBR and Poly R-478 gave very similar results, thus either one can be chosen for an indicator in future screening procedures. Reactions with guaiacol also correlated well with reactions on the polymeric dyes. However, tannic acid deviated substantially from the polymeric dyes and guaiacol. Tannic acid is one of the traditional screening reagents for laccases (Harkin and Obst 1973). Our results suggest, however, that colour reactions with synthetic dyes and guaiacol are more easily detectable, detect more laccase-positives, and these compounds can thus reliably be used for laccase activity screening.

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Laccase production by the fungal strains that were positive in the plate-test screening was further studied in liquid cultures. Using various growth media, nine strains were shown to produce detectable amounts of laccase in culture supernatant as detected by activity on ABTS. Some of the strains that showed positive reactions on indicator plates but did not produce laccase in liquid cultures may have produced other ligninolytic enzymes, such as manganese-dependent peroxidases or lignin peroxidases, because these enzymes are also capable of decolourizing the polymeric dyes (De Jong *et al.* 1992; Rodríguez Couto *et al.* 2000; Ryu *et al.* 2003). Differentiation among these activities cannot thus be made on plate-tests. The production level of laccases in liquid cultures were quite low (below 1 nkat ml^{-1}) in most of the media tested. However, four fungal strains, LLP12, LLP13, LLP17 and AH2, produced significant amounts of laccase (22–40 nkat ml^{-1}) in very rich media containing soya meal. This is in agreement with other results showing that laccase production is highly dependent on the medium and cultivation conditions (Niku-Paavola *et al.* 1990; Rogalski *et al.* 1991; Schlosser *et al.* 1997). The strain LLP12 was identified as *Peniophora* sp., LLP17 as *Peniophora* or *Lopharia* sp., AH2 as *Pholiota* sp., whereas LLP13 was an unidentifiable haploid Basidiomycete.

Preliminary characterization of four laccases from LLP13, LLP17, AH2 and LLP12 showed that these laccases were typical basidiomycete laccases with molecular weights around 60–70 kDa, isoelectric points around 4 and pH optima at an acidic pH range. In addition, the N-terminal amino acid sequence and two internal peptide sequences of AH2 (*Pholiota* sp.) showed high homology to other basidiomycete laccases. Interestingly, the thermal stabilities of these laccases were substantially high: the half-lives at 60°C ranged from 3 to 5 h. Typically, half-lives at this temperature for fungal laccases have been reported to be *ca* 0·5–1 h (Heinzkill *et al.* 1998; Tagger *et al.* 1998; Robles *et al.* 2000; Palonen *et al.* 2003). Good thermostability is an important factor in industrial applications. This study showed that interesting novel laccase producers can be discovered from environmental samples by very simple plate-test screening methods.

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L.-L. Kiiskinen · L. Viikari · K. Kruus

Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*

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Abstract A novel laccase from the ascomycete *Melanocarpus albomyces* was purified and characterised. The enzyme was purified using anion exchange chromatography, hydrophobic interaction chromatography and gel filtration, and the purified laccase was biochemically characterised. It had activity towards typical substrates of laccases including 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate), dimethoxyphenol, guaiacol, and syringaldazine. The laccase showed good thermostability and it had a pH optimum at neutral pH, both unusual properties for most known fungal laccases. The activity of the laccase from *M. albomyces* was highest at 60–70°C. With guaiacol and syringaldazine the pH optima were rather broad: 5–7.5 and 6–7, respectively. It retained 50% of its activity after 5 h incubation at 60°C. The molecular weight of the laccase was about 80 kDa and the isoelectric point 4.0. The ultraviolet-visible absorption and electron paramagnetic resonance spectra of the purified laccase indicated that the typical three types of copper were present.

Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. They catalyse oxidation of a surprisingly wide variety of organic and inorganic compounds, including diphenols, polyphenols, diamines, substituted phenols, and aromatic amines by a one-electron transfer mechanism. Molecular oxygen is used as the electron acceptor. The substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerisation (Thurston 1994).

Laccase or laccase-like activity has been demonstrated in higher plants, some insects and a few bacteria (Gianfreda et al. 1999). However, most known laccases are of fungal origin, especially from the class of white-rot fungi. Laccases are involved in several physiological functions, such as lignin biosynthesis, plant pathogenesis, insect sclerotisation, and degradation of lignocellulosic materials. It is well recognised that laccases are involved in both polymerisation and depolymerisation processes of lignin (Thurston 1994). Laccases are seen as industrially interesting enzymes because they have shown potential in various applications including pulp bleaching, textile dye bleaching, detergents, and enzymatic conversion of chemical intermediates (Xu 1999).

For many applications it is important to find an enzyme that is thermostable and works at relatively high pH-values. Thermophilic fungi could comprise a potential source of thermostable enzymes. A thermophilic ascomycete *Melanocarpus albomyces*, formerly known as *Myriococcum albomyces*, has previously been reported to produce xylanases and cellulases with pronounced thermal stability and activity at alkaline pH range (Jain et al. 1998; Prabhu and Maheshwari 1999). We report here that *M. albomyces* also produces a laccase with very interesting pH and temperature characteristics.

Materials and methods

Fungal strain

Melanocarpus albomyces (VTT D-96490, originally isolated from soil in Saudi Arabia), was maintained on oatmeal agar (Difco, Detroit, Mich.).

Media and culture conditions

M. albomyces was cultivated on liquid medium containing (per litre) 25 g glucose, 27.5 g yeast extract, 0.5 mg Indulin AT (Sigma, St. Louis, Mo.) and 0.04 l mineral stock solution containing (per litre) 1.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 1.0 g Na_2EDTA. Glucose was autoclaved separately. First, 100 ml of medium was inoculat-

L.-L. Kiiskinen · L. Viikari · K. Kruus (✉)
VTT Biotechnology, P.O. Box 1500, 02044 VTT, Finland
e-mail: Kristiina.Kruus@vtt.fi
Tel.: +358-9-4565143, Fax: +358-9-4552103

ed with agar pieces cut from well-grown mycelium on oatmeal agar. After 2 days cultivation at 37°C (160 rpm), the culture was homogenized and used to inoculate 1 l of culture medium in a 3 l Erlenmeyer flask. The flasks were incubated at 37°C on a rotary shaker (160 rpm). Laccase activity in the culture filtrate was measured daily and the enzyme was collected when laccase activity reached its maximum. The mycelium was removed by filtration through Whatman no. 1 filter paper.

Protein and enzyme activity determinations

Laccase activity was measured according to Niku-Paavola et al. (1988) using ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)], (Boehringer Mannheim; Mannheim, Germany) as a substrate. The laccase activity was also measured with syringaldazine (Sigma) according to Leonowicz and Grzywnowicz (1981), guaiacol (Sigma) according to Paszczynski et al. (1985), and 2,6-dimethoxyphenol (Sigma) according to Wariishi et al. (1992). All these activity assays were carried out in 25 mM succinate buffer (pH 4.5) at 25°C using a two-beam spectrophotometer (Lambda 20, Perkin-Elmer, Überlingen, Germany). The activity towards tyrosine was measured at a tyrosine concentration of 0.2 mM in 50 mM sodium phosphate buffer (pH 7) at 25°C and at 475 nm by monitoring dopachrome formation and using the molar extinction coefficient ε 3,600 M^{-1} cm^{-1} (Lerch and Ettlinger 1972). Activities were expressed as nanokatals. The protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as a standard.

Protein purification

The clear culture supernatant was concentrated and the buffer changed to 10 mM acetate buffer, pH 5, with an Amicon 8400 ultrafiltration unit using a PM30 membrane (Millipore, Bedford, Mass.). The subsequent purification steps were carried out at room temperature. The sample was applied to a DEAE Sepharose Fast Flow column (1.6×13 cm; Pharmacia, Uppsala, Sweden), which was pre-equilibrated with 10 mM acetate buffer, pH 5. Proteins were eluted with a linear 0–200 mM Na_2SO_4 gradient (90 ml) in acetate buffer. Laccase-positive fractions were pooled. Using Na_2SO_4, the pH and ionic strength of the pooled sample were adjusted to correspond to those of 400 mM Na_2SO_4 in 20 mM citrate buffer, pH 5. The sample was applied to a Phenyl Sepharose Fast Flow column (1.6×9 cm; Pharmacia) pre-equilibrated with 20 mM citrate buffer, pH 5, containing 400 mM Na_2SO_4. Proteins were eluted with a linear 400–0 mM Na_2SO_4 gradient (90 ml) in citrate buffer, thereafter with 2 mM citrate buffer (pH 5), and finally with distilled water. Laccase-positive fractions were pooled, concentrated, and applied to a Sephacryl S-100 HR column (1.6×90 cm; Pharmacia) equilibrated with 100 mM sodium phosphate buffer, pH 7. Active fractions were pooled and concentrated on an Amicon PM10 membrane (Millipore).

SDS-PAGE (12% Tris-HCl Ready Gel, Bio-Rad) was performed according to Laemmli (1970). Protein bands were visualised by staining with Coomassie Brilliant Blue (R350; Pharmacia) and compared with molecular weight markers (Prestained Protein Marker Broad Range Cat. no. 7708S; New England Biolabs, Beverly, Mass.).

Determination of isoelectric point

The isoelectric point of *M. albomyces* laccase was determined by isoelectric focusing within the pH range of 2.5–5.0 (Pharmalyte 2.5–5.0 for IEF, Pharmacia) on an LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia, Bromma, Sweden) according to the manufacturer's instructions. Bands containing laccase activity were visualised by staining the gel with 2 mM ABTS in 25 mM succinate buffer (pH 4.5) and proteins by Coomassie Blue staining.

Enzyme activity and stability with respect to pH and temperature

The pH optimum for purified *M. albomyces* laccase was determined in McIlvaine (Dawson et al. 1959) buffer within a pH range of 2.2–8.0 using ABTS, guaiacol and syringaldazine as substrates. The stability of the enzyme at different pH-values was determined in McIlvaine buffer by incubating the purified enzyme solution (20 nkat/ml) at different pH-values at room temperature. The residual laccase activity was determined by measuring the activity of the enzyme solutions with ABTS as described above. The effect of temperature on enzyme activity was determined by measuring the enzyme activity with ABTS and guaiacol within a temperature range of 25–80°C. With ABTS the measurements were performed in 25 mM succinate buffer at pH 4.5, and with guaiacol in 50 mM citrate buffer at pH 6. The temperature stability was determined by incubating the enzyme solution (200 nkat/ml) in 60 mM citrate buffer (pH 6) in different temperatures and determining the residual enzyme activity with guaiacol.

Spectra

The optical absorption spectrum of purified *M. albomyces* laccase was measured with a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker ESP 300 X-band spectrometer (Bruker, Karlsruhe, Germany) at 9.44 GHz and 12 K (cryostat from Oxford Instruments, Oxford, UK). Modulation frequency was 100 kHz, modulation amplitude 0.99 mT, sweep time/scan 168 s, sweep width 0.10 T, microwave power 1.00 mW, and protein concentration 50 μM.

Inhibition of laccase activity

The effect of various inhibitors on laccase activity was determined by measuring the oxygen consumption during the enzyme reaction with ABTS in sealed and fully filled Erlenmeyer flasks with an Orion Research 081010 oxygen electrode (software: SensorLink PCM800; Orion, Espoo, Finland). The oxygen consumption rates (nmol l^{-1} s^{-1}) were measured from solutions containing 15 μg of purified laccase (corresponding to an activity of about 15 nkat), 2 mM ABTS, and 0–5 mM of various inhibitors (added simultaneously with ABTS) in 50 mM citrate buffer (pH 5) in a 30 ml reaction volume. All measurements were taken in triplicate.

N-Terminal amino acid sequencing

The N-terminus of the protein and internal peptides were sequenced according to Edman degradation chemistry using PE Biosystems Procise Sequencer (PE Biosystems, Foster City, Calif.). For peptide preparation, the lyophilised protein was reduced with dithiothreitol, carboxymethylated with iodoacetamide and cleaved with sequencing grade trypsin (Promega, Madison, Wisc.) at an enzyme/substrate mass ratio of 1:100 for 12 h at 37°C in 0.1 M ammonium bicarbonate, pH 8.3 (Stone et al. 1988). The peptides generated were separated by reversed-phase high performance liquid chromatography (HP 1050, HP GmbH, Waldbronn, Germany; Vydac C-18 column, Grace Vydac, Hesperia, Calif.) with a linear acetonitrile gradient (0–60% acetonitrile in 0.1% trifluoroacetic acid). The amino acid sequence comparison of *M. albomyces* laccase with other laccase sequences was carried out with SIM-Alignment tool for protein sequences (Huang and Miller 1991).

Results

Enzyme purification

Laccase-positive fractions eluted as a single peak at about 100 mM salt concentration in anion exchange

Table 1 Purification of extracellular laccase from *Melanocarpus albomyces*

Purification step	Activity (nkat/ml)	Protein (mg/ml)	Volume (ml)	Specific activity (nkat/mg)	Activity yield (%)	Purification factor
Culture filtrate	23	5.9	250	3.9	100	1
Concentrated ultrafiltrate	35	3.4	170	10.2	103	2.6
Anion exchange chromatography	166	1.3	29	128	84	33
Hydrophobic interaction column	1,600	1.8	1.2	889	33	228
Gel filtration	1,250	1.1	0.8	1,136	17	292

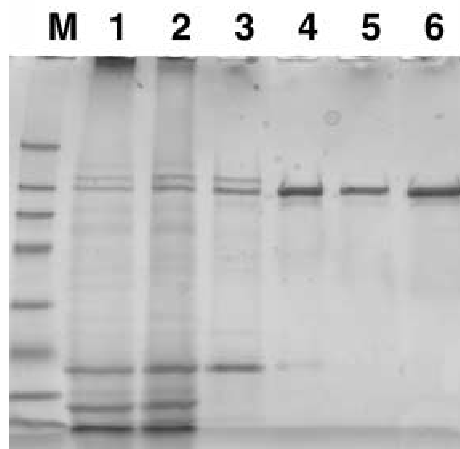

Fig. 1 SDS-PAGE of *Melanocarpus albomyces* laccase after different purification steps. Lanes: *M* Molecular weight marker (175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa), *1* culture supernatant (118 µg protein), *2* concentrated ultrafiltrate (68 µg), *3* pooled laccase fractions after DEAE Sepharose (9 µg), *4* pooled laccase fractions from phenyl Sepharose (4 µg), *5* purified *M. albomyces* laccase (2 µg), *6* purified *M. albomyces* laccase (4 µg)

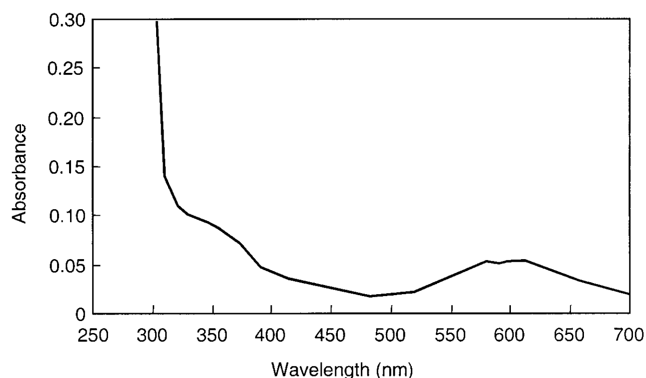

Fig. 2 The ultraviolet-visible absorption spectrum of *M. albomyces* laccase

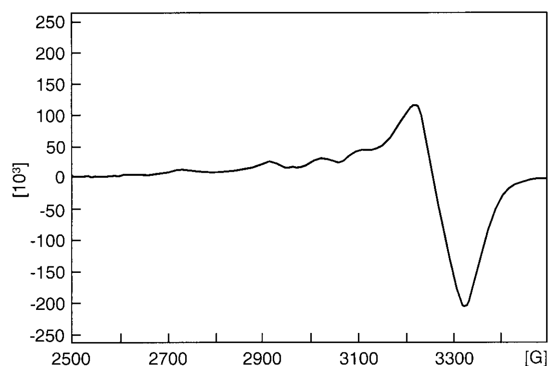

Fig. 3 The electron paramagnetic resonance (EPR) spectrum of *M. albomyces* laccase

chromatography. Laccase remained in the hydrophobic interaction column during decreasing salt gradient and eluted as a single peak with 2 mM citrate buffer. An overall 292-fold purification and activity recovery of 17% was achieved. Table 1 shows a summary of the purification. The purified laccase showed apparent homogeneity on SDS-PAGE (Fig. 1.). The molecular mass of the laccase as determined by SDS-PAGE was 80 kDa. Under nondenaturing conditions in isoelectric focusing both the culture filtrate and the purified laccase showed only one major band at pH 4.0 when stained with ABTS (results not shown).

Spectra

The purified laccase had a blue colour typical of copper-containing proteins. The ultraviolet-visible absorption spectrum of the laccase showed two peaks at 280 and 600 nm and a shoulder at 330 nm as shown in Fig. 2. The peak at 600 nm is typical for the type I Cu(II), and the shoulder at 330 nm suggests the presence of the type III binuclear Cu(II) pair (Eggert et al. 1996). Figure 3 presents the EPR absorption spectrum of purified *M. albomyces* laccase. The spectrum is typical of fungal laccases and it reveals the presence of type II (hyperfine line above 2,700 G) and type I (region 2,900–3,200 G) Cu(II) ions (Karhunen et al. 1990). The estimated parameters (g_{II} and A_{II}) are 2.18 and 0.010 cm^{-1} for the narrowly spaced signal and 2.25 and ≥0.019 cm^{-1} for the widely spaced signal.

Laccase activity with respect to pH and temperature

The pH optima for the purified laccase were determined with ABTS, guaiacol, and syringaldazine as substrates. The results are shown in Fig. 4. The lowest pH optimum, 3.5, was determined in oxidizing ABTS. Oxidation of guaiacol showed a very broad pH optimum from 5.0 to 7.5. The pH optimum for syringaldazine was within the

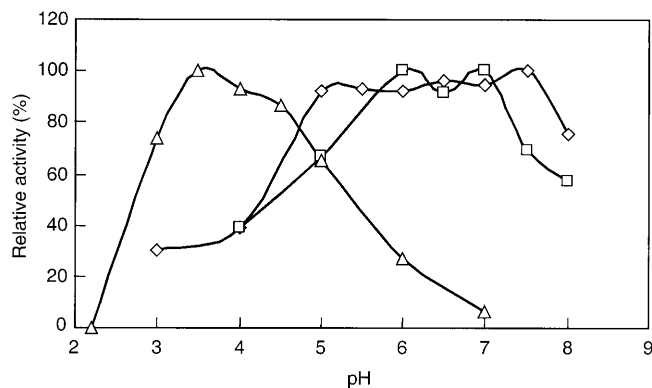

Fig. 4 pH activity profiles of purified *M. albomyces* laccase with 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (*triangles*), guaiacol (*diamonds*) and syringaldazine (*squares*) as substrates

N-terminus:

MaL	E P T C N T P S N R A C W S D G - F D I N T D Y E V S T P
NcL 55	G G G C N S P T N R Q C W S P G - F N I N T D Y E L G T P
PaL 46	Q S S C H T A A N R A C W A P G - F D I N T D Y E V S T P
CpL 33	Q P N C N T A S N R A C W I S G S Y D I T T D Y E V K T P

1st internal peptide:

MaL	D I D L G V F P I T D Y Y Y R
NcL 215	D V D L G P F P L T D Y Y Y D
PaL 209	D I D L G V F P I T D Y Y H K
CpL 197	D I D L G P L V L S D Y Y Y K

2nd internal peptide:

MaL	F V F D P A V D L A R
NcL 503	Y V F D P A V D M A R
PaL 503	Y R F N P A T D M A L
CpL no significant similarity	

Fig. 5 Comparison of the N-terminal amino acid sequence and two internal peptide sequences from *M. albomyces* laccase (*MaL*) with *Neurospora crassa* (*NcL*) (Germann et al. 1988), *Podospora anserina* (*PaL*) (Fernández-Larrea and Stahl 1996), and *Cryphonectria parasitica* (*CpL*) (Choi et al. 1992) laccase sequences

Table 2 Specific activity of purified *M. albomyces* laccase towards different substrates. *ABTS* [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)], *2,6-DMP* 2,6-dimethoxyphenol

Substrate	Concentration (mM)	Specific activity (nkat/mg)
ABTS	5	836
Guaiacol	1.5	90
2,6-DMP	0.8	290
Syringaldazine	0.06	382
Tyrosine	0.2	0

Table 3 Effect of various inhibitors on oxidation of ABTS by purified *M. albomyces* laccase

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	1	1
	5	5
KCN	0.1	97
	0.5	99
	1	100
Kojic acid	1	0
L-Cysteine	1	0
NaCl	1	0
NaF	0.5	38
	1	48
NaN_3	0.5	97
	1	99
p-Coumaric acid	0.5	0
	1	0
SDS	1	0

range of 6–7. Interestingly, the laccase still showed relatively high activity at pH 8, both with syringaldazine and guaiacol (60 and 75%, respectively). The purified laccase remained quite stable within the pH range of 4–8 after 22 h incubation. However, at pH-values lower than 4, the enzyme lost its activity totally.

