Molecular cloning of a laccase isozyme gene from *Pleurotus sajor-caju* and expression in the heterologous *Pichia pastoris* host

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The Psc *lac4* gene from *Pleurotus sajor-caju* has been cloned and expressed in the heterologous host *Pichia pastoris*, under the control of the *AOX1* methanol inducible promoter. The native *Ple. sajor-caju* laccase signal sequence was effective in directing the secretion of *lac4* expressed in *Pic. pastoris*. The control of media pH and temperature was found to be important in obtaining sufficient quantities of the protein to allow purification and subsequent biochemical characterization. The recombinant Psc Lac4 was purified to electrophoretic homogeneity and was shown to be immunologically related to *Pleurotus eryngii* Lac1. The purified laccase was estimated to have a molecular mass of around 59 kDa, to have a carbohydrate content of approximately 7% and a calculated pI of 4·38. The enzyme oxidized the substrates 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), 2,6-dimethoxyphenol, syringaldazine and guaiacol, exhibiting optimal pHs of 3·3, 6, 6·5 and 7 respectively. With ABTS as substrate the enzyme displayed optimal activity at 35 °C and pH 3·5. The enzyme was strongly inhibited by sodium azide and thioglycolic acid but not by EDTA.

Keywords: white-rot fungus, heterologous expression, laccase, biochemical characterization

INTRODUCTION

The white-rot fungus *Pleurotus sajor-caju* is a member of the oyster mushroom family. This edible basidiomycete secretes a range of enzymes, most notably laccases and peroxidases, which enable it to grow on a variety of different substrates (Toyama & Ogawa, 1974). Laccase is a copper-containing phenoloxidase catalysing the four-electron reduction of O_2 to H_2O with the concomitant oxidation typically of a phenolic substrate. This one-electron oxidation generates a free radical, which is typically unstable and may undergo a second enzyme-catalysed oxidation or a non-enzymic reaction such as hydration or spontaneous disproportionation and/or may partake in a polymerization reaction (Thurston, 1994).

Laccases are members of the blue copper oxidase enzyme family that typically contain four copper atoms per molecule. The distinctive spectroscopic and physical properties of the various copper centres within the protein allow for classification into three different kinds of copper sites: type 1 (T1), type 2 (T2) and type 3 (T3) (Malkin & Malmstrom, 1970). T1 copper is responsible for the intense blue colour, absorbs maximally in the visible range and is responsible for both abstracting electrons from the substrate and delivering them to the T2 and T3 sites. The type 2 copper exhibits lower visible absorbance and the type 3 site incorporates two copper centres and is responsible for a shoulder near 330 nm in the absorbance spectrum of native laccase. All these copper ions appear to be involved in the catalytic mechanism. Amino acid sequence analysis of different laccases indicates that the copper-binding sites, involving ten-histidine residues, are very highly conserved.

Various fungi, several insects (Thomas *et al.*, 1989), bacteria (Alexandre & Zhulin, 2000; Sanchez-Amat *et al.*, 2001; Martins *et al.*, 2002) and recently wasp venom (Parkinson *et al.*, 2001) have been shown to produce/contain laccase. The biological role for laccase has as yet not been fully elucidated and appears to vary depending on the type of organism (Thurston, 1994). In fungi

laccase has been shown to act as a ligninolytic enzyme (Eggert *et al.*, 1997), to be involved in fruiting body formation (Wood, 1980), in pigmentation (Leatham & Stahmann, 1981) and in pathogenicity (Choi *et al.*, 1992).

Laccases are remarkably substrate non-specific and can directly oxidize a broad range of diverse phenolic compounds including chlorophenols (Field *et al.*, 1993), methoxyphenols (Eriksson *et al.*, 1990), hydroquinones (Baker *et al.*, 1996) as well as di- and triamines (Harkin *et al.*, 1974). The ability of laccases to oxidize catechol, guaiacol, 2,6-dimethoxyphenol (DMP) and syringaldazine is considered diagnostic (Leatham & Stahmann, 1981). Syringaldazine has also been reported to act as a mediator (co-oxidant) facilitating the oxidation of non-phenolic subunits in lignin (Kawai *et al.*, 1989). Other mediators such as 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Bourbonnais *et al.*, 1995) and 1-hydroxy-benzotriazole (Bourbonnais *et al.*, 1997) have also been reported. In the presence of these mediators laccase can catalyse the oxidation of polycyclic aromatic hydrocarbons (Collins *et al.*, 1996) and benzyl alcohols (Potthast *et al.*, 1996), and can bleach textile dyes (Schneider & Pedersen, 1995). These characteristics of laccase have led to applications in selected bioremediation strategies (Gianfreda *et al.*, 1999), as a catalyst for regiospecific biotransformations (Agematu *et al.*, 1993), as a biosensor in immunoassays (Ghindilis *et al.*, 1995), in the treatment of wastewater (Shuttleworth & Bollag, 1986; Larsson *et al.*, 2001) and in the removal of natural phenols from oil (Tomati *et al.*, 1991).

