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

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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.*

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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.

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′-ACGTACGGTACCGAGCCGACGTGCAACACGCC-3′(forward) and 5′-ACGTACGGTACCTCAACGAACCAGCCACTCGC-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 $CuSO_4$.

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).

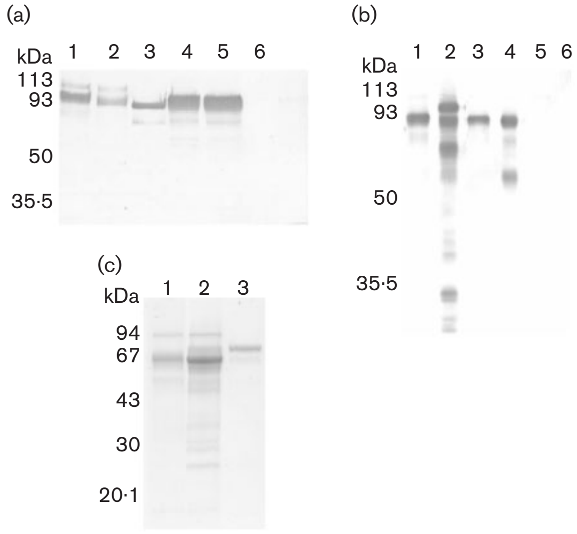

Fig. 1. SDS-PAGE and Western blot analyses of extra- and intracellular laccase production in *T. reesei*. (a) Western blot analysis of culture supernatants of HFBI–laccase fusion strains (lanes 1 and 2) and transformants producing non-fused laccase (lanes 4 and 5). Lane 3 contains 0·2 μg native *M. albomyces* 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).

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).

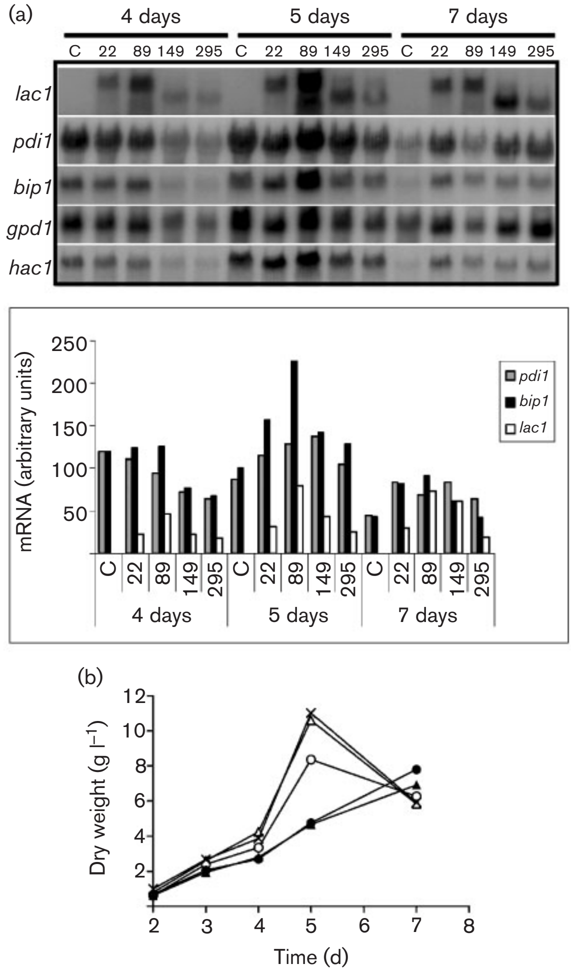

Fig. 2. Comparison of *T. reesei* transformants producing HFBI–laccase fusion protein or non-fused laccase. (a) Northern analysis of the control strain and two best-producing transformants from the HFBI–laccase fusion construct (transformants 22 and 89) and from the non-fusion construct (149 and 295) 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 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).

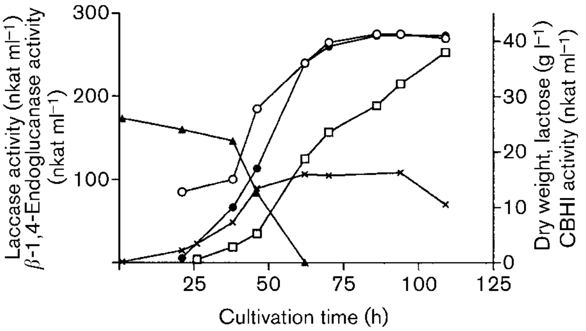

Fig. 3. Batch fermentation of *T. reesei* transformant pLLK13/295 producing laccase. Laccase activity was measured with ABTS (□), β-1,4-endoglucanase activity with hydroxyethylcellulose (○) and CBHI activity with methylumbelliferyl β-D-lactoside (●) as substrate. The lactose concentration (▲) and dry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

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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