The activity of purified *M. albomyces* laccase was determined at various temperatures (25–90°C) using ABTS and guaiacol as substrates. The enzyme showed highest activity at 60–70°C. Beyond 70°C the activity dropped sharply. The activity remained unaltered after prolonged incubation (over 100 h) at 40°C, whereas it showed a half-life of about 50 h, 5 h, and 12 min at 50, 60, and 70°C, respectively.

Substrates and inhibitors

Table 2 presents the specific activities of purified *M. albomyces* laccase with various substrates. The specific activity was highest with ABTS. The other typical substrates for laccases, 2,6-dimethoxyphenol, syringaldazine, and guaiacol, were also oxidized by this enzyme. However, tyrosine was clearly not a substrate, thus this laccase does not belong to the group of tyrosinase-type polyphenol oxidases (EC 1.10.3.1; EC 1.14.18.1). The inhibition of laccase activity with different inhibitors is shown in Table 3. Sodium azide and potassium cyanide were very effective inhibitors of *M. albomyces* laccase. At a concentration of 1 mM, potassium cyanide inhibited totally and sodium azide inhibited 99% of the laccase activity. Sodium fluoride and EDTA caused some inactivation, but sodium chloride, *p*-coumaric acid and SDS did not have any inhibition effect on this laccase under the test conditions.

N-Terminal amino acid sequence

The N-terminal and two internal amino acid sequences of *M. albomyces* laccase were determined. When compared to the amino acid sequences of known laccases, the highest identity was found with ascomycete laccases (Fig. 5), namely *Neurospora crassa* (Germann et al. 1988), *Podospora anserina* (Fernández-Larrea and Stahl 1996), and *Cryphonectria parasitica* (Choi et al. 1992). The overall identity based on these sequences was highest with the laccases of *N. crassa* and *P. anserina* (70%). With basidiomycete laccases, e.g. *Trametes versicolor*

(Ong et al. 1997), *Phlebia radiata* (Saloheimo et al. 1991), *Pleurotus ostreatus* (Giardina et al. 1995), and *Rhizoctonia solani* (Wahleithner et al. 1996), the identity was found to be low (below 30%). The amino acid identity between *M. albomyces* laccase and the laccase from the ascomycete *Aspergillus nidulans* (Aramayo and Timberlake 1990) was also remarkably low (below 20%).

Discussion

M. albomyces has previously been reported to produce several thermostable and alkaline xylanases, as well as cellulases (Vehmaanperä et al. 1997; Jain et al. 1998; Prabhu and Maheshwari 1999). According to Jain et al. (1998), *M. albomyces* xylanase has its maximum activity at 70°C and is stable at that temperature for more than 2 h. In addition, the xylanase retains more than 50% of its activity at pH 10. The results reported here are the first to indicate that this thermophilic fungus also produces a thermostable laccase.

The enzyme purified from *M. albomyces* was able to oxidize typical substrates for laccases: a variety of phenolic compounds and non-phenolic ABTS. As previously reported, many fungal laccases show highest activity towards ABTS. The relative activities towards ABTS, guaiacol, and 2,6-dimethoxyphenol for *Pycnoporus cinnabarinus* laccase were 100, 31, and 22%, respectively, for *Coriolus hirsutus* laccase, 100, 40, and 39% respectively (Eggert et al. 1996) and for *Trichophyton rubrum* laccase 100, 33, and 36%, respectively (Jung et al. 2002). The *M. albomyces* laccase also had highest activity towards ABTS followed by syringaldazine, 2,6-dimethoxyphenol, and guaiacol. Similar to other laccases the enzyme did not show tyrosinase activity (Thurston 1994). The UV-visible absorption spectrum of the purified enzyme from *M. albomyces* showed a shoulder at 330 nm, which corresponds to a type-3 binuclear copper and a peak at 600 nm corresponding to a type-1 or the blue copper atom. These characteristics, as well as the EPR absorption spectrum, indicate that all three types of copper atoms are present (Eggert et al. 1996; Xu et al. 1996). Based on the substrate specificity and spectral data we conclude that the enzyme purified from *M. albomyces* is a true laccase.

Many sulfhydryl-containing compounds, e.g. L-cysteine, are often referred to as laccase inhibitors. However, Johannes and Majcherczyk (2000) recently showed that the observed inhibitory effect is actually caused by the reduction of the oxidized substrate by the sulfhydryl compounds and not by true inhibition of the enzyme. That is why we tested the inhibitors by monitoring oxygen consumption in the reaction and not spectrophotometrically. From our results it is obvious that L-cysteine, often considered a substrate for laccases, did not inhibit the oxygen consumption by laccase. *M. albomyces* laccase was strongly inhibited by the typical laccase inhibitors potassium cyanide, sodium azide and sodium fluoride, but it was not sensitive to EDTA, SDS, coumaric acid or kojic acid.

The isoelectric focusing PAGE indicated the presence of only one isoform of laccase in the culture supernatant, whereas many laccases have been reported to be produced as multiple isoforms, e.g. *Trametes villosa* produces at least three laccase isoforms (Bourbonnais et al. 1995) and *Pleurotus ostreatus* four isoforms (Palmieri et al. 2000). The molecular weight of the purified laccase (ca. 80,000 Da) is consistent with the molecular weights of most other fungal laccases, which have been reported to be between 60,000 and 80,000 Da (Thurston 1994).

The pH optimum of the *M. albomyces* laccase depended very much on the substrate. The laccase exhibited a rather low pH optimum (3.5) with ABTS as a substrate, like many other fungal laccases (Xu 1997; Robles et al. 2000). With guaiacol and syringaldazine, the pH optima were very broad: 5–7.5 and 6–7, respectively. The difference in pH optima for ABTS and phenolic substrates is typical for laccases and it reflects the difference in oxidation mechanism with different substrates. Since the oxidation of ABTS does not involve protons, the only effect of elevated pH on oxidation rate is the increasing inhibition of laccase by OH^- ions (Xu 1997). The instability of the oxidation product of syringaldazine at pH-values above 7 may also affect the activity results obtained at alkaline pH-values. Compared to many other fungal laccases, e.g. those of *Trametes versicolor* (Schlosser et al. 1997), *Pycnoporus cinnabarinus* (Eggert et al. 1996), *Trametes villosa* (Xu 1997), and *Thermoascus aurantiacus* (Machuca et al. 1998), the noteworthy feature of the *M. albomyces* laccase is its activity at alkaline pH: the enzyme still showed 75% of maximum activity at pH 8 with guaiacol. In addition, the enzyme showed good stability at alkaline pH-values, over 90% of the activity remained after 22 h incubation at pH 8.

Besides high stability at elevated pH-values, the *M. albomyces* laccase showed good thermostability. Its activity remained virtually unaltered at 60°C for up to 2 h. This kind of combination of thermal- and pH-stability is very rare among fungal laccases. Many other laccases that have a pH optimum around 6–7 are not as thermostable as *M. albomyces* laccase. For example, laccases from *Coprinus friesii*, *Panaeolus papilionaceus* and *Panaeolus sphinctrinus* have unusually high pH optima (7–8) with dimethoxyphenol, but do not remain active at 60°C (Heinzkill et al. 1998). Similarly, the neutral laccases from *Myceliophthora thermophila* (Berka et al. 1997), *Chaetomium thermophilum* (Chefetz et al. 1998), and *Coprinus cinereus* (Schneider et al. 1999) retain less than 75% of maximum activity at 60°C after 1 h. In addition, thermal activation in which incubation at higher temperatures results in higher activity was clearly observed with *M. albomyces* laccase, as previously reported for laccases from *M. thermophila* and *S. thermophilum* (Xu et al. 1996).

The comparison of the N-terminal amino acid sequence and two internal peptide sequences of *M. al-*

bomyces laccase with other fungal laccases showed that the *M. albomyces* laccase clearly resembles other ascomycete laccases. In comparison, the identity with basidiomycete laccases was low. These results support the theory that the genes of ascomycete and basidiomycete laccases have evolved significantly after the phylogenetic divergence of the two classes of fungi (Fernández-Larrea and Stahl 1996; Berka et al. 1997). Currently, we are isolating the gene encoding the *M. albomyces* laccase in order to express the protein heterologously to achieve higher production levels as well as to further analyse the enzyme properties.

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Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site

Nina Hakulinen[1], Laura-Leena Kiiskinen[2], Kristiina Kruus[2], Markku Saloheimo[2], Arja Paananen[2], Anu Koivula[2] and Juha Rouvinen[1]

[1]*Department of Chemistry, University of Joensuu, PO BOX 111, FIN-80101, Joensuu, Finland.* [2]*VTT Biotechnology, PO BOX 1500, FIN-02044 VTT, Espoo, Finland.*

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We have crystallized the ascomycete laccase from *Melanocarpus albomyces* with all four coppers present and determined the crystal structure at 2.4 Å resolution. The enzyme is heavily glycosylated and consists of three cupredoxin-like domains, similar to those found in the Cu-depleted basidiomycete laccase from *Coprinus cinereus*. However, there are significant differences in the loops forming the substrate-binding pocket. In addition, the crystal structure of the *M. albomyces* laccase revealed elongated electron density between all three coppers in the trinuclear copper site, suggesting that an oxygen molecule binds with a novel geometry. This oxygen, required in the reaction, may enter the trinuclear site through the tunnel, which is open in the structure of the *C. cinereus* laccase. In contrast, the C-terminus on the *M. albomyces* laccase forms a plug that blocks this access.

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are widely distributed in Nature. Laccase activity has been demonstrated in higher plants, some insects and a few bacteria. However, most known laccases are of fungal origin. In plants, they participate in wound response and lignin synthesis[1]. The fungal laccases are involved in lignin degradation, as well as in several other functions including pigmentation, sporulation, pathogenicity and detoxification[2]. Laccases have been tested in many industrial applications, such as pulp bleaching, textile dye decolorization, detergents, bio-glueing and detoxification[3].

Laccases catalyze the oxidation of a wide variety of substrates, including various phenolic compounds and anilines[4,5]. The one-electron oxidation of the reducing substrate occurs concomitantly with the four-electron reduction of molecular oxygen to water. Laccases belong to the blue multi-copper oxidase family (reviewed in refs 6,7), which includes ascorbate oxidase and mammalian plasma ceruloplasmin. These proteins have a minimum of one mononuclear copper site containing one type-1 Cu (blue Cu) and a trinuclear copper site containing one type-2 Cu (normal Cu)[7] and two type-3 Cu (coupled binuclear Cu). Substrates are oxidized near the mononuclear site, and the electrons are transferred to the trinuclear site, where the molecular oxygen is reduced. Neither the electron transfer mechanism nor the oxygen reduction to water is fully understood.

Although laccases have been extensively studied, only one crystal structure is available so far: the type-2 Cu depleted laccase from *Coprinus cinereus*[8,9]. In addition, the crystallization of laccases from *Trametes versicolor* and *Pycnoporus cinnabarinus* have been reported[10,11]. Here we present the three-dimensional structure of the fully active laccase from *Melanocarpus albomyces* that contains all four copper ions. This ascomycete laccase shares only ~20–30% sequence identity with basidiomycete laccases,

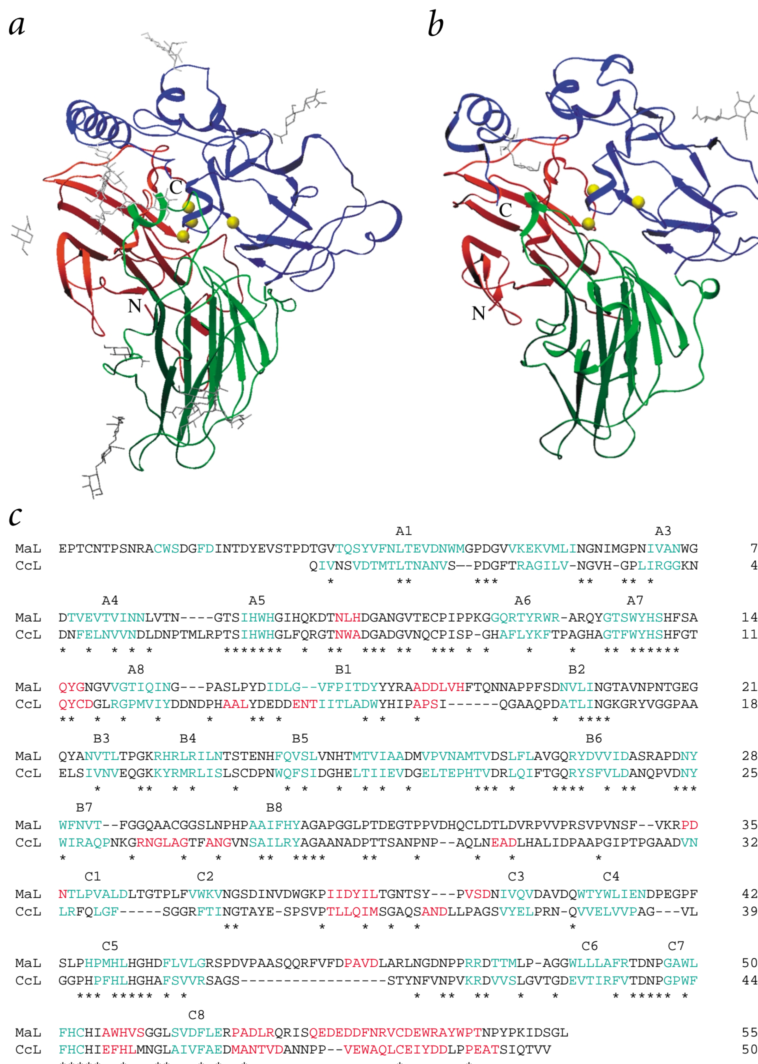

Fig. 1 Three-dimensional structures of ***a***, MaL and ***b***, CcL. Carbohydrates are shown as gray sticks. Domain A (red) includes residues that participate in the binding of coppers at the trinuclear site. Domain B (green) contains residues that take part in the substrate binding. Domain C (blue) contains residues that participate in the binding of coppers at the mononuclear and the trinuclear site, as well as in substrate binding. The mononuclear site is located entirely in domain C, and the trinuclear site is located at the interface between domains A and C. The diphenolic substrate-binding site is located in the cleft between domains B and C. ***c***, Structure-based sequence alignment. The amino acid sequence has been deduced from a laccase gene isolated from *M. albomyces* (L.-L.K. and M.S., unpub. results). Conserved amino acids are marked with asterisks. Secondary structure units are showed in colors: helices are in red and β-strands in green. The numbering of β-strands is based on the comparison of cupredoxin domains[34].

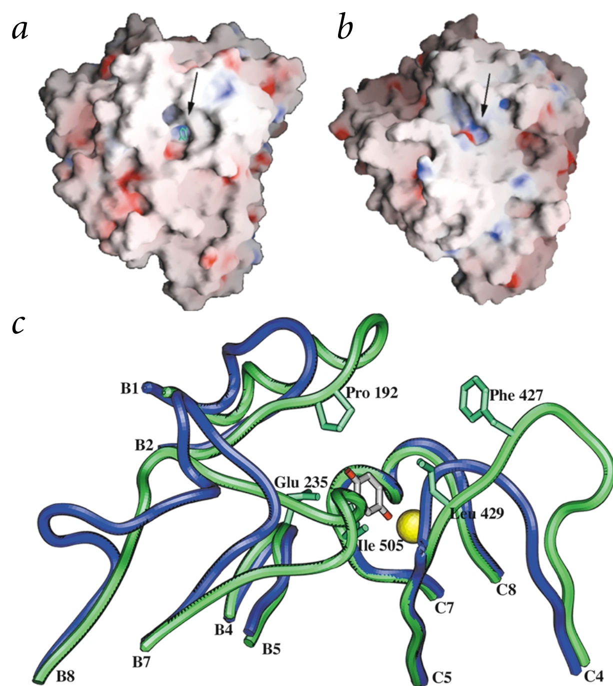

Fig. 2 Molecular surfaces of ***a***, MaL and ***b***, CcL. Red represents negative electrostatic potential; blue, positive electrostatic potential. A docked diphenol is shown in the binding site of MaL. ***c***, The loops of MaL (green) and CcL (blue) near the mononuclear site with docked diphenol. The putative substrate-binding residues of MaL are shown.

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such as those from *C. cinereus*, *T. versicolor* and *P. cinnabarius*. The laccase from *M. albomyces* shows high thermostability and has a pH optimum at neutral. These characteristics are rare among fungal laccases, making the *M. albomyces* laccase an interesting enzyme for different applications.

Overall structure

The crystal structure of the *M. albomyces* laccase (MaL) shows that the enzyme is a monomer consisting of three cupredoxin-like domains: A (1–157), B (158–341) and C (342–559) (Fig. 1*a*). MaL is heavily glycosylated, which substantially increases the molecular weight of the enzyme. Out of nine putative N-glycosylation sites, we found carbohydrates at seven sites in molecule A of the asymmetric unit and eight in molecule B of the asymmetric unit. For molecule A, the calculated molecular mass without the carbohydrates would be 62.0 kDa, but would increase to 65.9 kDa with observed carbohydrates. According to SDS-PAGE, the estimated mass is ~83 kDa; the additional mass is presumably from parts of the carbohydrates that are disordered and not visible in the electron density map. The structure is stabilized by three disulfide bridges: Cys 4–Cys 12, located in domain A; Cys 114–Cys 540, located between domains A and C; and Cys 298–Cys 332, located in domain B near the substrate-binding site.

The molecular architecture of laccase is common for all blue multi-copper oxidases, such as ascorbate oxidase[12,13] and mammalian plasma ceruloplasmin[14]. The overall fold of MaL is similar to that found in the type-2 Cu-depleted laccase from *C. cinereus* (CcL) (Fig. 1*b*). The sequence identity between MaL and CcL laccases is 26% (Fig. 1*c*). Despite this similarity, the structures are different in many respects. MaL has a longer N-terminus, which contains an additional disulfide bridge. Many loops, including some of the loops that form the substrate-binding pocket, are clearly different from CcL. The remarkable feature is that the C-terminal end of MaL is buried.

Substrate-binding pocket

To identify a possible binding pocket for the substrate, the solvent-accessible surfaces of MaL and CcL were calculated near the type-1 Cu. The binding pocket of MaL is narrower compared to that of CcL (Fig. 2*a*). Five loops that are potentially involved in substrate binding are located between β-strands B1 and B2, B4 and B5, B7 and B8, C4 and C5, and C7 and C8 (Figs 1*c*, 2*b*). Because β-strands B5 and B7, as well as C5 and C7, are adjacent, the loops are located near to each other. Only loop B1–B2 is topologically and structurally further away.

In domain B, the first putative substrate-binding loop B1–B2 is six residues longer in MaL compared to that in CcL. In MaL, a small helix located in this loop is longer and brings the loop much closer to the active site. Pro 192 in the active site of MaL probably packs against the substrate. The high-resolution structure of CcL lacks electron density for three residues in this loop, indicating mobility of the loop. In MaL, Glu 235 (Asp 205 in CcL), which is located in the β-hairpin between B4 and B5, is a good candidate for a ligand-binding residue. The structure of the third loop B7–B8 is completely different in MaL compared to CcL. In MaL, the loop is much closer to the active site and makes the substrate-binding pocket tighter. In addition, the loop B7–B8 forms a disulfide bridge (Cys 298–Cys 332) to another loop. The main chain Ala 297-Cys 298-Gly 299 in loop B7–B8 may pack against the substrate. In MaL, the fourth putative substrate-binding loop C4–C5 contains nine residues and is situated in domain C. The loop is three residues longer and clearly different from the corresponding loop in CcL. The putative substrate-binding residues of MaL in this loop are Phe 427 and Leu 429 (Fig. 2*c*). In addition, Ile 505 (present also in CcL), located in the fifth loop C7–C8 before the helix, may also interact with the substrate.