In an attempt to understand the functional role of fungal laccases, a number of laccase genes have been cloned and characterized including those from *Aspergillus nidulans* (Aramayo & Timberlake, 1990), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1997), *Rhizoctonia solani* (Wahleithner *et al.*, 1996), *Trametes versicolor* (Jönsson *et al.*, 1995), *Trametes villosa* (Yaver *et al.*, 1996) and *Trametes* I-62 (CECT 20197) (Mansur *et al.*, 1997). However, despite this, the biochemical characterization of these laccases has been difficult due to the low levels of expression in their native systems. A solution to this problem is the production of sufficient quantities of the recombinant protein in a heterologous host. In this regard the methylotrophic yeast *Pichia pastoris* has proven useful, with this yeast having previously been used to express laccases from *T. versicolor* (Jönsson *et al.*, 1997; Gelo-Pujic *et al.*, 1999; Brown *et al.*, 2002), *Trametes sanguinea* (Hoshida *et al.*, 2001) and *Pyc. cinnabarinus* (Otterbein *et al.*, 2000). In addition, *Saccharomyces cerevisiae* (Kojima *et al.*, 1990; Cassland & Jönsson, 1999), *Trichoderma reesei* (Saloheimo & Niku-Paavola, 1991), *Aspergillus oryzae* (Yaver *et al.*, 1996) and *Aspergillus niger* (Record *et al.*, 2002) have also been used for the heterologous expression of fungal laccases. Recently the bacterial laccase gene *cotA* from *Bacillus subtilis* has been expressed in *Escherichia coli* (Martins *et al.*, 2002).

We have previously described the cloning of four laccase isozyme genes from *Ple. sajor-caju* (Soden & Dobson, 2001). Here we report on the expression of one of these isozyme genes, namely Psc *lac4* in the heterologous *Pic. pastoris* system and demonstrate the use of this system to produce sufficient quantities of recombinant pLac 4 protein to allow its biochemical characterization.

METHODS

Organisms and vectors for cloning and expression. The basidiomycete *Pleurotus sajor-caju* strain P32-1 was maintained as previously described (Soden & Dobson, 2001). *Pichia pastoris* and the strains GS115 (*his4*) and KM71 (*arg4 his4*) were purchased from Invitrogen. Cloning procedures were performed using a laboratory stock of *E. coli* DH5α and One Shot INVαF′ Chemically Competent *E. coli* (Invitrogen). PCR products were cloned into the pCR2.1-Topo vector (Invitrogen). The vectors pPIC3.5 and pPIC9 (Invitrogen) were used for expression in *Pic. pastoris*. Expression of inserts in both vectors is controlled by the methanol-inducible AOX promoter. pPIC9 possesses the α-secretion factor from *Sac. cerevisiae* while pPIC3.5 does not contain a secretion signal. All media and protocols for *Pichia* are as described in the *Pichia* expression manual (Invitrogen).

DNA sequencing and analysis. Sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Dye Terminator Cycle Sequencing Ready Reaction with Ampli*Taq* DNA polymerase (Applied Biosystems) on a GeneAmp PCR system 2400 (Perkin Elmer) and run on an automated DNA sequencer (model 373 stretch, Applied Biosystems). The sequence data were assembled and processed using the DNASTAR software package. The BLAST algorithm (Altschul *et al.*, 1990) was used to search DNA and protein databases for similarity. The CLUSTAL program (Higgins & Sharp, 1989) was used for alignment of amino acid sequences.