Copper sites

The mononuclear site contains one type-1 Cu, which is trigonally coordinated to two ND atoms from a His residue and a SG atom from a Cys (Fig. 3*a*; Table 1). There is an axial SD atom from Met 2.9 Å away from Cu 1 in ascorbate oxidase[12]. However, in laccases, the Met is replaced by a Leu residue (or a Phe in some laccases), which can occur in a different conformation. In CcL, Cu 1 is 3.5 Å from the CD2 of Leu 462, but in MaL the Cu 1 is 3.7 Å away from the CD1 of the corresponding Leu 513. No residue occupies the axial position on the other side, so this position is free for the substrate.

So far, information about the trinuclear copper site has been inadequate because the only available laccase structure lacks the type-2 Cu, which changes the geometry of the trinuclear site considerably. Our MaL structure reveals new information about the trinuclear copper site. In this site, two type-3 Cu atoms (Cu2 and Cu3) are coordinated to six His N atoms, and type-2 Cu (Cu 4) is coordinated to two His N atoms and to one atom that is probably a chloride ion (Fig. 3*a*; Table 1). Thus, the basic architecture is similar to that observed in ascorbate oxidase, but the distance between the two type-3 coppers in MaL is 4.8 Å, which is ~1.1 Å longer than the oxidized form of ascorbate oxidase (Table 1). Interestingly, the distances between all three copper atoms of MaL are closer to the values obtained for the reduced form of ascorbate oxidase, in which the distance between two type-3 coppers is 5.1 Å.

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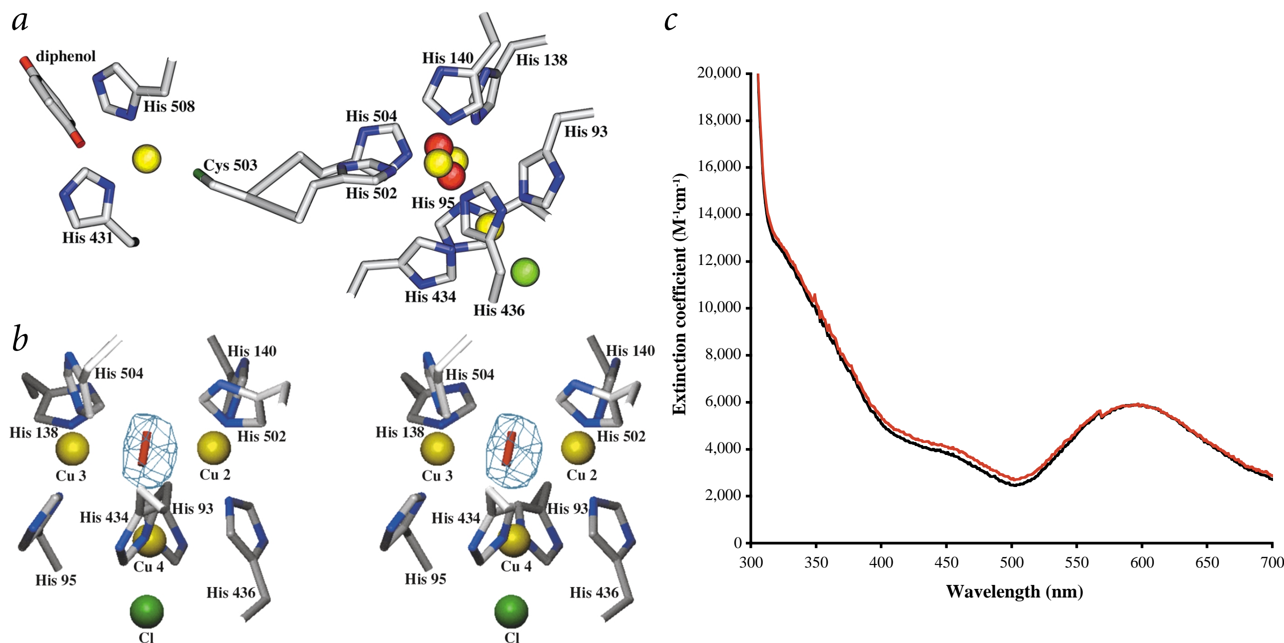

Fig. 3 Copper and oxygen binding to MaL ***a***, Copper sites. The mononuclear site is on the left and the trinuclear site on the right. Oxygen atoms are represented by red balls; a chloride atom, by a green ball. ***b***, A $F_o - F_c$ difference-Fourier omit map for an oxygen molecule in the trinuclear site. The contour level is 5.0 σ. The next highest feature appeared at the 2.0 σ level in the map. The dioxygen is shown as a red stick. ***c***, Absorption spectra of MaL measured under aerobic (black) and anaerobic (red) conditions. T1 copper causes a major band at 590 nm in both spectra. In addition, absorption peaks at ~360 (shoulder), 450 and 670 (shoulder) nm are also seen in both spectra.

Dioxygen binding

We observed an elongated electron density amidst the three copper atoms in the trinuclear site (Fig. 3*b*). The oxygen molecule is located in the middle of the two type-3 coppers, and there is no evidence for a bridging OH group between these coppers, as observed for ascorbate oxidase in oxidized form[12]. All distances between the O1 and O2 atoms of the dioxygen and the type-3 coppers (Cu2 and Cu3) are 2.4–2.6 Å. The distance between the O1 atom of the dioxygen and the type-2 copper (Cu4) is also 2.5 Å (Table 1). The coordination of type-3 coppers could be described as a distorted tetrahedral and of type-2 copper as a square planar geometry. In addition, an absorption spectrum of MaL (Fig 3*c*) supports our proposal that a dioxygen molecule is bound to the trinuclear center, because the spectral values are similar to the reported *Rhus vernicifera* 'oxygen-centered laccase intermediate', where peaks at 370, 420 and 670 nm could be detected[15–17]. Furthermore, both MaL spectra in aerobic and anaerobic conditions are similar, and some activity against guaiacol could be measured under anaerobic condition (data not shown), also suggesting stable dioxygen binding.

Recently the crystal structure of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*, has been determined[18]. In this structure, the distance between the type-3 coppers is 4.7 Å, close to that which was observed in MaL. In addition, the structure showed single oxygen-atom binding among all three coppers within the distances 2.3–3.1 Å. Thus, the binding geometry resembles the binding of the dioxygen molecule in MaL. The binding of molecular oxygen between the two type-3 copper atoms has been reported for *Limulus polyphemus* hemocyanin[19], but the distance between the two copper atoms was 3.6 Å and the distances between the oxygen and the copper atoms were 1.7–2.2 Å. Hemocyanin does not contain type-2 copper, which may be the reason for stronger bonds

Table 1 Copper–copper and copper–ligand distances of MaL compared to CcL, CueO and ascorbate oxidase

	MaL[1]	CcL[2]	CueO[3]	Ascorbate oxidase[1] Oxidized[4]	Reduced[5]
Mononuclear site					
Cu1–Cys 503 SG	2.2	2.2	2.2	2.1	2.2
Cu1–His 431 ND1	1.9	2.1	2.0	2.1	2.1
Cu1–His 508 ND1	1.9	2.0	2.0	2.1	2.1
Trinuclear site					
Cu2–Cu3	4.8	5.3	4.7	3.7	5.1
Cu4–Cu2	3.9	–	3.6	3.9	4.5
Cu4–Cu3	4.0	–	4.0	3.7	4.1
Cu2–His 140 NE2	2.0	2.0	2.0	2.2	2.2
Cu2–His 436 NE2	1.9	2.0	1.9	2.1	2.1
Cu2–His 502 NE2	2.0	2.0	2.0	2.1	2.1
Cu3–His 138 NE2	2.0	2.0	2.0	2.2	2.0
Cu3–His 95 ND1	1.9	2.0	2.0	2.1	2.1
Cu3–His 504 NE2	2.0	2.1	2.1	2.1	2.1
Cu4–His 93 NE2	1.9	–	1.9	2.0	2.0
Cu4–His 434 NE2	1.9	–	1.8	2.1	2.1
Cu4–O/Cl^-	2.5 (Cl^-)	–	3.0 (O)	2.0 (O)	2.2 (O)
Cu2–O1	2.5	–	2.3	–	–
Cu2–O2	2.4	2.2	–	2.0	–
Cu3–O1	2.5	–	2.4	–	–
Cu3–O2	2.6	3.3	–	2.1	–
Cu4–O1	2.5	–	3.1	–	–

[1]Averaged values of A and B molecules.
[2]Ref. 9.
[3]Ref. 18.
[4]Ref. 12.
[5]Ref. 13.

Table 2 Crystallographic data collection and refinement statistics

Data collection	
Space group	P1
Unit cell dimensions	
a (Å)	62.51
b (Å)	72.30
c (Å)	88.94
α (°)	110.40
β (°)	95.23
γ (°)	109.74
Molecules per asymmetric unit	2
Resolution (Å)[1]	99–2.4 (2.49–2.40)
Unique reflections[1]	49,561 (4,518)
Completeness(%)[1]	95 (87)
R_{sym} (%)[1]	9.2 (28.0)
I / I (σ)[1]	7.5 (2.0)
Multiplicity[1]	1.9 (1.8)
Refinement	
Number of reflections[1]	
Working set	44,578 (5,168)
Test set	4,983 (557)
R_{work} (%)[1]	18.9 (25.4)
R_{free} (%)[1]	24.8 (33.0)
R.m.s. deviation	
Bond length (Å)	0.006
Bond angle (°)	1.3
Number of atoms	
Total	10,060
Protein	8,738
Water	795
Copper	8
Oxygen	4
Chloride	2
Sulfate	15
Carbohydrate	498
Average B-factors (Å²)	
Overall	24.6
Protein	22.7
Water	26.5
Copper	25.9
Oxygen	22.9
Chloride	28.9
Sulfate	71.6
Carbohydrate	52.3

[1]Values in parentheses are for the highest resolution shell.

between the oxygen and the type-3 copper atoms. Short bond lengths (1.8–2.0 Å) between the copper atoms and dioxygen have also been found in many organometallic small molecule complexes[20]. However, there is no trinuclear copper model structure in which the mutual geometry of copper atoms would resemble the trinuclear copper site observed in multi-copper oxidases.

Because the mutual distances of the three copper atoms in MaL are only slightly shorter than the distances measured for the reduced form of ascorbate oxidase, the measured structure of MaL could be at least partially reduced and, in this arrangement, is able to bind the oxygen molecule. The existence of dioxygen in the crystal structure of MaL further suggests that this form is rather stable. To our knowledge, this is the first crystal structure that shows the oxygen molecule bound to the trinuclear copper site. In addition, the binding geometry is completely new.

C-terminal plug

Messerschmidt *et al.*[12] found two channels, a broad and a narrow, that provide access for the solvent molecules to the trinuclear site in ascorbate oxidase. Similar tunnels may also exist in laccases. In both MaL and CcL, a narrow tunnel leading to the type-2 copper (Cu 4) may provide access for the solvent molecules. In CcL, the broad tunnel, which is ~10 Å long and leads to one of the type-3 coppers (Cu2), is located between the trinuclear center and protein surface. A tunnel of similar shape and length could, in principle, also exist in MaL; however, the structure reveals a plug by the C-terminal residues Asp-Ser-Gly-Leu. The packing of C-terminus against the tunnel is extensive and there are no water molecules between the C-terminal residues and the residues forming the tunnel surface. In addition, the C-terminal carboxylate group makes a hydrogen bond to a side chain of His 140, which is also bound to Cu2.

The gene sequence of MaL codes for a 623-residue polypeptide; however, the secreted mature protein lacks 50 residues at the N-terminus and 14 residues at the C-terminus (L.-L.K. and M.S., unpub. results). The last 14 residues are cleaved at a conserved cleavage site, similar to other ascomycete laccases[21,22]. Although the processed C-termini of these enzymes are not identical, they have a conserved Asp-Ser-Gly-(Leu/Val/Ile) sequence preceding the cleavage site. The reason for and significance of C-terminal processing is not yet understood. Our MaL structure suggests that the processed C-terminus may bind to the tunnel leading to the trinuclear copper site. The C-terminal processing and the conserved cleavage site also suggest that C-terminal blocking might be a general feature among ascomycete laccases. The function of the tunnel is uncertain, but it could form an access route for the oxygen molecule to enter the trinuclear copper site. The entrance of an oxygen molecule and exit of a water molecule through this tunnel would require a conformational change in the C-terminus of MaL to open the tunnel. Moreover, the C-terminal blocking of the tunnel might be the reason the oxygen molecule is found in the active site of the MaL crystal structure. In any case, the closure of this tunnel certainly affects the function of the trinuclear copper site. Whether this feature is possible among basidiomycete laccases, which have different C-terminal residues, is not known. Interestingly, Gelo-Pujic *et al.*[23] have noticed that the redox potential of the type-1 Cu of a truncated basidiomycete laccase from *Trametes versicolor* (produced in *Pichia pastoris*) changed when 11 amino acids at C-terminus were replaced with a single Cys residue. This suggests that C-terminal amino acids might have a role in the function of all fungal laccases.

Methods

Absorption spectroscopy. The absorption spectra were measured with Varian Cary100 spectrophotometer (Varian Inc.). Protein concentration of 6.8 μM of MaL in 100 mM Tris buffer, pH 7.0, was used in measurements. A vacuum was applied to the anaerobic cell and the laccase protein solution was flushed with argon for 10 min before measuring the absorption spectrum at room temperature. In addition, dithionite ($Na_2S_2O_2$) under argon was added to the cell in several different concentrations.

Crystallization, data collection and structure determination. MaL was purified as described[24] and crystallized at 22 °C in hanging drops by combining 2 μl of protein (concentration 6 mg ml^{-1} in 100 mM sodium phosphate buffer, pH 7) with 2 μl of reservoir solution. The reservoir solution contained 25% (w/v) polyethylene gly-

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col monomethyl ether (PMME) 2000, 0.2 M lithium sulfate and 0.1 M sodium acetate, pH 4.2. Initially, the crystals were not well ordered but the microseeding method[25] gave better quality crystals.

Diffraction data were collected on a RAXIS-IIC imaging plate detector using a Rigaku RU-200HB rotating anode source with Osmic mirror optics. The crystal was transferred into the cryoprotectant solution containing 25% (w/v) PMME 2000, 0.2 M lithium sulfate, 0.1 M sodium acetate, pH 4.2, and 15% (w/v) PEG 400. The crystal was then flash-cooled in a cold nitrogen stream at 120 K. Data were processed and scaled using DENZO and SCALEPACK[26]. The data processing statistics are showed in Table 2.

A molecular replacement solution was found using AMoRe[27]. The search model was laccase from *C. cinereus* (PDB entry 1A65)[28]. The asymmetric unit contained two molecules, designated A and B. The structure was refined by iterative cycles of manual refitting with O[29] and simulated annealing and positional refinement with CNS[30]. The parameters of Quanta 3.0 (ref. 31) were used as constraints for the metal sites. The resulting map showed good connectivity throughout the polypeptide chain, and the final model had a good stereochemistry. Data statistics are listed in Table 2. Figures were prepared with SETOR[32] and GRASP[33].

During refinement, we observed an elongated electron density between all three copper atoms in the trinuclear copper center and concluded that this best corresponds to a dioxygen molecule. To test whether the density corresponds to one water, hydroxyl or single oxygen atom occupying two positions, we refined the structure with a water molecule occupying both positions by half. This calculation showed clear residual electron density in the $F_o - F_c$ map and the B-factors for the oxygen atoms were low (2–4 Å^2). This suggests that two oxygen atoms are covalently bound to each other in the trinuclear copper center. The alternatives for this molecule would be either neutral oxygen or dihydrogen peroxide. A negatively charged hydroperoxide ion would probably make a covalent bond (2.0 Å) with one of the copper atoms, as observed in ascorbate oxidase[13]. The protonation states of these two oxygens are difficult to determine. However, the oxygen–oxygen distance corresponds better with the dioxygen molecule, where it is 0.3 Å shorter that in dihydrogen peroxide. Therefore, we suggest that the molecule is the dioxygen. By using the dioxygen in the refinement, we obtained B-factors of 19–26 Å^2 for the oxygen atoms. The molecule bound to the type-2 copper (Cu4) was reasoned to most likely be a chloride ion because the water molecule in this position showed a low B-factor.

Coordinates. The coordinates and the structure factors have been deposited in the Protein Data Bank (accession code 1GW0).

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence should be addressed to J.R. *email: Juha.Rouvinen@joensuu.fi*

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Molecular Cloning and Expression in *Saccharomyces cerevisiae* of a Laccase Gene from the Ascomycete *Melanocarpus albomyces*

Laura-Leena Kiiskinen* and Markku Saloheimo

VTT Biotechnology, Espoo, Finland

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The *lac1* gene encoding an extracellular laccase was isolated from the thermophilic fungus *Melanocarpus albomyces*. This gene has five introns, and it encodes a protein consisting of 623 amino acids. The deduced amino acid sequence of the laccase was shown to have high homology with laccases from other ascomycetes. In addition to removal of a putative 22-amino-acid signal sequence and a 28-residue propeptide, maturation of the translation product of *lac1* was shown to involve cleavage of a C-terminal 14-amino-acid extension. *M. albomyces lac1* cDNA was expressed in *Saccharomyces cerevisiae* under the inducible *GAL1* promoter. Extremely low production was obtained with the expression construct containing laccase cDNA with its own signal and propeptide sequences. The activity levels were significantly improved by replacing these sequences with the prepro sequence of the *S. cerevisiae* α-factor gene. The role of the C-terminal extension in laccase production in *S. cerevisiae* was also studied. Laccase production was increased sixfold with the modified cDNA that had a stop codon after the native processing site at the C terminus.

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases. They catalyze the oxidation of a variety of phenolic compounds, as well as diamines and aromatic amines, with concomitant reduction of molecular oxygen to water (43). Laccases are widely distributed in higher plants and fungi, and laccase or laccase-like activity has also been demonstrated in some insects and bacteria (18, 31, 12). In fungi, laccases are involved in several physiological functions, such as plant pathogenesis (3, 11), pigment production (2), and degradation of lignocellulosic materials (6). Because of their surprisingly wide variety of substrates, laccases are considered industrially interesting enzymes for various applications, including textile dye bleaching, pulp bleaching, detergents, and enzymatic conversion of chemical intermediates (47).

Despite intensive research, the molecular basis of laccase-catalyzed reactions is still partially unknown. In order to determine the function of laccases and to produce them heterologously in large quantities, several laccase genes have been cloned, especially the genes from basidiomycetous fungi, including *Phlebia radiata* (39), *Cryptococcus neoformans* (46), *Pleurotus ostreatus* (19), *Trametes versicolor* (25), *Trametes villosa* (49), *Pycnoporus cinnabarinus* (13), and *Coprinus cinereus* (48). Some laccase genes have also been cloned from ascomycetes, including *Neurospora crassa* (17), *Aspergillus nidulans* (1), *Podospora anserina* (14), and *Myceliophthora thermophila* (5). Generally, the laccase sequences of members of a fungal class exhibit levels of amino acid identity of 50% or more, whereas the levels of identity between sequences of members of different classes are around 30%.

Heterologous expression of laccase genes has been studied in *Saccharomyces cerevisiae* (27, 10, 29), *Trichoderma reesei* (38), *Aspergillus oryzae* (49, 5), *Pichia pastoris* (24, 16, 34, 7), *Aspergillus sojae* (22), and *Aspergillus niger* (36). Especially in *S. cerevisiae*, reasonable expression levels have proven to be very difficult to achieve. In most previous studies of laccase expression in *S. cerevisiae*, highly sensitive measurement methods had to be used in order to detect laccase activity. For example, Larsson et al. reported activity measurements that were monitored for 24 h in order to detect satisfactory changes in absorbance, even after optimization of the fermentation conditions for *S. cerevisiae* expressing a laccase from *T. versicolor* (29). Reasonable laccase activity levels are essential for using *S. cerevisiae* in high-throughput screening of directed evolution experiments. *P. pastoris* has been used as a heterologous host for laccases more often than *S. cerevisiae*, and higher production levels have been obtained, but the results of use of this organism in high-throughput screening have not been favorable because of a lower transformation frequency and integration of the expression constructs. Successful use of *S. cerevisiae* expressing *M. thermophila* laccase in directed evolution was recently reported by Bulter et al., who obtained very promising results (8).

The thermophilic ascomycete *Melanocarpus albomyces* produces an industrially interesting laccase with substantial thermal stability and activity at alkaline pH values (26). Based on the N-terminal sequence and two internal amino acid sequences of *M. albomyces* laccase, this enzyme was found to be related to other ascomycete laccases. In this paper, we describe cloning and sequence analysis of the gene encoding *M. albomyces* laccase. In addition, we expressed the laccase gene in *S. cerevisiae* so that directed evolution studies can be performed in the future.

MATERIALS AND METHODS

Strains and reagents. The fungal strains used in this study were *M. albomyces* VTT D-96490 and *P. anserina* ATCC 26003. The *S. cerevisiae* strain used for laccase expression was INVSc1 (MAT**a** *his3Δ1 leu2 trp1-289 ura3-52*/MATα *his3Δ1 leu2 trp1-289 ura3-52*), which was obtained from Invitrogen (Carlsbad, Calif.), and the yeast strain used for plasmid construction by the in vivo recombination technique was W3031a (*ade2 can1 his3 leu2 trp1 ura3*), which was

* Corresponding author. Mailing address: VTT Biotechnology, P.O. Box 1500, Fin-02044 VTT, Finland. Phone: 358-9-4565984. Fax: 358-9-4552103. E-mail: Laura-Leena.Kiiskinen@vtt.fi.

obtained from Hans Ronne, Agricultural University, Uppsala, Sweden. The *Escherichia coli* strains used were XL1-Blue MR and XL10-Gold from Stratagene (La Jolla, Calif.), TOP10F′ from Invitrogen, and DH5α from Gibco BRL (Gaithersburg, Md.). The enzymes used to manipulate RNA or DNA were obtained from New England Biolabs (Beverly, Mass.) or Boehringer (Mannheim, Germany), unless otherwise stated.

Isolation of the genomic laccase gene. *M. albomyces* was cultivated as previously described (26). Total cellular DNA was extracted from the cells by the method of Raeder and Broda (35). The genomic DNA was digested with *Eco*RI and *Hin*dIII in two separate reactions, and the fragments were analyzed by Southern hybridization (40). The laccase-specific probe fragment comprised the portion of the *P. anserina lac2* gene that encodes the mature laccase protein (14). The probe fragment was prepared by PCR from *P. anserina* genomic DNA isolated with an Easy DNA kit (Invitrogen) by using primers 5′-TGCCACACT GCCGCCAACCGTGCT-3′ (forward) and 5′-GTTCTTGATATACCAATCAG GATG-3′ (reverse). The probe was radiolabeled with a random primed DNA labeling kit (Boehringer) by using [α-^{32}P]dCTP. The hybridization solution contained 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), 1× Denhardt's solution (40), 0.5% sodium dodecyl sulfate (SDS), and 100 μg of herring sperm DNA per ml. To test for optimal hybridization conditions for library screening, hybridization was performed at four different temperatures (48, 50, 55, and 60°C) overnight. Following incubation, the filters were washed with 2× SSC–0.1% SDS twice at room temperature for 5 min and once at the hybridization temperature for 30 min. The hybridization signals were detected by exposing the filters to X-ray film at −70°C.

A genomic library of *M. albomyces* was constructed in the SuperCos I cosmid (Stratagene). DNA was digested partially with *Sau*3AI and was dephosphorylated with calf intestinal alkaline phosphatase (Finnzymes, Espoo, Finland). DNA fragments were size fractionated by 15 to 30% sucrose density gradient centrifugation. DNA fragments that were more than 20 kb long were ligated with SuperCos I cosmid vector arms that had been digested with *Xba*I, dephosphorylated with calf intestinal alkaline phosphatase, and finally digested with *Bam*HI. The ligation mixture was packaged into λ particles with a Gigapack III Gold packaging extract (Stratagene). The synthesized DNA library was amplified in *E. coli* XL1-Blue MR cells.

Approximately 5×10^5 clones from the genomic DNA library were plated and grown overnight and then transferred to Protran nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany), and the DNA was fixed by using standard procedures (40). The membranes were hybridized with the labeled *P. anserina lac2* gene as described above at 57°C overnight.

To find a suitable fragment for subcloning, the cosmids isolated from strongly hybridizing colonies were digested with 19 different restriction enzymes, and the fragments obtained were analyzed by Southern hybridization with the labeled *P. anserina lac2* gene as described above. As a 4.5-kb *Eco*RI fragment was shown to hybridize with the probe, the cosmid was digested with *Eco*RI, and the fragment was purified by agarose gel electrophoresis. The fragment was ligated into the vector pBluescriptSK(−) (Stratagene) to obtain plasmid pLLK1. The *M. albomyces lac1* gene was sequenced from pLLK1 by using the primer walking technique with a DNA sequencing kit, the dRhodamine terminator cycle sequencing ready reaction (PE Biosystems, Warrington, United Kingdom), and an ABI Prism 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems, Foster City, Calif.).

cDNA cloning. The cDNA encoding the laccase was cloned by rapid amplification of cDNA ends (RACE)-PCR. Total RNA was extracted from *M. albomyces* cells with TRIzol reagent (Life Technologies, Gaithersburg, Md.), dephosphorylated, and reverse transcribed according to the instructions of a FirstChoice RLM-RACE kit (Ambion, Inc., Austin, Tex.). The 5′ and 3′ ends of the *lac1* cDNA were amplified in two separate nested PCRs by using two gene-specific primers and two primers specific for adapters ligated into the cDNA. The nested gene-specific primers were as follows: for the 5′ end, 5′-GCCGGTGAGGATG TAGTCGATGAT-3′ (outer primer) and 5′-AGGTGACGTTGAACCAGTAG TTGTC-3′ (inner primer); and for the 3′ end, 5′-CTGGTGCACTTCACGCA GAACAA-3′ (outer primer) and 5′-AGAACCACTTCCAGGTGTCGCT-3′ (inner primer). Thirty five cycles of 94°C for 30 s, 60°C (62°C for the 3′ end) for 30 s, and 72°C for 2 min were performed. The resulting fragments were cloned into the pCR2.1-TOPO vector (TOPO TA cloning kit; Invitrogen). The inserts were sequenced and confirmed to correspond to the laccase gene and to have no PCR mistakes. The two separate cDNA ends were ligated into the same vector in the correct orientation by using a unique *Aat*II restriction site in the overlapping region to obtain plasmid pLLK4.

Southern hybridization of *M. albomyces* genomic DNA with *lac1* cDNA. In order to demonstrate the presence of other laccase genes in the *M. albomyces* genome, Southern hybridization of *M. albomyces* genomic DNA with *M. albomyces lac1* cDNA was performed. The DNA was digested with *Eco*RI, *Hin*dIII, and *Pvu*II in three separate reactions, and the fragments were analyzed by Southern hybridization at three different temperatures (50, 55, and 60°C) as described above.

Northern hybridization of *M. albomyces* RNA with *lac1* DNA. Total RNA was extracted with the TRIzol reagent from a shake flask culture and from a fermentor culture of *M. albomyces* at a growth stage at which laccase activity was produced. A 594-bp portion of the *M. albomyces lac1* gene (bp 218 to 811 from the translation start site) was used as a probe. The hybridization solution contained 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, and 125 μg of herring sperm DNA per ml. Hybridization was performed at 42°C overnight. Following incubation, the filter was washed once with 5× SSPE and twice with 1× SSPE–0.1% SDS for 15 min at 42°C (1× SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 1.3 mM EDTA [pH 7.4]).

Construction of expression vectors for *S. cerevisiae*. *M. albomyces lac1* cDNA from a *Sac*I site 49 bp upstream of the translation start site to the polyadenylation site was cloned into the *Eco*RI sites of two yeast expression vectors, pYES2 (Invitrogen) containing the inducible *GAL1* promoter and pAJ401 (37) containing the constitutive *PGK1* promoter. This created plasmids pLLK10 and pLLK7, respectively.

An expression vector in which the native laccase signal and propeptide sequences were replaced with the prepro sequence of the *MFα1* gene of *S. cerevisiae* was constructed. A DNA fragment in which the yeast α-factor prepro region was fused with the 5′ end of the mature laccase was constructed by overlap extension PCR. The 5′ end of this fragment had a 40-bp overlap with the *GAL1* promoter, added to the 5′ end primer. The primers used in the PCR were 5′-CTACTAGCAGCTCTAATACGACTCACTATAGGGGAATATTAAGCT TATGAGATTTCCTTCA-3′ (5′ end primer with an overlap with the *GAL1* promoter), 5′-AGAAGGGGTATCTTTGGATAAAAGAGAGCCGACGTGC AACACGCCGAGCA-3′ (forward primer for constructing an overlap between α-factor and laccase), 5′-TGCTCGGCGTGTTGCACGTCGGCTCTCTTTTAT CCAAAGATACCCCTTCT-3′ (reverse primer for constructing an overlap between α-factor and laccase), and 5′-AGCGGTACGTCCGCTGGCCG-3′ (3′ end primer). The plasmid was constructed by in vivo recombination in *S. cerevisiae*. Strain W3031a was transformed with the laccase expression plasmid pLLK10 digested with *Hin*dIII and *Bst*EII and the PCR fragment described above which had overlaps at both ends with digested pLLK10. Plasmids were rescued into *E. coli* from the yeast transformants obtained, and plasmid pMS174 was confirmed by restriction enzyme digestion and DNA sequencing.

To study the significance of C-terminal processing for laccase production in yeast, a version of pMS174 was made in which a translation stop codon was added after the C-terminal processing site. This was done by site-directed mutagenesis with a QuickChange mutagenesis kit (Stratagene) and primers 5′-CC CAAGATCGACTCGGGCCTG**T**AGCGTCGCCGCTGGGTGGAGG-3′ (forward) and 5′-CCTCCACCCAGCGGCGACGCT**A**CAGGCCCGAGTCGATC TTGGG-3′ (reverse) (the mutated bases are indicated by boldface type). The right mutation in the plasmid constructed, pMS175, was confirmed by sequencing.

S. cerevisiae strain INVSc1 was transformed with the expression vectors pLLK7, pLLK10, pMS174, and pMS175 and the corresponding empty control vectors by using the lithium acetate procedure (20). Production of laccase by the transformants was first assayed on SC-Ura plates (41) supplemented with glucose (for transformants carrying pLLK7) or galactose (for other transformants) by soaking the well-grown colonies on plates with 2 ml of 20 mM ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate] (Roche Diagnostics GmbH, Mannheim, Germany). To study laccase production in liquid cultures, three transformants of each type were grown in parallel in SC-Ura medium (41) buffered to pH 6 with succinate and supplemented with 2% glucose (transformants with pLLK7) or 2% raffinose (transformants with pLLK10, pMS174, or pMS175) and 0.5 mM $CuSO_4$. All the shake flask cultures were inoculated to obtain an initial optical density at 600 nm of 0.2, incubated at 30°C, and shaken at 250 rpm. After 2 days of cultivation, cells from transformants carrying pLLK10, pMS174, or pMS175 were separated by centrifugation (4,000 × g, 3 min) and resuspended in the same volume of induction medium (SC-Ura medium supplemented with 2% galactose and 0.5 mM $CuSO_4$). Extracellular laccase activity was monitored daily by measuring the oxidation of 5 mM ABTS in 25 mM succinate buffer (pH 4.5) at 436 nm with an extinction coefficient of 29,300 M^{-1} cm^{-1} (33).

The presence of laccase in yeast culture supernatants and inside the cells was detected by Western blotting with polyclonal antibodies raised in rabbits against the laccase purified from *M. albomyces* cultures. After 3 days of growth in the induction medium, the cells in 1 ml of culture were collected by centrifugation (4,000 × g, 3 min). The cells were lysed by intensive mixing with glass beads (diameter, 0.45 mm; Sigma) in a 2% SDS solution containing protease inhibitors

(Complete Mini protease inhibitor cocktail; Roche). Samples of cell lysates and culture supernatants were separated by SDS-polyacrylamide gel electrophoresis (Mighty Small II SE250; Hoefer Pharmacia Biotech Inc., San Francisco, Calif.) performed as described by Laemmli (28). Proteins were electroblotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) by using a Trans-blot cell (Bio-Rad Laboratories Inc., Hercules, Calif.). *M. albomyces* laccase was detected by using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Bio-Rad).

Nucleotide sequence accession number. The nucleotide sequence of the *M. albomyces lac1* gene has been deposited in the EMBL nucleotide sequence database under accession number AJ571698.

RESULTS

Cloning and characterization of the *M. albomyces lac1* gene. Whether the *P. anserina lac2* gene could be used as a laccase probe in heterologous Southern hybridization was tested with *M. albomyces* total DNA digested separately with *Eco*RI and *Hin*dIII. The results showed that the *P. anserina lac2* gene hybridized with a 4.5-kb *Eco*RI fragment of *M. albomyces* DNA at all temperatures tested (48 to 60°C) (data not shown), which suggested that *M. albomyces* had a gene with relatively high conservation with *P. anserina lac2*. Thus, *P. anserina lac2* was used to screen the *M. albomyces* genomic DNA library that was constructed. Several colonies that gave positive hybridization signals were found, and cosmids from six strongly hybridizing colonies were isolated. The restriction fragment suitable for subcloning the laccase gene from the cosmid was found by mapping the cosmid by digestion with 19 different restriction enzymes, followed by Southern hybridization. A 4.5-kb *Eco*RI fragment was again shown to hybridize with the labeled *P. anserina lac2* gene. This fragment was subcloned into the vector pBluescriptSK(−) to obtain plasmid pLLK1, and the laccase gene region of the insert was sequenced. All three peptide sequences obtained previously for *M. albomyces* laccase (26) were identified in the amino acid sequence deduced from the gene (Fig. 1), confirming that the right gene was cloned. When the promoter region was sequenced, an *Eco*RI site was found only 285 bp upstream of the translation start codon. Thus, altogether, 1,619 bp of the promoter region was sequenced from the cosmid instead of pLLK1. A putative TATA box (TATATAAT) was found in the promoter region at position −170 bp with respect to the translation start codon.

In order to determine the intron-exon structure of the *M. albomyces lac1* gene and to facilitate heterologous expression, the cDNA copy of the gene was cloned by RACE-PCR by using a method that amplified only capped mRNA molecules from the 5′ end. This enabled determination of the transcription start site based on the 5′ end fragment length. The resulting fragments from the RACE-PCR were 1,194 bp (5′ end) and 1,322 bp (3′ end) long. The positions of five introns (lengths, 78, 73, 87, 86, and 82 bp) (Fig. 1) in the *lac1* gene were verified by comparing the cDNA sequence and the sequence of the genomic copy. The 5′ end RACE fragment suggested that there was an ACCAGG transcription start site at bp −120 from the translation start site.

Northern hybridization was performed by using a shake flask culture and a fermentor culture of *M. albomyces* at a growth stage at which laccase activity was produced. Probing of the Northern blot with a 594-bp portion of the *M. albomyces lac1* gene resulted in a 2.0-kb signal, which corresponded well to the size of the cDNA isolated (Fig. 2B).

The sequence encoded by the open reading frame of the *M. albomyces lac1* gene is 623 amino acids long, which is typical for fungal laccases. The regions involved in copper binding are well conserved (14, 48). The amino acid sequence was compared to other known laccase sequences, and it exhibited high levels of identity with other ascomycete laccase sequences (Fig. 1). The levels of amino acid identity were 73% with *M. thermophila* laccase (4), 68% with *P. anserina* laccase 2 (14), and 63% with *N. crassa* laccase (17). On the other hand, the levels of homology with basidiomycete laccases were quite low. For example, the levels of identity of the *M. albomyces* laccase with laccases from *T. versicolor* and *T. villosa* were 27 to 28% (25, 49), and the level of identity with *C. cinereus* laccase Lcc1 was 31% (48).

The first 22 N-terminal amino acids of *M. albomyces* laccase consist of a predicted signal sequence typical of eukaryotic proteins (32). The signal sequence is followed by a cleavable 28-amino-acid propeptide, as shown by comparison of the deduced amino acid sequence to the N-terminal peptide sequence of the purified protein (26). In addition, posttranslational removal of the last 14 predicted C-terminal residues was shown by sequencing the C terminus of the laccase purified from *M. albomyces*. Ser, Gly, and Leu were identified as the last three amino acids of the mature protein.

Southern blot hybridization of *M. albomyces* genomic DNA with *M. albomyces lac1* cDNA showed the expected strong signals corresponding to the *lac1* gene. In addition, weaker but clearly detectable signals were observed with genomic DNA digested with *Pvu*II and with *Hin*dIII (Fig. 2A). *Pvu*II digestion resulted in one weaker signal in addition to the expected signals, whereas two weaker signals were detected after *Hin*dIII digestion. The signals were seen in hybridization experiments done at all temperatures tested. This indicates that there are at least two laccase genes in the *M. albomyces* genome.

Heterologous expression. Four different expression vectors were constructed for expression of *M. albomyces* laccase in *S. cerevisiae* (Fig. 3). In two of them, the laccase cDNA was alone under the yeast *PGK1* promoter or under the *GAL1* promoter (pLLL7 and pLLK10, respectively). In the third vector, the region encoding the mature laccase was fused with the yeast α-factor prepro region (pMS174), and the rest of the laccase cDNA was intact. The fourth vector, pMS175, had the α-factor prepro-laccase fusion and a stop codon after the C-terminal processing site of the laccase. All the expression plasmids and the corresponding vectors without an insert were transformed into yeast strain INVSc1. Production of laccase was first assayed with a plate test in which ABTS was the substrate. Formation of a green color around yeast colonies was detected after overnight incubation for transformants carrying pMS175. No color changes were observed for other transformants.

Laccase production was then studied in liquid cultures grown in synthetic complete media with 0.5 mM $CuSO_4$ added to support copper incorporation into the laccase. The yeast strains in which laccase production was driven by the *GAL1* promoter were first grown in raffinose medium, and the cells were subsequently transferred into a medium with galactose to induce the promoter. Laccase production from the pLLK7 vector with the *PGK1* promoter was not detectable with an

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MaL -MKTFTSALALVVGMLAPGAVVAAPPSTPAQRDLVELREARQE--GGKDLRPREPTCNTP 57
PaL MMKSFFSAAALLLGLVAPSAVLAAPSLPGVP------REVTRD--LLRPVEERQSSCHTA 52
NcL -MK-FLGIAALVAGLLAPPLVLAAPAPGTEGVNLLTPVDKRQDSQAERYGGGGGGCNSP 58
     **:* .  **: *::**  *:***.      .     :  ::    :        *::.

MaL SNRACWSDGFDINTDYEVSTPDTGVTQSYVFNLTEVDNWMGPDGVVKEKVMLINGNIMGP 117
PaL ANRACWAPGFDINTDYEVSTPNTGVTRTYTLTLTEVDNWLGPDGVVKQKVMLVNGDIFGP 112
NcL TNRQCWSPGFNINTDYELGTPNTGKTRRYKLTLTETDNWIGPDGVIKDKVMMVNDKIIGP 118
    :** **: **:******:.**:** *: * :.***.***:*****:*:***::*..*:**

MaL NIVANWGDTVEVTVINNLVTNGTSIHWHGIHQKDTNLHDGANGVTECPIPPKGGQRTYRW 177
PaL TITANWGDWIQVNVINNLRTNGTSIHWHGLHQKGTNMHDGANGVTECPIPPKGGSRIYRF 172
NcL TIQADWGDYIEITVINKLKSNGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGGSKVYRW 178
    .* *:*** :::.***:* :*********:**:.:*::**.**********:**.: **:

MaL RARQYGTSWYHSHFSAQYGNGVVGTIQINGPASLPYDIDLGVFPITDYYYRAADDLVHFT 237
PaL RAQQYGTSWYHSHFSAQYGNGVVGTIVVNGPASVPYDIDLGVFPITDYYHKPADVLVEET 232
NcL RATQYGTSWYHSHFSAQYGNGIVGPIVINGPASANYDVDLGPFPLTDYYYDTADRLVLLT 238
    ** ******************:**.* :*****  **:*** **:****: .** **  *

MaL QNNAPPFSDNVLINGTAVNPNTGEGQYANVTLTPGKRHRLRILNTSTENHFQVSLVNHTM 297
PaL MNGGPPPSDTVLFKGHGKNPQTGAGKFANVTLTPGKRHRLRIINTSTHDHFQLKLQNHTM 292
NcL QHAGPPPSNNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLLLVNHSM 298
     : .** *:.**::* . :* ** *::*.*:** **:****::***..:***: * **:*

MaL TVIAADMVPVNAMTVDSLFLAVGQRYDVVIDASRAPDNYWFNVTFGGQAACGGSLNPHPA 357
PaL TIIAADMVPVQAQTVDSLFLAVGQRYDVTIDANKSVGNYWFNATFGGGLACGASLNPHPA 352
NcL TIISADLVPVQPYKVDSLFLGVGQRYDVIIDANQAVGNYWFNVTFGGSKLCGDSDNHYPA 358
    *:*:**:***:. .******.******* ***.:: .*****.****   ** * * :**

MaL AIFHYAGAPGGLPTDEGTPPVDHQCLDTLDVRPVVPRSVPVNSFVKRPDNTLPVALDLTG 417
PaL AVFRYQGAPNTLPTNIGTPAADANCMDLNNLTPVVSRSVPTSGFTPRPNNTLPVSLTLGG 412
NcL AIFRYQGAPKALPTNQGVAPVDHQCLDLNDLKPVLQRSLNTNSIALNTGNTIPITLDG-- 416
    *:*:* ***  ***: *....* :*:*  :: **: **: ...:. ...**:*::*   .