RNA-PCR. Total RNA was prepared by a modification of the method of Gromroff *et al.*, 1989 and as described previously (Soden & Dobson, 2001). Reverse transcription was carried out using Superscript II RNase H (Gibco-BRL and Invitrogen) using the following protocol. Total RNA (1·5 μg) and 80 pmol of the oligo dT primer (Table 1) in a reaction volume adjusted to 10·5 μl with HPLC grade dH$_2$O were heated to 72 °C for 2 min. This was added to 1×RT buffer, 1 mM DTT, 1 mM each deoxynucleoside triphosphates (dNTPs), 20 U RNasin inhibitor and 1 μl Superscript II RNase H reverse transcriptase, in a total volume of 20 μl. The reaction was heated to 42 °C for 150 min and placed on ice. PCR amplification was carried out with the Ampli*Taq* Gold proof reading DNA polymerase (Perkin Elmer, Roche Molecular Systems). Amplification of the *lac4* cDNA from *Ple. sajor-caju* was performed using the primers, pLac4 ATG Fw and pLac4 Rev (Table 1). A 5 μl volume from the RT reaction mixture was mixed with 10 pmol of each primer, 1× Ampli*Taq* PCR gold buffer, 1·5 mM MgCl$_2$, 200 μM (each) dNTPs and 1·25 U of Ampli*Taq* gold DNA polymerase. Reaction volumes were adjusted to 100 μl with HPLC-grade water. Amplification was performed in a PTC-100 programmable thermal controller (MJ Research) with 1 cycle of 94 °C for 3 min followed by 10 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C) and extension (100 s at 72 °C), 10 cycles of 30 s at 94 °C, 30 s at 55 °C and 150 s at 72 °C, 10 cycles of 30 s at 94 °C, 30 s at 55 °C and 4 min at 72 °C, and a final step of 72 °C for 10 min. PCR products were loaded on a 1·5 % agarose gel and electrophoresed in TAE buffer (40 mM Tris/acetate, 1 mM EDTA) for 2 h at 90 V. The gel was stained with ethidium bromide, visualized under UV light and a single PCR

Table 1. Gene-specific primer sequences and annealing temperatures

Name	Primer sequence	Annealing temp. (°C)
oligo dT primer	TTTTTTTTTTTTTTT	42
pLac4 ATG Fw	CTGCTACACCTACCAACGATGGTTC	55
pLac4 Rev	GCAAATGGCATTCTGAC	55
pLac4-SS Fw	CGAATTCTGCCATTCGGCCCGCTGGCA	55
pLac4-SS Rev	GCAAATGGCATTCTGACATCC	55
5′ Aox Fw	GACTGGTTCCAATTGACAAGC	55
3′ Aox Rev	GCAAATGGCATTCTGACATCC	55

product of ∼1·7 kb was cut and purified using the QiaQuick purification kit (Qiagen).

Gene cloning/construction of vectors. The purified laccase cDNA was cloned into the pCR2.1-Topo vector, subsequently excised using *Eco*RI, followed by purification from an agarose gel and insertion into pPIC3.5, resulting in the vector pPIC3.5-*lac4*. For cloning into pPIC9, PCR amplification of the *lac4* cDNA in the pCR2.1-Topo vector was performed with the primers, pLac4–SS Fw and pLac4–SS Rev (Table 1). The primer pPIC9-SS Fw does not contain a Kozak consensus sequence like pPIC3.5-*lac4* Fw, as it is present in the α-secretion signal for pPIC9. The PCR conditions employed were as previously described for *lac4* cDNA. The resultant PCR product, *lac4*–SS was cloned into pPIC9 in the same manner as for pPIC3.5, resulting in the vector pPIC9-*lac4*-SS. After transformation of *E. coli* and isolation of plasmid DNA, the presence and orientation of the inserts was determined both by PCR and by restriction enzyme digestion followed by agarose-gel electrophoresis. The primers 5′ Aox Fw and 3′ Aox Rev (Table 1) were used to determine the presence of inserts in pPIC3 and pPIC9 using a PCR protocol as described in the *Pichia* expression manual. Sequence analysis was ultimately performed on pPIC3.5-*lac4* and pPIC9-*lac4*-SS, to ensure the integrity of the constructs. All restriction enzymes used for cloning and restriction analysis were purchased from Roche. Techniques for cloning were as described by Ausubel *et al.* (1996). All chemicals were of analytical grade unless otherwise stated.