MaL TPLFVWKVNGSDINVDWGKPIIDYILTGNTSYPVSDNIVQVDAVDQWTYWLIENDPEGPF 477
PaL TPLFVWKVNGSSINVDWDKPIVDYVIAQNTSYPPQANVITVNSVNQWTYWLIENDPTGPF 472
NcL ---FVWRVNGTAININWNKPVLEYVLTGNTNYSQSDNIVQVEGVNQWKYWLIENDPDGAF 473
    :. ***:***: **::*.**:::*::: **.*.  .*:: *:.*:**.******** *.*

MaL SLPHPMHLHGHDFLVLGRSPDVPAAS-QQRFVFDPAVDLARLNGDNPPRRDTTMLPAGGW 536
PaL SIPHPMHLHGHDFLVVGRSPDQPAGVPQTRYRFNPATDMALLKSSNPVRRDVAMLPANGW 532
NcL SLPHPIHLHGHDFLILGRSPDVTAIS-QTRYVFDPAVDMARLNGNNPTRRDTAMLPAKGW 532
    *:***:********::***** .*   * *: *:**.*:* *:..** ***.:**** **

MaL LLLAFRTDNPGAWLFHCHIAWHVSGGLSVDFLERPADLRQRISQEDEDDFNRVCDEWRAY 596
PaL LLIAFKSDNPGAWLFHCHIAWHVSGGLSVQYLERPNDLRNGFSQADKNQHNNNCNAWRAY 592
NcL LLIAFRTDNPGSWLMHCHIAWHVSGGLSNQFLERAQDLRNSISPADKKAFNDNCDAWRAY 592
    **:**::****:**:************* ::***. ***: :*  *:. .*  *: ****
                  ↓            .
MaL WPTN-PYPKIDSGL KR-RRWVEES-EWLVR- 623
PaL WPTN-PFPKIDSGL KV-KKWVGEHPDWYIKN 621
NcL FPDNAPFPKDDSGL RSGVKAREVKMKW---- 619
    :* * *:** **** :   :      .*
```

FIG. 1. Alignment of laccase sequences from *M. albomyces* (MaL), *P. anserina* (PaL; accession number P78722) (14), and *N. crassa* (NcL; accession number P06811) (17) as determined with the Clustal W multiple-sequence alignment program (42). An asterisk indicates that the residues at a position are identical in all sequences in the alignment, a colon indicates that conserved substitutions have been observed, and a period indicates semiconserved substitutions. Putative signal sequences are indicated by italics, the propeptides are underlined, and the conserved C-terminal cleavage site is indicated by an arrow. The conserved residues involved in copper binding are enclosed in boxes, and the peptide sequences obtained from purified *M. albomyces* laccase (26) are overlined. The possible N-glycosylation sites are indicated by boldface type, and the shaded amino acids indicate the locations of introns in the DNA sequence.

activity assay in which ABTS was the substrate. In the case of pLLK10, very low laccase activity was detected after 2 days of growth in the galactose medium. Detection of this activity required overnight incubation of the culture supernatant with ABTS. On the other hand, the activity assay was clearly disturbed by some agent present in the yeast culture broth that reduced the color of ABTS at the beginning of the activity measurement. This agent was produced by yeast cells in all the cultures, including controls, since the color of ABTS was not removed by fresh culture medium. Thus, with low laccase activities, the activity assay as such did not give reliable values for production. We therefore attempted to obtain rough estimates

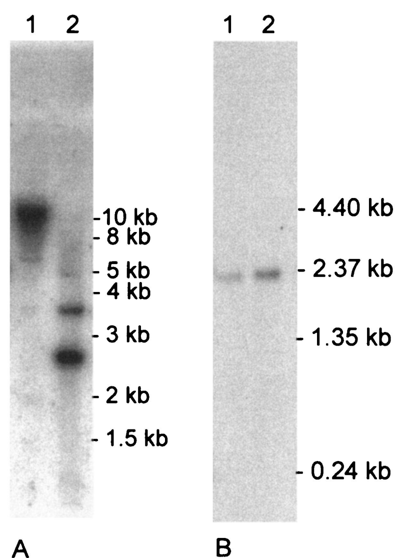

FIG. 2. (A) Southern blot analysis of *M. albomyces* genomic DNA. A 20-μg portion of total DNA was digested with *Hin*dIII (lane 1) and with *Pvu*II (lane 2). *M. albomyces lac1* cDNA was used as a probe. (B) Northern blot analysis of *M. albomyces* shake flask (lane 1) and fermentor (lane 2) cultures. Five micrograms of total RNA was hybridized with a portion of the *M. albomyces lac1* gene.

for laccase production from pLLK10 by adding known amounts of purified *M. albomyces* laccase to the activity assay mixture together with the culture supernatant of the control strain. The absorbance changes observed were compared to those of the culture supernatants of the pLLK10 strain. The estimated level of production was around 2 pkat/ml. The higher laccase activities produced by the pMS174 and pMS175 strains could override the effect of the reducing agent.

Expression of the *M. albomyces* laccase from pMS174, in which the mature laccase was fused with the yeast α-factor signal sequence and propeptide, resulted in an approximately 200-fold improvement in production compared to the production with laccase cDNA with its own signal and propeptide sequences in the same vector. The maximum laccase activity in pMS174 cultures was 0.45 nkat/ml. When the C terminus of the laccase was removed from the expression construct (i.e., pMS175), laccase production was further improved about sixfold to the maximum activity, 2.8 nkat/ml. The time course of laccase production by yeast transformants is shown in Fig. 4. Laccase activity for pMS175 strains increased until day 3, whereas for pMS174 the activity was nearly maximal on the first day after induction. Continuing the cultivations for more than 3 days did not markedly increase the laccase activity (data not shown). Figure 4 also shows the growth of yeast transformants and the control strain carrying the empty expression plasmid pYES2 after induction of laccase expression. The control strain carrying pYES2 grew slightly faster than the laccase-producing strains, indicating that laccase production resulted in stress in the cells.

The laccase produced by *S. cerevisiae* was studied by Western blotting of samples from the culture supernatants and yeast cell lysates with polyclonal antibodies (Fig. 5). No laccase bands were detected in samples from the vector control or transformants carrying pLLK10. A major band at about 95 kDa was observed for the supernatants of transformants carrying pMS174 or pMS175, whereas the native laccase had a molecular mass of about 80 kDa. In addition to the 95-kDa band, there was a smear of larger proteins, which indicates that there was heterogeneous overglycosylation by *S. cerevisiae*. Two smaller bands which indicated that there was proteolytic degradation were also detected. In the cell lysates of pMS174 and pMS175 strains, the band at 95 kDa, the smear of larger proteins, and a large number of degradation products were detected. The laccase with the C-terminal extension from pMS174 appeared to be more degraded by *S. cerevisiae* than the product of the truncated laccase gene (pMS175). The supernatant and cell lysate samples used for Western blotting corresponded to the same culture volume. As a major part of the laccase was detected inside the cells, secretion of this enzyme appeared to be one of the steps limiting production.

DISCUSSION

A novel laccase with extremely interesting technical properties was recently isolated from the fungus *M. albomyces* (26). This laccase showed good thermostability, retaining full activity for 2 h at 60°C, as well as high pH optima at a neutral pH, both of which are unusual properties for most known fungal laccases. In this paper, we describe isolation and heterologous expression of the laccase gene. The amino acid sequence of the laccase was shown to exhibit high levels of homology with the sequences of laccases from other ascomycetes, such as *M. thermophila*, *N. crassa*, and *P. anserina*, and, as expected, low levels of identity with the more widely studied basidiomycete laccases. This finding further strengthens the hypothesis that the fungal laccases can be separated into two divergent classes, ascomycete laccases and basidiomycete laccases (14, 5). The laccase of *A. nidulans* differs remarkably from all the other fungal laccases, presumably reflecting a functionally different role of the laccase in the formation of conidiophores (2).

pLLK7:	*PGK1* promoter	Lacc. ss & propeptide	Laccase cDNA
pLLK10:	*GAL1* promoter	Lacc. ss & propeptide	Laccase cDNA
pMS174:	*GAL1* promoter	α-factor ss & propeptide	Laccase cDNA
pMS175:	*GAL1* promoter	α-factor ss & propeptide	Lacc. cDNA without the 3'-end extension

FIG. 3. Laccase gene expression cassettes created in this study. ss, signal sequence; Lacc., laccase.

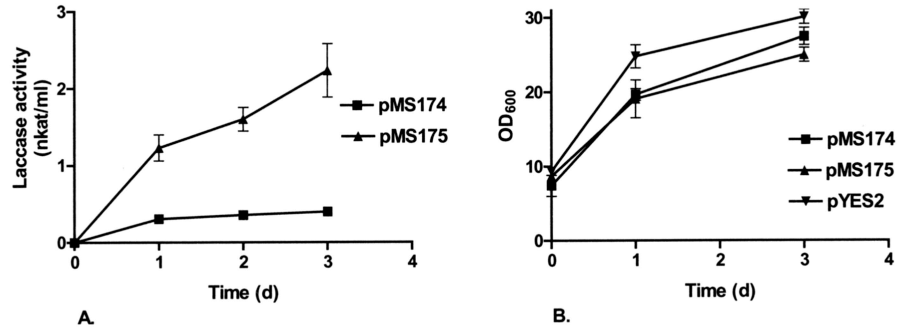

FIG. 4. (A) Extracellular laccase production by yeast transformants carrying expression plasmid pMS174 or pMS175. (B) Growth of yeast transformants producing laccase (pMS174 and pMS175) and the control strain (pYES2). OD_{600}, optical density at 600 nm.

The deduced *M. albomyces* laccase contains nine potential N-linked glycolysation sites (Asn-X-Thr/Ser) (Fig. 1). This protein is, in fact, highly glycosylated, since analysis of the crystal structure of the protein showed that altogether there are seven glycosylated sites (21). In addition to glycosylation, formation of mature *M. albomyces* laccase requires posttranslational removal of a signal sequence, propeptide cleavage at the N terminus, and removal of a C-terminal extension. A C-terminal truncation was also seen in the crystal structure of MaL as no electron density was observed for the last 14 predicted C-terminal residues (21). This finding was also confirmed in this study by C-terminal sequencing of the purified laccase. Similar C-terminal processing has also been proposed for laccases from the ascomycetes *N. crassa* (17), *P. anserina* (14), and *M. thermophila* (8), and the processing site Asp-Ser-Gly-Leu is conserved in these laccases. Interestingly, the same kind of sequence is found at the C-terminal ends of some other ascomycete laccases with no C-terminal extensions. In the laccases of *Cryphonectria parasitica* (11), *Botrytis cinerea* (9), *Glomerella lagenarium* (44), and *Gaeumannomyces graminis* var. *tritici* (30) the last four C-terminal amino acids are Asp, Ser, Gly, and Leu/Ile/Val. We believe that this C-terminal end is conserved because it is related to the catalytic activity of ascomycete laccases. In the crystal structure of *M. albomyces* laccase, the last four amino acids of the protein were shown to pack tightly inside the protein. In addition, the C-terminal carboxylate group is hydrogen bonded to the side chain of His-140, which is also bound to type 2 copper in the active center (21).

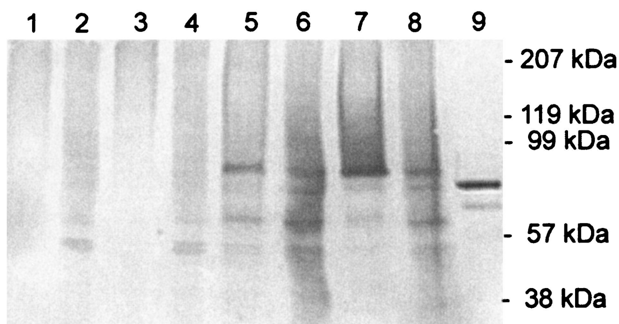

FIG. 5. Western blot analysis of culture supernatants (lanes 1, 3, 5, and 7) and cell lysates (lanes 2, 4, 6, and 8) of *S. cerevisiae* transformants after 3 days of induction with galactose. *S. cerevisiae* was transformed with pYES2 (lanes 1 and 2), pLLK10 (lanes 3 and 4), pMS174 (lanes 5 and 6), and pMS175 (lanes 7 and 8). Fifteen microliters of the supernatant and a corresponding amount of cell lysate were loaded on the gel. Lane 9 contained 30 ng of native *M. albomyces* laccase.

The maximal level of production of *M. albomyces* laccase in *S. cerevisiae* was 2.8 nkat/ml. If the enzyme produced by yeast had the same specific activity as the enzyme produced by *M. albomyces*, yeast cells would produce about 3 mg of laccase per liter. Previously, substantial laccase activities in *S. cerevisiae* have been detected with the laccase of *Coriolus hirsutus* (27) or *M. thermophila* (8). For *C. hirsutus* laccase no data on how the activity was measured are available. For *M. thermophila* laccase, the initial level of production obtained with the laccase cDNA alone under a galactose-inducible promoter was low, around 0.6 U/liter, corresponding to 0.01 nkat/ml. Production was improved by directed evolution of the *M. thermophila* laccase gene. The study of Bulter et al. (8) and our work are the first examples of ascomycete laccase expression in *S. cerevisiae*. It is possible that laccase production in *S. cerevisiae* is easier with laccases from the ascomycetous fungi, which are phylogenetically more closely related to the ascomycete *S. cerevisiae*, than with laccases from basidiomycetes.

The very low laccase activity levels obtained with a construct having the intact native laccase cDNA were significantly improved by replacing the native laccase signal and propeptide sequences with the *S. cerevisiae* α-factor secretion signal sequence and propeptide. The prepro sequence of the *MFα1* gene of *S. cerevisiae* has been used as a secretion signal in different yeast expression systems (45, 24, 23, 34, 7), but to our knowledge, this is the first time that it has been used in *S. cerevisiae* expressing a laccase. Use of the α-factor secretion signal and propeptide for laccase expression has previously been reported only in *P. pastoris*, and studies with *T. versicolor lcc1* (24), *P. cinnabarinus lac1* (34) and *T. versicolor lccIV* (7) showed no improvement in laccase production with the α-factor secretion signal and propeptide. Signal sequences are generally interchangeable between eukaryotic species, and therefore it is suspected that the propeptide cleavage step of *M. albomyces* laccase is the problematic step for production of this enzyme in yeast. In fact, the propeptide of the laccase is cleaved after Pro-Arg, but the KEX2 protease of *S. cerevisiae* has been shown to cleave the propeptides specifically after two basic amino acids (15).

Based on the production kinetics and the Western analysis of the yeast strains producing *M. albomyces* laccase, the factors

limiting production appear to include inefficient secretion and proteolytic degradation. Analysis of cell lysate samples by Western blotting showed that there were abundant multiple degradation products, and the most abundant products were also detected outside the cells. These data also suggested that the C-terminal extension makes the protein more susceptible to degradation.

The role of the C-terminal extension in heterologous laccase production was studied by producing *M. albomyces* laccase in yeast in two different forms, with and without the extension. The activity levels were about sixfold higher with the 3′-end-truncated laccase gene. This suggests that *S. cerevisiae* is not able to process the C terminus correctly or that the cleavage activity is limiting. Our results are in contrast to the results of Bulter et al., who observed a significant decrease in laccase activity with the truncated laccase mutant gene of *M. thermophila* (8). However, it is worth noting that the *M. thermophila* laccase gene had already gone through a series of mutations at the time that the C-terminal point mutation was made, and these mutations may have affected the activity levels observed. Germann et al. (17) and Bulter et al. (8) have suggested that the C-terminal extension might be essential for production by inactivating the laccase during early posttranslational processing steps. Our data, however, do not support this theory.

In this work, we isolated the gene encoding a novel ascomycete laccase. The recent determination of the crystal structure of this enzyme (21) makes this laccase very interesting for studies of the basic mechanisms of oxidative enzymes. The crystal structure revealed some very interesting novel features, especially concerning the C-terminal end of the mature protein. This study showed that the C-terminal extension is also of special interest with respect to production of this enzyme. The expression of *M. albomyces* laccase in yeast established in this work, together with the structure, should facilitate our future studies of the mechanism of action and improvement of this enzyme.

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Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme

Laura-Leena Kiiskinen,† Kristiina Kruus, Michael Bailey, Erkko Ylösmäki, Matti Siika-aho and Markku Saloheimo

VTT Biotechnology, PO Box 1500, Fin-02044 VTT, Finland

Correspondence
Markku Saloheimo
Markku.Saloheimo@vtt.fi

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Previous studies on *Melanocarpus albomyces* laccase have shown that this enzyme is very interesting for both basic research purposes and industrial applications. In order to obtain a reliable and efficient source for this laccase, it was produced in the filamentous fungus *Trichoderma reesei*. Two approaches were used: production of a non-fused laccase and a hydrophobin–laccase fusion protein. Both proteins were expressed in *T. reesei* under the *cbh1* promoter, and significantly higher activities were obtained with the non-fused laccase in shake-flask cultures (corresponding to about 230 mg l^{-1}). Northern blot analyses showed rather similar mRNA levels from both expression constructs. Western analysis indicated intracellular accumulation and degradation of the hydrophobin–laccase fusion protein, showing that production of the fusion was limited at the post-transcriptional level. No induction of the unfolded protein response pathway by laccase production was detected in the transformants by Northern hybridization. The most promising transformant was grown in a fermenter in batch and fed-batch modes. The highest production level obtained in the fed-batch culture was 920 mg l^{-1}. The recombinant laccase was purified from the culture supernatant after cleaving the major contaminating protein, cellobiohydrolase I, by papain. The recombinant and wild-type laccases were compared with regard to substrate kinetics, molecular mass, pH optimum, thermostability, and processing of the N- and C-termini, and they showed very similar properties.

INTRODUCTION

Laccases (EC 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases. They catalyse oxidation of a wide variety of organic and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines and aromatic amines. One electron at a time is removed from the substrate by a type-1 blue copper ion and transferred to a trinuclear copper cluster (Messerschmidt, 1997). Molecular oxygen is used as the electron acceptor. The substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalysed oxidation or non-enzymic reactions including hydration, disproportionation and polymerization (Thurston, 1994). Laccases are common enzymes in nature, especially in plants and fungi (Gianfreda *et al.*, 1999). Recently, some novel bacterial laccases have also been reported (Martins *et al.*, 2002; Arias *et al.*, 2003). The laccases most studied hitherto are of fungal origin, especially from the class of white-rot fungi. Several physiological roles have been proposed for fungal laccases, such as participation in plant pathogenesis, pigment production and degradation of lignocellulosic materials (Thurston, 1994; Gianfreda *et al.*, 1999).

Laccases are currently seen as highly interesting industrial enzymes because of their wide variety of potential substrates. Proposed applications for laccases include textile dye bleaching, pulp bleaching, effluent detoxification, biosensors and bioremediation (Gianfreda *et al.*, 1999; Xu, 1999). However, a serious problem often encountered with industrial exploitation of laccases is the low production level by the native hosts. This problem may be overcome by heterologous production of laccases in fungal hosts that are capable of producing high amounts of extracellular enzymes, generally *Trichoderma reesei* or *Aspergillus* spp. Expression of *Phlebia radiata* laccase has previously been reported in *T. reesei* (Saloheimo & Niku-Paavola, 1991), whereas laccases from *Trametes villosa* (Yaver *et al.*, 1996), *Myceliophthora thermophila* (Berka *et al.*, 1997) and *Coprinus cinereus* (Yaver *et al.*, 1999) have been expressed in *Aspergillus oryzae.*

†Present address: Finnzymes Oy, Keilaranta 16 A, Fin-02150 Espoo, Finland.

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate); CBHI, cellobiohydrolase I; 2,6-DMP, 2,6-dimethoxyphenol; ER, endoplastic reticulum; HFBI, hydrophobin I; UPR, unfolded protein response.

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Recently, expression in *Aspergillus niger* has also been reported for laccases from *Pycnoporus cinnabarinus* (Record *et al.*, 2002) and *Ceriporiopsis subvermispora* (Larrondo *et al.*, 2003). The yields of fungal laccase production have generally been in tens of milligrams per litre.

We have previously described a novel laccase from the thermophilic fungus *Melanocarpus albomyces* (Kiiskinen *et al.*, 2002). This laccase was shown to have very interesting properties relating to potential industrial applications of laccases as well as to studies on structure–function relationships. Compared to many other laccases studied, *M. albomyces* laccase is more thermostable and has a higher pH optimum, both of which are useful properties for many applications (Kiiskinen *et al.*, 2002). The three-dimensional structure of *M. albomyces* laccase has been solved as one of the first complete laccase structures (Hakulinen *et al.*, 2002). The crystal structure revealed novel properties of *M. albomyces* laccase concerning molecular oxygen binding to the active site and the C-terminus entering inside the enzyme. Heterologous expression in *Saccharomyces cerevisiae* was also recently reported for this laccase, and the results showed that the C-terminal end of the protein is of special interest also with respect to production of this enzyme (Kiiskinen & Saloheimo, 2004). Because these results further emphasized the potential of this laccase for research and industrial purposes, we have now expressed the laccase gene in *T. reesei*, a filamentous fungus that is well known for its ability to produce high amounts of extracellular enzymes (Mäntylä *et al.*, 1998).

METHODS

Microbial strains and enzymes used for cloning. *Escherichia coli* strains used for vector propagation were DH5α from Gibco-BRL and TOP10F′ from Invitrogen. *T. reesei* RutC-30 (Montenecourt & Eveleigh, 1979) was used as a host for laccase production. Enzymes used to manipulate DNA or RNA were obtained from New England Biolabs or Boehringer Mannheim.

Vector construction. The *T. reesei* expression vector pAMH110 (Saloheimo *et al.*, 1989) was digested with *Ksp*I and *Nde*I to remove a spacer fragment between the *cbh1* promoter and terminator sequences. *M. albomyces lac1* cDNA was released from the plasmid pLLK4 (Kiiskinen & Saloheimo, 2004) by *Sac*I and *Eco*RI digestion and ligated into pAMH110 by blunt-end ligation, to obtain the plasmid pLLK8. The expression cassette for hygromycin resistance consisting of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator and the *E. coli* hygromycin resistance gene was taken from the plasmid pBluekan7-1.NotI (from P. J. Punt, TNO Nutrition and Food Research, the Netherlands) by *Not*I digestion. It was cloned into the *Eco*RI site of pLLK8 by blunt-end ligation to obtain the final expression plasmid pLLK13.

In order to construct a vector for production of a fusion protein between the *T. reesei* hydrophobin I (HFBI) and *M. albomyces* laccase, the cDNA encoding mature laccase was amplified by PCR with a programme of 26 cycles of 94 °C for 45 s; 57 °C for 30 s; 72 °C for 2 min. The PCR primers were 5′-ACGTACGGTACCGAGCCGAC-GTGCAACACGCC-3′(forward) and 5′-ACGTACGGTACCTCAAC-GAACCAGCCACTCGC-3′ (reverse). *Asp*718 sites incorporated into the primers are underlined. The PCR product was cloned into pCR2.1-TOPO-vector (TOPO TA Cloning Kit, Invitrogen) and sequenced. As the cDNA itself contained an *Asp*718 site, the cDNA was released from the vector by partial digestion with *Asp*718 and ligated into the *Asp*718 site of the plasmid pTNS29. pTNS29 is a pUC19-based expression vector containing the *T. reesei cbh1* promoter and a genomic copy of the *hfb1* open reading frame (Nakari-Setälä *et al.*, 1996), followed by an artificial linker and the *cbh1* terminator sequence. The *Asp*718 site of pTNS29 is located after the linker sequence. The final HFBI–laccase expression construct was pLLK12.

Transformation of *T. reesei* and screening of the transformants. *T. reesei* RutC-30 was transformed with linearized expression vector pLLK13 essentially as described by Penttilä *et al.* (1987). As pLLK12 did not contain the hygromycin resistance cassette, it was digested with *Sph*I and *Stu*I to release the expression cassette, and the cassette was cotransformed with pBluekan7-1.NotI carrying a hygromycin resistance cassette. The transformants were plated on minimal medium (Penttilä *et al.*, 1987) containing 20 g glucose l^{-1} and 125 mg hygromycin l^{-1}. Well-growing transformants were purified to uninuclear clones by plating single spores on selective medium. To test for laccase production on plates, the transformants were grown on plates with minimal medium containing 20 g lactose l^{-1} and 125 mg hygromycin l^{-1} for 5 days. Two millilitres of 15 mM ABTS (Roche Diagnostics) in 25 mM succinate buffer (pH 4·5) was pipetted onto the plates and the formation of green colour around fungal colonies was monitored visually for 2 h. Selected laccase-positive transformants were cultivated in shake-flasks in minimal medium (Penttilä *et al.*, 1987) supplemented with 40 g lactose l^{-1}, 20 g grain-based carbon and nitrogen source l^{-1} (Suominen *et al.*, 1993), 0·1 mM $CuSO_4$, and 10 g potassium hydrogen phthalate l^{-1} for buffering at pH 6. For monitoring the growth properties of the fungi, selected transformants were also grown in a soluble medium where the grain-based carbon and nitrogen source was replaced by 2 g peptone l^{-1}. All the cultivations were performed at 28 °C and 200 r.p.m. The effect of copper concentration on laccase production was studied by cultivating selected transformants in the minimal medium with 0·1, 0·5, 1, 2 or 3 mM $CuSO_4$.

Western, Northern and Southern blot analyses. The recombinant laccase was studied by Western blotting with polyclonal antibodies raised in rabbits against the native *M. albomyces* laccase. Samples from culture supernatants and cell lysates of shake-flask cultures grown in the medium with lactose and grain-based nitrogen and carbon source were analysed. After 7 days of growth, the supernatant samples and cells were collected. The cells were washed twice with 20 mM sodium succinate buffer (pH 4·8), ground under liquid nitrogen and suspended into the same buffer with protease inhibitors (Complete Mini protease inhibitor cocktail, Roche). Samples were separated by SDS-PAGE (Mighty Small II SE250, Hoefer Pharmacia Biotech) and proteins were electroblotted onto nitrocellulose filters (Schleicher & Schuell). *M. albomyces* laccase was detected using alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad). The supernatant and cell lysate samples analysed corresponded to the same culture volume.

Total *T. reesei* RNA was extracted with the TRIzol reagent (Life Technologies). The RNA was treated with glyoxal, run in a 1 % agarose gel (Sambrook *et al.*, 1989), transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) and hydridized according to the manufacturer's instructions. The filters were probed with *M. albomyces lac1* cDNA and with the *pdi1* gene encoding protein disulphide isomerase (Saloheimo *et al.*, 1999), the *bip1* encoding the major endoplasmic reticulum (ER) chaperone (Pakula *et al.*, 2003) and the *hac1* gene encoding the transcription factor of the unfolded protein response (Saloheimo *et al.*, 2003). Signal intensities were normalized with respect to the signals of the *gpd1* gene encoding glyceraldehyde-3-phosphate dehydrogenase. The Northern signals were quantified with a Typhoon 8600 phospho/fluoroimager (Molecular Dynamics).

The copy number of the expression construct in positive *T. reesei* transformants was studied by Southern hybridization. *T. reesei* genomic DNA was extracted with Easy DNA Kit (Invitrogen) and hybridized with radiolabelled *M. albomyces lac1* cDNA in stringent conditions (Sambrook *et al.*, 1989).

Fermenter cultivations. The transformant pLLK13/295 producing the highest level of laccase in shake-flasks was cultivated in a Braun Biostat C-DCU 3 fermenter (B. Braun Biotech) in 20 litres of a medium containing (g l^{-1}): lactose 40, peptone 4·0, yeast extract 1·0, KH_2PO_4 4·0, $(NH_4)_2SO_4$ 2·8, $MgSO_4.7H_2O$ 0·6, $CaCl_2.2H_2O$ 0·8, $CuSO_4.5H_2O$ 0·025, and 2·0 ml 2× trace elements solution l^{-1} (Mandels & Weber, 1969). pH was adjusted to 5·5–6 with NH_4OH and H_3PO_4, and the cultivation temperature was 28 °C. Dissolved oxygen level was kept above 30 % with agitation at 400–500 r.p.m., aeration at 2–10 litres min^{-1} and 0–20 % O_2-enrichment of incoming air. Foaming was controlled by automatic addition of Struktol J633 polyoleate antifoam agent (Schill & Seilacher). Samples were taken daily to measure dry weight, lactose and total protein concentration, laccase activity and β-1,4-endoglucanase activity. Activity of the major cellulase cellobiohydrolase I (CBHI) was also measured (Bailey & Tähtiharju, 2003). In the fed-batch fermentation, lactose was fed into the fermenter according to the algorithm described by Bailey & Tähtiharju (2003).

Purification of recombinant *M. albomyces* laccase. The culture supernatant from the batch fermentation was first clarified by adding 10 g bentonite l^{-1} (Steetley Bentonite and Absorbents). The solution was mixed at 4 °C for 1 h and centrifuged at 2300 ***g*** for 10 min. To enable the separation of recombinant *M. albomyces* laccase from the major extracellular protein CBHI, the clear culture supernatant was treated with papain (from papaya latex, Sigma). The ratio of papain concentration to CBHI concentration was 15 : 100. The reaction was carried out in 100 mM acetate buffer (pH 5·0) containing 10 mM cysteine and 2 mM EDTA at 37 °C for 2 h. After papain digestion, the solution was loaded on a Phenyl-Sepharose Fast Flow column (5 × 17 cm; Pharmacia) equilibrated with 600 mM Na_2SO_4 in 5 mM citrate buffer (pH 5·0). Proteins were eluted with a linear 600–0 mM Na_2SO_4 gradient within four column volumes. Laccase-containing fractions were pooled and the buffer was changed to 20 mM sodium acetate (pH 5) by gel filtration through a Sephadex G-25 Coarse column (5 × 17 cm; Pharmacia). Laccase was further purified by anion-exchange chromatography with a DEAE-Sepharose Fast Flow column (5 × 23 cm; Pharmacia) equilibrated with 20 mM sodium acetate buffer (pH 5). Proteins were eluted with a linear 0–400 mM Na_2SO_4 gradient within 2·5 column volumes. Active fractions were pooled, concentrated on an Amicon PM10 membrane (Millipore) and applied to a Sephacryl S-100 HR gel filtration column (5 × 82 cm; Pharmacia) equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 150 mM NaCl. Laccase-positive fractions were concentrated and the buffer was exchanged to 50 mM Tris/HCl (pH 7) with disposable desalting columns (PD-10; Amersham).

Enzyme activity and protein concentration measurements. Laccase activity was calculated by measuring the oxidation of 5 mM ABTS in 25 mM succinate buffer (pH 4·5) at 436 nm and using an absorption coefficient (ε) of 29 300 M^{-1} cm^{-1} (Niku-Paavola *et al.*, 1988). The kinetic parameters for purified laccase were also measured with syringaldazine (525 nm; ε 65 000 M^{-1} cm^{-1}; Leonowicz & 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 40 mM MES/NaOH buffer (pH 6). The Michaelis–Menten curves for determining K_m and V_{max} were obtained with the GraphPad Prism 3.02 program (GraphPad Software).

β-1,4-Endoglucanase activity was measured with hydroxyethyl cellulose as substrate at pH 5 using a standard method (IUPAC, 1987), and CBHI activity was measured according to Bailey & Tähtiharju (2003). Biomass dry weight was measured gravimetrically. Lactose concentration was measured enzymically with an assay kit (Boehringer Mannheim). Protein concentrations were determined by the Lowry method after precipitation of proteins with an equal volume of 10 % trichloroacetic acid. Total protein concentration during purification of laccase was determined with the Bio-Rad DC Protein Assay kit. Bovine serum albumin was used as a standard in both methods. Purification was monitored with SDS-PAGE (12 % Tris/HCl Ready Gel, Bio-Rad), in which protein bands were visualized by staining with Coomassie brilliant blue (R 350; Pharmacia).

Molecular mass, pH optimum, thermostability and redox potential of recombinant laccase. The molecular masses of native and recombinant *M. albomyces* laccases were determined by MALDI-TOF mass spectrometry on a Ultraflex time-of-flight instrument (BrukerDaltonics) as previously described (Palonen *et al.*, 2003).

The pH optimum of recombinant *M. albomyces* laccase was determined in the universal McIlvaine buffer (Dawson *et al.*, 1959) within a pH range of 2·2–8·0, using guaiacol as substrate. The thermal stability of recombinant laccase was determined by incubating the enzyme solution (0·3 g l^{-1}) in 85 mM citrate buffer (pH 6) at 40, 50 and 60 °C. The residual enzyme activities were measured at room temperature with ABTS or 2,6-DMP as substrates. Isoelectric focusing was performed on an LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia) as previously described (Kiiskinen *et al.*, 2002). The redox potentials of the T1 coppers of native and recombinant *M. albomyces* 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 $K_3Fe(CN)_6/K_4Fe(CN)_6$.

RESULTS

Expression of *M. albomyces* laccase cDNA in *T. reesei*

Two expression plasmids for laccase production in *T. reesei* were constructed. In pLLK13 the full-length laccase cDNA was alone between the *cbh1* promoter and terminator, whereas in pLLK12 the region encoding the mature laccase was fused with the *T. reesei* hydrophobin gene *hfb1*. pLLK12 was constructed for expression from the *cbh1* promoter of an HFBI–laccase fusion protein with HFBI at the N-terminus. This was done for two reasons. Firstly, it has been shown that fusion of a foreign protein with a secreted native protein can enhance its production (e.g. Nyyssönen *et al.*, 1993), and secondly, HFBI as a fusion partner can facilitate the purification of recombinant proteins in aqueous two-phase purification (Linder *et al.*, 2001; Selber *et al.*, 2001).

T. reesei RutC-30 was transformed with the two *M. albomyces* laccase expression vectors and transformants were selected on plates for hygromycin resistance and purified to uninuclear clones through a single-spore culture. About 40 transformants that grew well on hygromycin plates were selected for laccase production from both transformations. Laccase expression was studied by applying ABTS solution on fungal streaks grown on plates containing selective medium with lactose as the sole carbon source, and laccase activity was observed as the appearance of green colour

around the streaks. Thirty laccase-positive clones were found among pLLK13 transformants and 33 positive clones for pLLK12. The time of green colour appearance ranged from about 2 min to several hours and the best transformants from both constructs gave approximately similar results.

Several laccase-positive transformants from both expression constructs were grown in shake-flask cultures, and the laccase activities were measured in the culture supernatants. This activity typically peaked on the eighth day of culture. The non-fused laccase from pLLK13 was produced in significantly higher amounts than the HFBI–laccase fusion protein The two best transformants from the non-fusion construct pLLK13 produced 193 (transformant 295) and 160 nkat ml^{-1} (transformant 149) and the two best transformants from the fusion construct pLLK12 produced 42 (transformant 89) and 26 nkat ml^{-1} (transformant 22) of laccase. As estimated from the specific activity of the purified laccase produced in *T. reesei*, the laccase level produced by pLLK13/295 corresponds to about 230 mg l^{-1}.

The effect of copper concentration on laccase production by *T. reesei* was tested in shake-flask cultivations. The results showed that addition of Cu^{2+} to the *T. reesei* minimal medium was beneficial. Addition of 0·1 mM copper to the medium improved the production levels about fourfold (data not shown). Increasing the concentration of copper up to 3 mM did not improve production levels any further. The laccase yields shown in this paper were obtained in media supplemented with 0·1 mM $CuSO_4$.

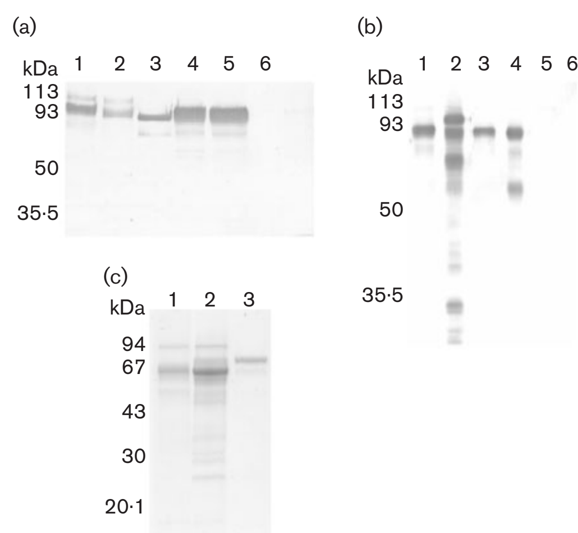

Fig. 1. SDS-PAGE and Western blot analyses of extra- and intracellular laccase production in *T. reesei*. (a) Western blot analysis of culture supernatants of HFBI–laccase fusion strains (lanes 1 and 2) and transformants producing non-fused laccase (lanes 4 and 5). Lane 3 contains 0·2 µg native *M. albomyces* laccase. Lane 6 is the control strain *T. 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 and 2), non-fusion laccase strains (lanes 3 and 4), and the control strain (lanes 5 and 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 transformant pLLK13/295 (lane 2), and purified recombinant *M. albomyces* laccase (lane 3).

Characterization of laccase-producing transformants

The *T. reesei* transformants producing *M. albomyces* laccase were characterized by SDS-PAGE and Western blot analysis with laccase antiserum. The culture supernatants of transformants producing non-fused laccase showed a somewhat heterogeneous laccase band with slightly higher molecular mass than that of native *M. albomyces* laccase (Fig. 1a, lanes 4 and 5). However, mass spectrometric analysis of the purified recombinant laccase discussed below showed that the recombinant laccase was essentially homogeneous and had a molecular mass very close to that of the native laccase. The different mobility of the recombinant laccase in SDS-gels could be explained by the high content of other proteins in the *T. reesei* supernatant (see Fig. 1c). Western blotting from culture supernatants of the HFBI–laccase fusion construct transformants showed a minor band of the expected fusion protein size (Fig. 1a, lanes 1 and 2). Most of the HFBI–laccase fusion protein was probably cleaved by a protease between the fusion partners, as the antiserum detected a major laccase band similar in size to that produced by the non-fusion construct transformants. In some culture supernatant samples the fusion protein band was not detected at all, indicating total cleavage of the protein (Fig. 1b, lane 1). Analysis of the culture supernatant samples in a Coomassie-stained SDS-gel showed that the recombinant laccase was one of the major secreted proteins in the transformants (Fig. 1c).

Cell lysate samples of the laccase-producing transformants were also studied by Western blotting. The efficiency of laccase secretion from the *T. reesei* cells was investigated by analysing supernatant and cell lysate samples corresponding to the same culture volume. For both expression constructs, a major proportion of the recombinant protein was detected inside the cell, but for the fusion protein the intracellular proportion was much higher (Fig. 1b, lanes 2 and 4), suggesting less efficient secretion of this protein. Both the HFBI–laccase fusion protein and several cleavage products were detected in the intracellular sample of the fusion construct pLLK12 transformant. This indicates that the fusion protein is more susceptible to intracellular protease attack in general than the non-fused laccase.

The laccase gene copy numbers of the five best-producing transformants from both expression constructs were studied by Southern hybridization. All the analysed transformants from the non-fusion construct appeared to have a single

copy of the expression plasmid, whereas all the transformants derived from the fusion construct had two or three copies (data not shown).

Northern analysis was performed from the parental strain and the two best-producing transformants from both constructs. These strains were grown in shake-flasks in a medium inducing high laccase production, and mycelium samples for Northern analysis were taken on the fourth, fifth and seventh day of growth. The Northerns were probed with *M. albomyces* laccase cDNA to determine the expression level of the transformed constructs. In addition, possible activation of the unfolded protein response (UPR) pathway by laccase expression was studied by probing the Northerns with two target genes of this signalling pathway: *pdi1* encoding protein disulphide isomerase (Saloheimo *et al.*, 1999) and *bip1* encoding the major ER chaperone (Pakula *et al.*, 2003). The signal intensities quantified for these genes were normalized with respect to signal intensities of the *gpd1* gene encoding glyceraldehyde-3-phosphate dehydrogenase. In addition, probing with the *hac1* gene encoding the UPR transcription factor was performed. It has been shown that under UPR-inducing conditions a truncated *hac1* mRNA is found in the cells (Saloheimo *et al.*, 2003).

The laccase gene probing showed mRNAs of the expected size, derived from the two expression constructs (Fig. 2a). Quantification of the signals showed that the mRNA levels obtained from the fusion construct were at the same level as those derived from the non-fusion construct, or even at a higher level (Fig. 2a). The *pdi1* and *bip1* mRNA levels did not show major differences between the laccase-producing strains and the control. On the fourth culture day they were similar or lower in the transformants compared with the parental strain and at the other time points they were slightly higher (Fig. 2a). The only exception was the *bip1* transcript level of the transformant pLLK12/89 on the fifth day (about twofold difference compared to the control). The truncated form of *hac1* mRNA was not found in any of the samples analysed. Taken together, these results show that the UPR pathway is not induced by *M. albomyces* laccase production in *T. reesei.*