Transformation and detection of expression in *Pic. pastoris*. *Pic. pastoris* was transformed by the electroporation method. pPIC3.5-*lac4* and pPIC9-*lac4*-SS plasmid DNA was digested with *Stu*I prior to transformation into electrocompetent cells of *Pic. pastoris*. The vectors pPIC3.5 and pPIC9 without inserts were simultaneously transformed into *Pic. pastoris* and used as controls. Transformant colonies were picked from minimal glucose (MD) plates and transferred to plates with either MD or minimal methanol (MM) as the carbon source, for examination of methanol utilization. Colonies were spotted onto MM plates supplemented with 0·2 mM ABTS and 0·1 mM CuSO₄. Laccase-producing transformants were identified by the presence of a dark green colour around the *Pichia* colonies.

Production of laccase in liquid media. Colonies producing a green zone/halo were further analysed in liquid cultures. Laccase activity in these cultures was determined spectrophotometrically using 600 μl cell-free supernatant, 250 μl dH₂O, 100 μl 1 M NaOAc buffer, pH 6; with the reaction being initiated by the addition of 50 μl 10 mM ABTS. The reaction was monitored at 420 nm and 25 °C with a Beckman Du® 640 spectrophotometer. Ten transformants of both

pPIC3-*lac4* and pPIC9-*lac4*-SS in both *Pic. pastoris* GS115 and KM71 along with the controls for pPIC3.5 and pPIC9 were cultivated first at 28 °C in 15 ml volumes (50 ml baffled shake flasks) of buffered glycerol minimal medium (BGM) until the optical density at 600 nm (OD$_{600}$) was approximately 10. The culture was centrifuged (1200 *g* at 4 °C for 10 min.) and washed with 0·9 % (w/v) NaCl. The sample was recentrifuged and the cell pellet was diluted with buffered methanol minimal (BMM) medium to an OD of ∼1 in 100 ml baffled shake flasks. Transformants were cultivated at 20 °C in BMM medium, shaking at 160 r.p.m. (supplemented with 0·1 mM CuSO₄, 0·8 % alanine) and methanol (0·5 % of the total volume of medium) was added daily to maintain the induced production of laccase. Samples were analysed daily for laccase activity using the ABTS assay and cell growth was also measured by taking OD$_{600}$ readings (1 OD$_{600}$ = ∼5·0 × 10⁷ cells). Experiments involving comparison of strains were performed with triplicate transformants to allow for variations in yield.

During preliminary studies on laccase production by the transformants it was noted that the pH of the cultures decreased rapidly from pH 6 to pH 3. The BMM media was supplemented with 0·8 % alanine, allowing the pH to remain at 6.

Enzyme purification. The liquid culture (1·8 l) was centrifuged at 10 000 *g* for 30 min and the subsequent cell-free supernatant was further filtered through a 0·45 μm filter (Millipore) to remove any remaining cell debris. The sample was then concentrated on an Amicon ultrafiltration stirred cell using a PM-10 membrane and buffered with 100 mM sodium acetate, pH 5. The subsequent purification steps were carried out at 4 °C. Partial purification of the laccase was initially performed using a Sephadex G-75 chromatography column (2·6 × 10 cm, Amersham) with 100 mM sodium acetate buffer, pH 5, at 0·11 ml min⁻¹. Fractions containing laccase activity were pooled and reconcentrated using the Amicon PM-10 membrane. The concentrate was dialysed against 20 mM Tris buffer, pH 8·5, and loaded onto a Q-Sepharose (2·75 × 11 cm) (Pharmacia Biotech AB) anion exchange column (Bio-Rad). The dialysis tubing had a molecular mass cutoff of 3 kDa. Ten column volumes of the same buffer were passed through the column before elution with a step-wise increase in the NaCl gradient (0 to 0·6 M) in the same buffer. The fractions containing laccase activity were pooled and concentrated. The concentrate was dialysed against 20 mM Tris, pH 7. The purified laccase was stored at 4 °C. The degree of enzyme purity was confirmed by SDS-PAGE.

Electrophoresis. Polyacrylamide (12 %) gel slab electrophoresis in 0·1 % SDS was carried out as described by Laemmli (1970). For molecular mass determinations, the gel was

calibrated with α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48·5 kDa), lactic dehydrogenase (36·5 kDa) and triosephosphate isomerase (26·6 kDa). Proteins were visualized after staining with Coomassie brilliant blue R-250 (0·1 %).