To analyse whether the growth of *T. reesei* was affected by expression of the laccase gene, a shake-flask cultivation of the pLLK12 and pLLK13 transformants and the parental strain was carried out in a soluble medium and mycelial dry weight was measured. Growth of the non-fusion construct pLLK13 transformants was clearly retarded compared with the control (Fig. 2b). Growth of the HFBI–laccase fusion transformants appeared to be only slightly or not at all affected. *M. albomyces* laccase is susceptible to low pH (Kiiskinen *et al.*, 2002) and thus the shake-flask cultures for which the growth curves are shown were buffered to pH 6·0. This is not optimal for the growth of *T. reesei.* In other cultures in which the medium was buffered to pH 5·5, the laccase transformants grew equally well as the parental strain (data not shown).

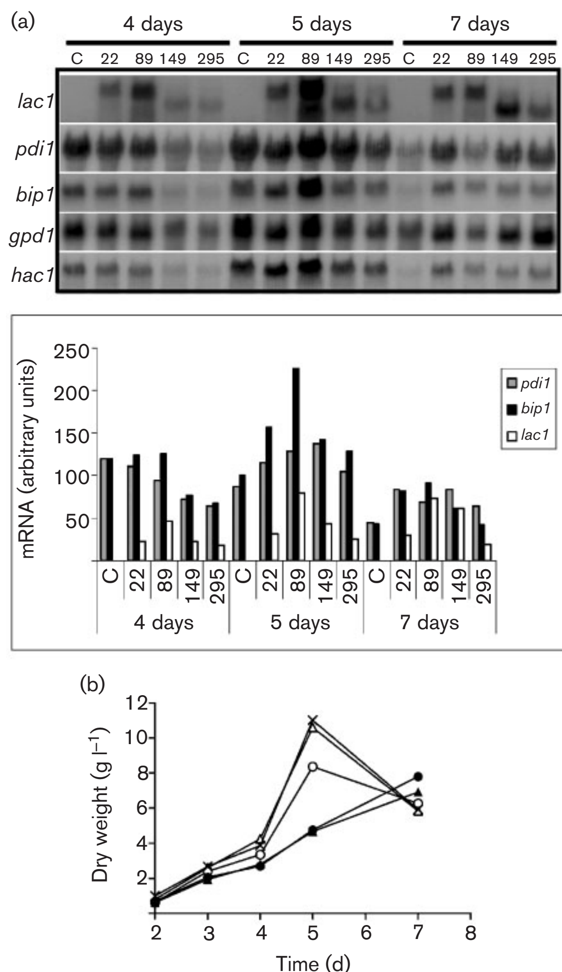

Fig. 2. Comparison of *T. reesei* transformants producing HFBI–laccase fusion protein or non-fused laccase. (a) Northern analysis of the control strain and two best-producing transformants from the HFBI–laccase fusion construct (transformants 22 and 89) and from the non-fusion construct (149 and 295) was performed by probing the Northerns with *lac1* and the UPR-related probes *pdi1*, *bip1* and *hac1* after 4, 5 and 7 days of cultivation. The signal intensities were quantified and normalized to *gpd1* signal. (b) Growth curves for *T. reesei* transformants 22 (○) and 89 (△) producing HFBI–laccase fusion protein, 295 (●) and 149 (▲) producing non-fused *M. albomyces* laccase, and the control strain (×).

Laccase production and purification

The transformant pLLK13/295 that produced the highest laccase activity in shake-flasks was cultivated in a 20 litre fermenter. The batch fermentation was carried out in a medium with lactose as the carbon source and 0·1 mM added $CuSO_4$ to support incorporation of copper into the laccase. The pH of the medium was adjusted to 5·5–6 because *M. albomyces* laccase has been shown to be unstable at low pH (Kiiskinen *et al.*, 2002). Laccase production was detectable after about 1 day of cultivation, and it continued

up to the end of the fermentation. The highest activity, 250 nkat ml^{-1}, was obtained at the time when the fungus was already autolysing, as indicated by the decrease in dry weight (Fig. 3). At this point, the fermentation had to be ended due to increasing difficulties in foam control. The production curves for cellulases were typical for this kind of batch fermentation: the most efficient cellulase production occurred at the time when exhaustion of lactose started to limit fungal growth (Bailey & Tähtiharju, 2003).

The recently published medium-feeding method for continuous cellulase production with *T. reesei* (Bailey & Tähtiharju, 2003) was tested with the laccase-producing transformant. In a fed-batch fermentation, lactose was added into the fermenter according to an algorithm that calculates the decrease in the rate of base addition for pH control. It has been shown that the production of cellulases is highest at the time when the rate of base addition required for pH control starts to decrease, i.e. when the growth rate of the fungus is decreasing (Bailey & Tähtiharju, 2003). In this first attempt to adapt the strategy for laccase production, the fermentation was continued for 215 h. The laccase production phase of the fermentation was significantly prolonged, as the highest laccase activity in the culture supernatant was measured after 169 h (data not shown). Laccase activity at that point was 780 nkat ml^{-1}, corresponding to about 920 mg l^{-1}.

Purification of recombinant laccase was hindered by the presence of high amounts of the major cellulase CBHI in the culture supernatant. Laccase and intact CBHI could not be separated by the chromatographic methods used because the isoelectric points, hydrophobicities and sizes of these proteins were very similar. However, purification of laccase was successful after treating the culture supernatant with papain. Papain cleavage altered the hydrophobic properties of CBHI so that it lost its binding capacity on Phenyl-Sepharose at 600 mM Na_2SO_4. The activity or size of *M. albomyces* laccase did not change during papain treatment, suggesting that papain did not digest the laccase. This was also verified by N- and C-terminal sequencing of the purified laccase. Both termini were similar to those of native *M. albomyces* laccase (Kiiskinen & Saloheimo, 2004). After papain digestion, CBHI and laccase were separated by hydrophobic interaction chromatography, because laccase

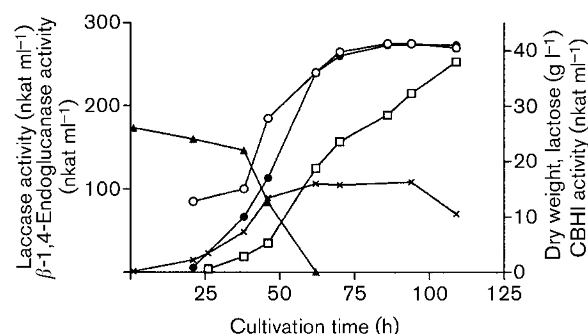

Fig. 3. Batch fermentation of *T. reesei* transformant pLLK13/295 producing laccase. Laccase activity was measured with ABTS (□), β-1,4-endoglucanase activity with hydroxyethylcellulose (○) and CBHI activity with methylumbelliferyl β-D-lactoside (●) as substrate. The lactose concentration (▲) and dry weight development (×) during the fermentation are also shown.

was bound on Phenyl-Sepharose. It eluted as two active peaks: the first peak eluted at about 200 mM salt concentration, and the second, smaller, peak eluted with buffer. The first laccase peak was further purified by DEAE Sepharose anion-exchange chromatography and Sephacryl S-100 HR gel filtration. The overall purification factor was 11 and the recovery of activity was 40 % (Table 1).

The kinetic parameters with four different substrates were determined for purified native and recombinant laccases. The K_m and k_{cat} values, as well as the specific activities, were very similar for both enzymes (Table 2). The molecular masses were determined by MALDI-TOF mass spectrometry, which gave a value of 71·3 kDa for the recombinant laccase and 72·2 kDa for the native laccase. In isoelectric focusing, both laccases gave one band with a pI of about 4·0 (data not shown). The pH optimum of the recombinant *M. albomyces* laccase was determined using guaiacol as substrate. The optimum was broad, ranging from 5 to 7, as also earlier described for the native laccase (Kiiskinen *et al.*, 2002). The thermal stability of the recombinant laccase measured at 40, 50 and 60 °C was also similar to that of the native laccase: the half-lives of both enzymes were >70 h at

Table 1. Purification of the recombinant *M. albomyces* laccase

Purification step	Vol. (ml)	Total activity (nkat)	Protein (mg)	Specific activity (nkat mg^{-1})	Activity yield (%)	Purification factor
Concentrated culture filtrate	100	170 000	3200	53	100	1
Papain treatment	89	120 000	ND	ND	71	ND
Phenyl-Sepharose	680	136 000	1090	125	80	2·4
DEAE-Sepharose	37	105 000	200	540	62	10
Sephacryl S-100	18	67 200	120	560	40	11

ND, Not determined.

Table 2. Kinetic parameters and specific activities of native (MaL) and recombinant (rMaL) *M. albomyces* laccases

ABTS measurements were performed at pH 4·5, other assays at pH 6.

Substrate	K_m (μM)		k_{cat} (min^{-1})		k_{cat}/K_m (μM^{-1} min^{-1})		Specific activity (nkat mg^{-1})	
	rMaL	MaL	rMaL	MaL	rMaL	MaL	rMaL	MaL
ABTS	270 ± 10	280 ± 20	4700	4500	18	16	850	930
Syringaldazine	1·8 ± 0·1	1·3 ± 0·1	5500	4700	3000	3600	1070	1030
2,6-DMP	5·2 ± 0·1	5·2 ± 0·2	4000	3400	780	660	400	390
Guaiacol	910 ± 80	890 ± 80	1900	1900	2·1	2·2	150	200

40 °C, 50 h at 50 °C and 3·5 h at 60 °C. The redox potential of the T1 copper of *M. albomyces* laccase expressed in *T. reesei* was 0·47 ± 0·01 V, which is in accordance with the redox potential of the wild-type enzyme: 0·46 ± 0·01 V.

DISCUSSION

The first laccase was described in 1883 from the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883). Since then several laccases have been studied with respect to their biological function, substrate specificity, copper-binding structure and industrial applications (Thurston, 1994; Gianfreda *et al.*, 1999; Xu, 1999). Despite the long history of laccase research, many biochemical and functional aspects of these enzymes still remain unclear. In order to understand the mechanisms of substrate binding, electron-transfer reactions and the suitability of laccases for various applications, well-characterized model enzymes are needed. *M. albomyces* laccase is a very interesting enzyme for laccase research due to its temperature and pH characteristics, suitable for industrial applications (Kiiskinen *et al.*, 2002), and its novel structural properties concerning oxygen binding (Hakulinen *et al.*, 2002). However, the production levels of laccase by *M. albomyces* have been low and the cultivations have been difficult to reproduce. In order to obtain a more reliable source for this enzyme, *M. albomyces* laccase cDNA was expressed in *T. reesei.*

M. albomyces laccase cDNA was expressed in *T. reesei* from two plasmids with the strong *cbh1* promoter. One of the constructs contained the laccase cDNA alone with its own signal sequence and propeptide region. In the other construct, the laccase cDNA region encoding the mature enzyme was fused to the *T. reesei* gene encoding the hydrophobin HFBI. Both expression constructs yielded transformants that produced significant laccase activities. In shake-flasks, the highest production level was 230 mg l^{-1} from the non-fusion construct, whereas the activity levels from the fusion construct were about five times lower. Fusion to a secreted host protein has improved the heterologous production of, for example, murine Fab fragments (Nyyssönen *et al.*, 1993) and a bacterial xylanase (Paloheimo *et al.*, 2003) in *T. reesei*, but in our case this effect was not observed. On the other hand, this was the first time that HFBI was used as a production carrier protein. Based on Southern hybridization, the differences in production levels between the two expression constructs did not depend on expression construct copy numbers. The fusion construct was expressed to higher or similar mRNA levels compared with the non-fusion construct, and therefore the difference between the constructs cannot be explained by transcription efficiency or mRNA stability (Fig. 2a). The cause of the lower production with the fusion construct most probably occurred at the post-translational level, because the fusion protein was retained inside the cells and degraded much more than the non-fused laccase (Fig. 1b). Two efficiently secreted proteins thus did not produce a well-secreted fusion protein in this case. One reason might be the presence of a linker sequence that might not have been optimal. Although a laccase protein species with about the same mobility as the mature laccase was observed inside the cells, along with multiple other degradation products, the fusion protein might not have been proteolytically cleaved precisely at the N-terminus of the mature laccase. This may have predisposed the remaining laccase to proteolysis.

The addition of copper to the *T. reesei* minimal medium had a positive effect on heterologous laccase production. Because the laccase was expressed under the *cbh1* promoter, which is not activated by copper, the improved production levels were most probably not caused by higher transcription rates. In addition, no effect of copper addition on fungal growth was detected, which implies that the higher laccase yields may have been caused by improved folding of the active enzyme in the presence of elevated copper concentrations. The ability of added copper to improve correct folding of recombinant laccase has previously been detected in *A. nidulans* and *A. niger* expressing a laccase from *Ceriporiopsis subvermispora* (Larrondo *et al.*, 2003). In addition, the importance of adequate copper levels for efficient heterologous laccase production has been reported in *S. cerevisiae*, where the overexpression of two copper-trafficking enzymes from *Trametes versicolor* led to significantly improved recombinant laccase yields (Uldschmid *et al.*, 2003).

Unfolded protein response (UPR) is a signal-transduction pathway that reacts to accumulation of unfolded proteins in

the ER and induces genes involved in folding, degradation of proteins in the ER or their further transport from this compartment (Mori, 2003). Heterologous proteins often do not fold as efficiently as native ones, and thus their production can cause induction of the UPR pathway (Saloheimo *et al.*, 1999). The possible induction of UPR, in other words the possible exposure of the host cells to secretion stress, by laccase expression in *T. reesei* was studied by Northern hybridization with the genes *pdi1*, *bip1* and *hac1* as probes (Fig. 2a). The mRNAs of the UPR target genes *pdi1* and *bip1* were not present at elevated levels as compared with the control, and the truncated form of the *hac1* mRNA was not found in any of the strains, indicating that laccase expression did not cause severe secretion stress in *T. reesei.* In the case of the HFBI–laccase fusion strain this was somewhat unexpected, since rather extensive intracellular accumulation of the protein and its degradation products was detected (Fig. 1b). A possible explanation for this result could be that the fusion protein is not trapped in the ER but rather in other parts of the secretory pathway, e.g. the vacuole. The growth curves measured for the laccase-producing transformants in non-optimal pH conditions show that laccase expression from the non-fusion construct is somewhat harmful to the host (Fig. 2b), although no secretion stress response appears to be triggered in the transformant cells. The reduced growth at pH 6 could indicate that laccase production causes some additional stress for the host but this can only be seen under non-optimal growth conditions where other stress factors are also present. Another possibility is that the *M. albomyces* laccase already secreted to the growth medium may inhibit growth at higher pH values, because the laccase is most active at pH values above 5 (Kiiskinen *et al.*, 2002).

It has been shown that *M. albomyces* laccase is processed at both its N- and C-termini (Kiiskinen & Saloheimo, 2004). The C-terminal processing is of special interest, because the truncated C-terminus protrudes inside the enzyme, potentially forming a plug to an O_2/H_2O exchange tunnel leading to the active site (Hakulinen *et al.*, 2002). N- and C-terminal peptide sequence analyses of the purified recombinant laccase produced by *T. reesei* showed that this host is able to perform both of the processing steps correctly. When *M. albomyces* laccase was expressed in *S. cerevisiae*, the production levels were enhanced both by using yeast alpha-factor prosequence in the expression construct and by introducing a stop codon into the laccase cDNA at the C-terminal processing site (Kiiskinen & Saloheimo, 2004). This indicated indirectly that baker's yeast was not able to perform either of the processing steps of the laccase efficiently. Interestingly, the production curve of laccase in the *T. reesei* batch fermentation (Fig. 3) was rather different from that of cellulase production, even though the laccase gene was expressed from the major cellulase gene *cbh1* promoter. Laccase activity in the culture supernatant increased for 40 h after cellulase production and fungal growth had ended due to exhaustion of the carbon source. This increase in laccase level could be related to activation of laccase by processing at the C-terminus. This is consistent with our finding that laccase activity increased in *T. reesei* shake-flask culture supernatant samples during storage at 4 °C. The involvement of proteolysis in activation of laccases has previously been shown with a *Pleurotus ostreatus* laccase (Palmieri *et al.*, 2001). Another possible reason for the increase in laccase level at the late stage of the fermentation is that some of the laccase may have been trapped inside the cells and released as they started to autolyse.

Recombinant laccase was purified from the fermentation culture supernatant of *T. reesei.* The separation of laccase and the major secreted protein CBHI was achieved by hydrophobic interaction chromatography after cleaving the CBHI with papain. Papain cleaved CBHI between the cellulose-binding domain and the core protein (van Tilbeurgh *et al.*, 1986), which resulted in altered hydrophobic properties. Papain digestion did not change the activity, size, or N- and C-terminal peptide sequences of *M. albomyces* laccase, confirming that this method was suitable for laccase purification. According to mass spectrometry, the molecular mass of recombinant laccase was only 0·9 kDa lower than that of native laccase, which indicates that *T. reesei* glycosylated laccase to the same extent as *M. albomyces.* In contrast, significant overglycosylation was observed when *M. albomyces* laccase was expressed in *S. cerevisiae* (Kiiskinen & Saloheimo, 2004).

The purified recombinant laccase was biochemically characterized and compared to the wild-type laccase. The results for specific activities, pH optima, thermostabilities and redox potentials showed that *M. albomyces* laccase produced in *T. reesei* was similar to the native laccase. This was further verified by kinetic analysis with four different substrates. The parameters K_m and k_{cat} for both laccases were very similar. When compared to other laccases, *M. albomyces* laccase has a rather high K_m value (0·28 mM) for the nonphenolic ABTS, as K_m values around 0·03–0·05 mM have been observed for most fungal laccases (Xu *et al.*, 1996; Chefetz *et al.*, 1998; Garzillo *et al.*, 1998; Schneider *et al.*, 1999; Record *et al.*, 2002). On the other hand, the K_m values for the phenolic 2,6-DMP (0·005 mM) and syringaldazine (0·003 mM) were very low, indicating that phenolic compounds are better substrates for *M. albomyces* laccase than the commonly used ABTS. This was also observed when the rate of electron transfer was taken into account and the values for k_{cat}/K_m were compared between different substrates; the order of k_{cat}/K_m was: syringaldazine > 2,6-DMP > ABTS (Table 2). Guaiacol proved to be a poor substrate for *M. albomyces* laccase, as its K_m value was as high as 0·9 mM. Relatively high K_m values (0·4–5 mM) for guaiacol have also been measured with other fungal laccases (Chefetz *et al.*, 1998; Garzillo *et al.*, 1998).

Laccases have generally been rather difficult to produce in large amounts in heterologous hosts. For example, only about 20 mg *P. radiata* laccase l^{-1} was previously produced in a fermenter cultivation of *T. reesei* (Saloheimo & Niku-Paavola, 1991). In shake-flasks, 19 mg *Mt. thermophila*

laccase l^{-1} was produced in *A. oryzae* (Berka *et al.*, 1997), and 70 mg *P. cinnabarinus* laccase l^{-1} in *A. niger* (Record *et al.*, 2002). The highest reported production level of heterologous laccase hitherto was 135 mg *C. cinereus* laccase l^{-1} in a shake-flask cultivation of *A. oryzae* (Yaver *et al.*, 1999). Laccase expression in the yeasts *S. cerevisiae* and *Pichia pastoris* has resulted in significantly lower yields than in filamentous fungi, and the highest laccase production levels in *Pichia pastoris* have been around 5–8 mg l^{-1} (Otterbein *et al.*, 2000; Soden *et al.*, 2002). The production levels reported in this work for *M. albomyces* laccase in *T. reesei* in shake-flask cultures (230 mg l^{-1}), batch fermentations (290 mg l^{-1}) and in the fed-batch fermentation (920 mg l^{-1}) are thus the highest heterologous laccase expression levels reported so far. Comparable laccase yields have previously been achieved with homologous laccase production systems in a shake-flask cultivation of *P. cinnabarinus* which yielded 1000–1500 mg laccase l^{-1} (Lomascolo *et al.*, 2003) and a fermenter cultivation of *Trametes pubescens* which yielded 700 mg laccase l^{-1} (Galhaup *et al.*, 2002). The wild-type *M. albomyces* is not an efficient laccase producer and therefore heterologous expression of the *lac1* gene was required in order to obtain high laccase amounts.

In addition to efficient production in *T. reesei*, expression of the *M. albomyces* laccase gene in *S. cerevisiae* has also given a relatively good yield as compared to other laccases produced in *S. cerevisiae* (Kiiskinen & Saloheimo, 2004). It is known that proteins from organisms closely related to the host are generally better produced than those from more distantly related organisms. Most of the laccases previously studied originate from the white-rot basidiomycetes, whereas the protein production hosts *T. reesei*, *Aspergillus* sp., *P. pastoris* and *S. cerevisiae* are ascomycetes. Therefore, the fact that *M. albomyces* is an ascomycete may be an important reason for the good production yields of its laccase. On the other hand, the highest reported expression levels of the ascomycetous *Mt. thermophila* laccase in *A. oryzae* were very modest (Berka *et al.*, 1997). Thus it will be interesting to follow new data on ascomycete laccase production in ascomycetous production hosts as results become available. In this paper we have demonstrated a high level of expression of *M. albomyces* laccase in *T. reesei*, providing a reliable and economical means of producing this interesting enzyme for future studies.

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Laccase from *Melanocarpus albomyces* binds effectively to cellulose

Laura-Leena Kiiskinen[1], Hetti Palonen, Markus Linder, Liisa Viikari, Kristiina Kruus*

VTT Biotechnology, P.O. Box 1500, Fin-02044 VTT, Finland

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Abstract A laccase from the thermophilic fungus *Melanocarpus albomyces* was shown to bind to softwood and pure microcrystalline cellulose. The binding isotherm fitted well the Langmuir type one-site binding model. The adsorption parameters indicated that *M. albomyces* laccase binds with high affinity to cellulose with a relatively low maximum binding capacity, as compared to the values for various cellulases. The binding was shown to be reversible and not influenced by non-specific protein or 0.1–0.5 M Na_2SO_4. No binding was detected with laccases from *Trametes hirsuta* or *Mauginiella* sp., which suggests that binding to cellulose is typical for only some laccases.
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Keywords: Cellulose binding; Bacterial microcrystalline cellulose; *Melanocarpus albomyces* laccase

1. Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases catalyzing oxidation of various phenolic compounds, aromatic amines, and even certain inorganic compounds by a one-electron transfer mechanism. The electron withdrawn from the substrate is transferred via four copper atoms to molecular oxygen [1]. Laccases are very common in nature, especially in plants and fungi [2,3]. Fungal laccases participate in plant pathogenesis, pigment production, and lignin biodegradation [2,4]. Mainly because of the broad substrate specificity range of laccases, they possess great biotechnological potential. Promising applications for laccases include e.g., textile dye bleaching, pulp bleaching and bioremediation [2,5].

We have recently purified and characterized from the thermophilic fungus *Melanocarpus albomyces* a laccase, which has high thermostability and a pH optimum at a neutral and slightly alkaline pH range [6]. The three-dimensional structure of *M. albomyces* laccase has been solved as one of the first complete laccase structures including all four coppers [7]. In this article, a novel feature for *M. albomyces* laccase is demonstrated: its effective binding to cellulose. Binding to cellulose has been shown for many enzymes involved in modification of lignocellulose, including various cellulases (for reviews, see [8,9]), hemicellulases [10–12], a β-glucosidase [13], and some cellobiose dehydrogenases [14–17]. In addition to cellulose-binding enzymes, binding to solid substrates has been reported with several chitinases [18,19] and glucoamylases [20,21]. We demonstrate in this article for the first time that a laccase binds to cellulose with high affinity.

2. Materials and methods

2.1. Enzymes and cellulosic materials

The enzymes used in this study were: recombinant *M. albomyces* laccase purified from *Trichoderma reesei* [22], native *M. albomyces* laccase [6], *Trametes hirsuta* laccase [23] and *Mauginiella* sp. laccase [24]. Steam-pretreated softwood (SPS) from *Picea abies* [25], Avicel PH 101 (SERVA Electrophoresis) and bacterial microcrystalline cellulose (BMCC) from *Acetobacter xylinum* [26] were used as cellulosic adsorbents.

2.2. Adsorption studies with steam-pretreated softwood

Laccases were added into 1 ml of 10 g l^{-1} steam-pretreated softwood (SPS) suspension in 50 mM citrate buffer (pH 5) giving initial enzyme dosages of 100 and 1000 nkat g^{-1}. After gentle mixing in an end-over-end rotary shaker at 22 °C for 1 h, the samples were centrifuged (10 000×g, 22 °C, 5 min). The remaining laccase activity in the supernatant was measured spectrophotometrically with 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) [ABTS, Roche Diagnostics] as substrate [27]. All measurements described in this study were repeated two to four times and the results were calculated as mean values from the parallel samples.

2.3. Adsorption of *M. albomyces* laccase on pure cellulose

Recombinant *M. albomyces* laccase (0.09–5 μM) was added into 0.25–1 ml of cellulose suspension containing 10 g l^{-1} Avicel or 0.1 g l^{-1} BMCC in 50 mM sodium citrate buffer (pH 5) at 22 °C. In order to reduce non-specific adsorption of laccase, 0.5% of bovine serum albumin (BSA; Sigma) was added to the BMCC-containing reaction mixtures [28,29]. The remaining laccase activity in the supernatant was measured after 1 h as described above. The corresponding protein concentrations were calculated by using the specific activity of 600 nkat mg^{-1} (measured on ABTS in citrate buffer) and the molecular weight of 71 000 Da for recombinant *M. albomyces* laccase [22]. The amount of bound laccase was calculated from the difference between the initial and free enzyme concentrations. A control sample without cellulose was also measured each time to ensure that the laccase remained active during the treatment. The non-linear regression curves for binding isotherms were calculated with GraphPad Prism 3.02 program (GraphPad Software).

The activity of bound laccase was measured with an end-point activity assay after binding. The supernatant was removed and the cellulose fraction containing the bound laccase was suspended in citrate buffer to restore the initial volume. The cellulose suspension was

* Corresponding author. Fax: 358-9-4552103.
E-mail address: Kristiina.Kruus@vtt.fi (K. Kruus).

[1] Present address: Finnzymes Oy, Keilaranta 16 A, Fin-02150 Espoo, Finland.

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate); BMCC, bacterial microcrystalline cellulose; BSA, bovine serum albumin; CBD, cellulose-binding domain; SPS, steam-pretreated softwood

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Table 1
Melanocarpus albomyces, *Mauginiella* sp. and *Trametes hirsuta* laccase activities in supernatant after mixing with 1% steam-pretreated softwood (SPS) for 1 h at 22 °C, pH 5

Laccase	Initial activity (nkat ml^{-1})	Activity in the supernatant after 1 h treatment (nkat ml^{-1})	
		Sample with SPS	Control without SPS
Melanocarpus albomyces	1	0.02 ± 0.01	1.0 ± 0.2
	10	0.14 ± 0.01	10.0 ± 0.8
Mauginiella sp.	1	0.74 ± 0.01	<0.01
	10	7.0 ± 0.6	6.3 ± 0.6
Trametes hirsuta	1	0.9 ± 0.1	<0.01
	10	10.0 ± 0.2	8 ± 1

further diluted and ABTS (20 mM solution) was added to give a final concentration of 5 mM [27]. The reaction was allowed to proceed for exactly 2 min, after which the solution was filtrated through a 0.2 μm sterile FP 30 filter (Scleicher and Schuell) and the absorbance at 436 nm was immediately measured.

Reversibility of binding was determined by dilution experiments. *M. albomyces* laccase was first allowed to adsorb on BMCC for 1 h. Subsequently, the mixture was diluted fivefold with the sample buffer. Formation of a new equilibrium was monitored by removing small samples from the mixture after 1, 15, 30, 60 and 120 min. The samples were filtrated through a 0.2 μm GHP filter (Scleicher and Schuell), and the concentration of laccase at the new equilibrium was determined by activity measurements as described above.

The effect of non-specific adsorption on binding to BMCC was studied by omitting BSA in the binding experiments and the role of ionic interactions in adsorption was analyzed by adding 0.1 or 0.5 M Na_2SO_4 into the reaction mixture. The adsorption of *T. hirsuta* laccase on BMCC was studied at two protein concentrations (0.1 and 0.4 μM) as described above.

3. Results

The adsorption of *M. albomyces*, *T. hirsuta* and *Mauginiella* sp. laccases on steam-pretreated softwood was tested at two enzyme concentrations, 1 and 10 nkat ml^{-1}. The difference in binding between different laccases was very obvious; only *M. albomyces* laccase showed extensive binding, whereas *T. hirsuta* and *Mauginiella* laccases did not adsorb on softwood (Table 1). Interestingly, SPS seemed to stabilize *Mauginiella* and *T. hirsuta* laccases, as it prevented significant loss of activity that was detected in the control samples containing low concentrations of these laccases in buffer without SPS. Steam-pretreatment of softwood generates swollen wood fibers consisting of cellulose (42%) and lignin (51%) [25]. The stabilizing effect of SPS may be related to the exposed lignin, because various lignin-derived phenolic compounds have been shown to enhance the stability of *Trametes versicolor* laccase in citrate buffer [30]. In the case of *M. albomyces* laccase, the loss of activity was not observed even in very low protein concentrations.

Binding of *M. albomyces* laccase was also analyzed on relatively pure cellulose, Avicel, to elucidate whether the binding to SPS was truly caused by cellulose present in wood fibres. The adsorption studies with 1% Avicel clearly indicated that *M. albomyces* laccase was effectively bound to purified cellulose (Table 2). Activity of the Avicel-bound laccase was also

Table 2
Amount of *Melanocarpus albomyces* laccase in the supernatant and in the solid fractions after mixing with 1% Avicel at 22 °C for 1 h at pH 5

Initial dosage (nkat)	Supernatant fraction (nkat)	Cellulose fraction (nkat)
10	0.2 ± 0.1	10 ± 1
0.8	<0.01	0.8 ± 0.1

determined in order to clarify whether the bound laccase was still active. As shown from the results (Table 2), all laccase activity could be recovered from the Avicel fraction, indicating that binding does not inactivate the enzyme.

The macroscopic structure of cellulose in Avicel preparations is relatively heterogeneous [26,31], therefore more thorough binding analyses were conducted with bacterial microcrystalline cellulose (BMCC). The adsorption isotherm of *M. albomyces* laccase on BMCC is shown in Fig. 1. In comparison, the adsorption of *T. hirsuta* laccase was studied at two points on the isotherm. No adsorption of *T. hirsuta* laccase occurred, since all laccase activity was detected in the supernatant after 1 h mixing with BMCC (Fig. 1).

The data points on the binding isotherm of *M. albomyces* laccase fitted well the classical Langmuir-type binding model represented by the Eq. (1)

$$Y = B_{max}[L]/(K_d + [L]), \quad (1)$$

where [L] is the concentration of free enzyme in equilibrium, B_{max} is the maximum binding capacity and K_d is the dissociation constant (= the reciprocal of the association constant K_a) [32]. B_{max} and K_d were solved by non-linear regression, and 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) was calculated using the slope of the isotherm in low enzyme concentrations. The slope was calculated from the first derivative of the isotherm Eq. (1) as the concentration of free enzyme approaches zero [33]. Thus, K_p was calculated by substituting the values for B_{max} and K_d into the Eq. (2)

$$K_p = \lim_{[L]\to 0} Y'([L]) = B_{max}/K_d. \quad (2)$$

Eq. (2) yielded $K_p = 320 \pm 80$ l g^{-1}.

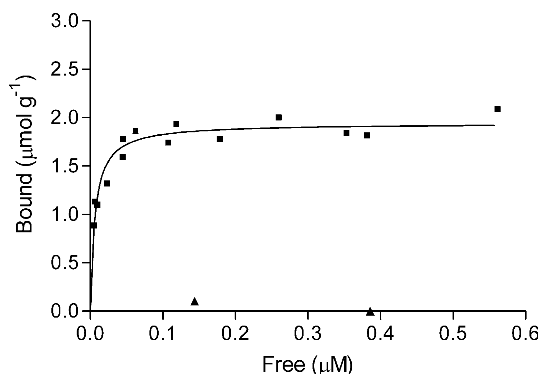

Fig. 1. The adsorption isotherm of *Melanocarpus albomyces* (■) and *Trametes hirsuta* (▲) laccases obtained with bacterial microcrystalline cellulose at 22 °C, pH 5.

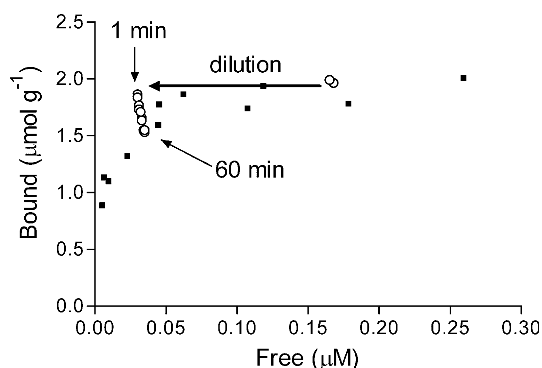

Fig. 2. Reversibility of *Melanocarpus albomyces* laccase by dilution when bound to bacterial microcrystalline cellulose at 22 °C, pH 5. (■) isotherm points, (○) dilution studies.

Data points on the isotherm were also measured with higher enzyme concentrations than presented in Fig. 1 to ensure that the saturation level evident in Fig. 1 was truly valid [34]. No increase in the amount of bound enzyme per gram of cellulose was seen with data points of up to free enzyme concentration of 4.3 μM (data not shown). In high enzyme concentrations (>1 μM) the random error of data points substantially increased, when the relative proportion of bound enzyme versus free enzyme decreased, as has previously been discussed by Bothwell and Walker [32].

The reversibility of binding was determined by dilution experiments. The equilibrium between laccase and cellulose was unbalanced by adding buffer, and the desorption of laccase was monitored by activity measurements of the supernatant fraction. The desorption data showed that a new equilibrium was established on the same isotherm (Fig. 2). 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 studied. BSA was added to the reaction mixtures in adsorption studies to prevent non-specific adsorption of the laccase [28,29], and the effect of non-specific adsorption was studied by omitting BSA in the binding experiments at three points on the isotherm. Omission of BSA did not cause changes in the adsorption behaviour of *M. albomyces* laccase (Fig. 3). In addition, the effect of 0.1 and 0.5 M Na_2SO_4 on adsorption was studied at three points on the isotherm. The results showed that the addition of salt did not alter the adsorption of *M. albomyces* laccase on BMCC (Fig. 3).

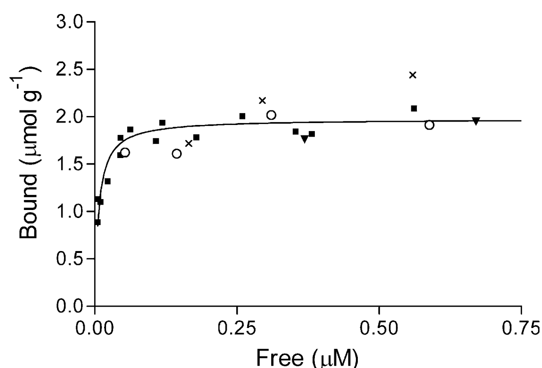

Fig. 3. The effect of 0.1 M (○) or 0.5 M (▼) Na_2SO_4 or omission of bovine serum albumin (BSA) (×) on binding of *Melanocarpus albomyces* laccase to bacterial microcrystalline cellulose. (■) isotherm points with BSA and without salt.

4. Discussion

The adsorption of a laccase on cellulose was demonstrated for the first time in this study; laccase from the ascomycete *M. albomyces* was shown to effectively bind to steam-pretreated softwood, Avicel and bacterial microcrystalline cellulose. Furthermore, it was demonstrated that the cellulose-bound laccase retained its full activity. The adsorption on cellulose was also studied with two other fungal laccases from *T. hirsuta* and *Mauginiella* sp. The results clearly indicated that neither of these laccases were able to bind to cellulose. These results thus suggest that laccases can be divided into two groups based on their affinity for cellulose.

The adsorption isotherm of *M. albomyces* laccase on BMCC was found to fit the Langmuir model, which is commonly used to describe the binding of cellulases to cellulose [35–38]. The curve fitting facilitated the calculation of the adsorption parameters for maximum binding capacity and binding affinity. The maximum binding capacity of *M. albomyces* laccase (1.94 μmol g^{-1}) was relatively low as compared to the values for various cellulases with binding capacities for BMCC ranging from 6 to 20 μmol g^{-1} [39,40]. However, similar binding capacity of 2.1 μmol g^{-1} has previously been reported for a cellobiose dehydrogenase from *Phanerochaete chrysosporium* [14]. On the other hand, the affinity of *M. albomyces* laccase represented by the partition coefficient of 320 l g^{-1} was clearly higher than reported for cellulases. For example, the partition coefficients of cellobiohydrolases Cel6A and Cel7A from *Trichoderma reesei* were 3.4 and 18 l g^{-1}, respectively [29], and of the cellulases CenA and Cex from *Cellulomonas fimi* 40.5 and 33.3 l g^{-1}, respectively [26]. The combination of high affinity and relatively low capacity of binding suggests that *M. albomyces* laccase is able to bind very effectively to BMCC, but only on relatively few binding sites.

The differences in binding parameters between cellulases and *M. albomyces* laccase may be related to the absence of a cellulose-binding domain (CBD) in the latter. A separate CDB, which in most cellulases dominates the binding characteristics, cannot be located either in the amino acid sequence [41] or the crystal structure of *M. albomyces* laccase [7]. In the case of cellulases, the available structures suggested specific sites on the protein that putatively interact with cellulose [42]. These were subsequently confirmed by experimental mutagenesis studies, which showed that the binding of CBDs is mediated by several aromatic amino acids forming a planar surface on the binding face of the domain [42,43]. An analysis of the *M. albomyces* laccase structure did not, however, reveal any hydrophobic surface patches on the protein that could obviously be assigned as the cellulose-binding site. Similarly to other laccases, the crystal structure of *M. albomyces* showed three cupredoxin-like domains, none of which contained regions with increased hydrophobicity on the outer surfaces. These observations are consistent with the results obtained with *P.*

chrysosporium cellobiose dehydrogenase, which binds effectively to cellulose but does not contain a separate CBD or any other evident cellulose-binding substructure [14,44].

One of the assumptions in a Langmuir-type binding model is full reversibility of adsorption. Our results from dilution studies of bound *M. albomyces* laccase showed that the binding was fully reversible, thus reinforcing the applicability of a Langmuir-type binding model for calculating binding constants from our data. Reversibility of binding is not a straightforward issue among enzymes adsorbing on cellulose, as both reversible and irreversible binding have been demonstrated [29,35,39,45,46]. The observed irreversible binding of cellulases may be related to their two-domain structure, because both domains participate in the binding [28,45]. Adsorption of *M. albomyces* laccase on cellulose was not affected by non-specific protein (BSA), which indicated that binding was not due to unspecific protein binding to solid substrate. In addition, the binding was unaltered by the presence of 0.1–0.5 M salt. As ionic interactions are weakened by increasing ionic strength, it can be concluded that electrostatic forces are not the main cause for the observed binding [46].

Interestingly, Paice et al. [47] reported a preliminary observation of *Myceliophthora thermophila* laccase adsorption on Kraft pulp that is mostly composed of cellulose. *Mt. thermophila* laccase is highly homologous to *M. albomyces* laccase, having a level of amino acid sequence identity of 73% [41]. The result suggests that the adsorption on cellulose might be a common feature among some fungal laccases. The role of the binding of *M. albomyces* laccase to cellulose may be related to total hydrolysis of lignocellulose, because *M. albomyces* is also known to produce several cellulose- and hemicellulose-degrading enzymes [48,49]. However, the detailed binding mechanism as well as the possible role of the binding need to be further elucidated. The ability of a laccase to bind to cellulose might be exploited in applications. It would be interesting, for example, to analyze whether the dosage of the enzyme can be decreased in textile or pulp applications, when a laccase that adsorbs on cellulose is used. On the other hand, the adsorption on inexpensive cellulosic materials could possibly be utilized for immobilization and recycling purposes.

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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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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 good technical properties 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.

A novel laccase from the ascomycete *Melanocarpus albomyces* was characterized. Interestingly, *M. albomyces* laccase showed good thermostability and it had a pH optimum at neutral pH. The crystal structure of *M. albomyces* laccase was resolved and the overall structure was shown to be similar to other blue copper oxidases. The gene encoding *M. albomyces* laccase was isolated and expressed heterologously in *Saccharomyces cerevisiae* and *Trichoderma reesei*. Finally, this work 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.

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