Protein determination. Protein concentration was determined using the Bio-Rad Protein Assay, based on the Bradford (1976) dye-binding procedure, with bovine serum albumin (BSA) as standard.

Immunoblot analysis. SDS-PAGE was performed on a 12 % (w/v) polyacrylamide gel. Electrotransfer of proteins was carried out according to the method of Towbin *et al.* (1979). The proteins were electrotransferred to a nitrocellulose membrane for 1 h at 170 mA in a Bio-Rad transblot apparatus. The membrane was blocked by incubation with 0·5 % BSA in TBST [10 mM Tris (pH 8·0), 150 mM NaCl, 0·05 % Tween 20] for 30 min at room temperature. The membrane was incubated in TBST containing anti-laccase (*Pleurotus eryngii*) antiserum at a 1:100 dilution overnight and then washed with TBST for 30 min. It was incubated again in TBST containing anti-mouse IgG (whole molecule) alkaline phosphatase conjugate at 1:10000 dilution for 90 min, washed with TBST for 30 min and developed with nitro blue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer [100 mM Tris (pH 9·5), 100 mM NaCl, 5 mM MgCl$_2$]. The laccase-specific antibodies used for immunological detection were generated against purified laccase from *Ple. eryngii* (Muñoz *et al.*, 1997).

Endoglycosidase treatment. N-linked glycans of Psc Lac4 were removed with *N*-glycosidase F (Roche Diagnostics). Purified Psc Lac4 ($\sim$5 µg) was denatured by heating at 100 °C for 3 min in 1·2 % (w/v) SDS, then diluted in buffer to 25 µl (final composition 10 mM sodium phosphate buffer, pH 6·8; 5 mM EDTA; 0·1 M β-mercaptoethanol; 0·3 % Nonidet P-40; 0·4 % SDS). After adding 1 U (1 µl) *N*-glycosidase F, the sample was incubated at 37 °C overnight and then analysed by SDS-PAGE. A control in which the glycosidase was substituted with deionized H$_2$O was prepared in an identical fashion. The carbohydrate content of the laccase was estimated by comparison of the migration of native and treated enzyme on SDS-PAGE gel.

Enzyme assays. Laccase activity was assayed at 25 °C using (a) ABTS, (b) DMP, (c) syringaldazine and (d) guaiacol as substrates as follows. (a) The assay mixture contained 0·5 mM ABTS and 0·1 M sodium acetate buffer, pH 3·3. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36\,000$ M^{-1} cm^{-1}). (b) The assay mixture contained 1 mM DMP and 0·1 M sodium phosphate buffer, pH 6·0. Oxidation of DMP was followed by absorbance increase at 477 nm ($\varepsilon = 14\,800$ M^{-1} cm^{-1}). (c) The assay mixture contained 0·5 mM syringaldazine (dissolved in ethanol) and 0·1 M sodium phosphate buffer, pH 6·5. Oxidation of syringaldazine was followed by an absorbance increase at 525 nm ($\varepsilon = 65\,000$ M^{-1} cm^{-1}). (d) The assay mixture contained 10 mM guaiacol (dissolved in 10 % acetone) and 0·1 M sodium phosphate buffer, pH 6·5. Oxidation was followed by absorbance increase at 460 nm. Tyrosinase activity was assayed at 25 °C using 3,4-dihydroxy-DL-phenylalanine (DL-dopa) as substrate. The assay mixture contained 0·2 mM DL-dopa and 0·1 M sodium phosphate, pH 6. The oxidation of substrate was followed by an absorbance increase at 475 nm ($\varepsilon = 3600$ M^{-1} cm^{-1}) (Palmieri *et al.*, 1997). All kinetic studies were performed at least three times and the kinetic data were fitted to hyperbola by using the Michaelis–Menton equation. All enzyme activity is expressed as international units (IU).

pH activity and stability. Laccase activity as a function of pH for ABTS, guaiacol, syringaldazine and DMP was measured using McIlvaine's citrate-phosphate buffer adjusted to different pH levels in the range 2·2–8·0 at 25 °C. To determine the pH stability, the purified laccase was incubated at either 4 °C or 25 °C and the activity measured at selected times up to 300 min, using ABTS as substrate. The same buffer was used to determine the pH stability.

Temperature activity and stability. Laccase activity as a function of temperature was measured after pre-incubation of 50 µg purified protein for 5 min at 10, 20, 30, 35, 40, 45, 50, 60, 70 and 80 °C in 100 mM sodium acetate buffer, pH 5. ABTS was used as substrate for determining activity. Thermal stability was estimated by incubating the purified laccase at 35, 45, 55 and 65 °C and measuring activity at selected times up to 240 min.

Inhibitor studies. The effects of sodium azide, EDTA, thioglycolic acid and hydroxylamine on enzyme activity were tested using ABTS as substrate after pre-incubating the enzyme for 10 min (at 25 °C) with the various inhibitors before the addition of substrate.

Absorption spectra. For the spectroscopic characterization 0·2 mg ml^{-1} purified laccase, in 25 mM sodium acetate buffer, pH 5 was used. The absorption spectra was determined between 190 and 1000 nm at 25 °C using a Beckman Du 640 spectrophotometer. The ratio of $A_{280/605}$ was used for characterization of the copper content of laccase.

Nucleotide sequence accession number. The sequence of the *Pleurotus sajor-caju* laccase 4 gene (*lac4*) reported here has been assigned the accession no. AF 297228 in the GenBank database.

RESULTS

Cloning and sequence analysis of the *Ple. sajor-caju* laccase isozyme Psc *lac4* gene

RNA-PCR using primers designed from the previously cloned *Pox1* laccase gene of *Pleurotus ostreatus* (pLac4 ATG Fw and pLac4 Rev; Table 1) (Giardina *et al.*, 1995) were used to clone the full-length cDNA *lac4* gene. A single cDNA PCR product of 1695 bp, with a 1603 bp open reading frame was obtained. The deduced amino acid sequence of Psc Lac4 encodes a protein of 533 amino acids. All of the expected copper ligands (10 His residues and one Cys residue) are present in the Psc Lac4. The translation initiation site for Psc Lac4 is ACGATGT, which is consistent with the consensus sequence for higher eukaryotes, RNNATGG (Kozak, 1981), where R is a purine. Translation of 69 bp starting at the ATG codon results in a 23 aa putative signal peptide, which shows the typical structure of sorting for secreted proteins in eukaryotes and is followed by a sequence that could act as a peptidase recognition site (Von Heijne, 1986). The core region of the putative signal peptide is predominantly hydrophobic, which is again characteristic of eukaryotic signal peptides. The predicted amino acid sequence of Psc Lac4 exhibited a high degree of similarity to Pox2 from *Ple. ostreatus* (Palmieri *et al.*, 1997), as well as a high degree of similarity with corresponding sequences of other basidiomycete laccases genes, including those of other *Pleurotus* and *Trametes* species, ranging from 52 to 99 % identity at the amino acid level (Soden, 2002).
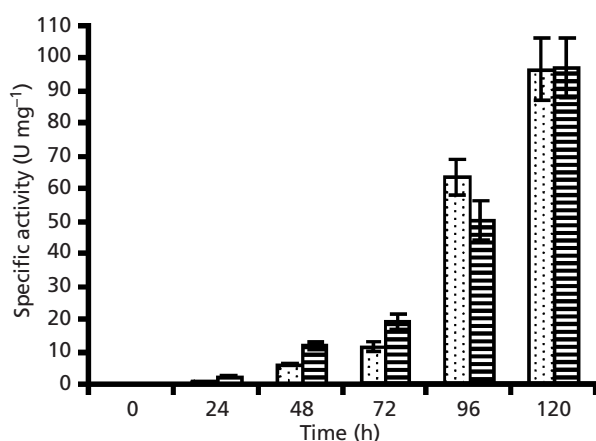
Fig. 1. Comparison of laccase expression from *Pic. pastoris* strains GS115 and KM71. Mean values were obtained by using three separate transformants for each strain. Methanol (0·5 %) was added daily to cultures. Dotted bars, GS115; lined bars, KM71.

Identity to the laccase genes of the ascomycete fungi was lower at between 14 and 24 %, while the *ppoA* 'bacterial laccase' gene from the prokaryote *Mariomonas mediterranea* (Sanchez-Amat *et al.*, 2001) exhibited 11 % identity.

Heterologous expression

Both the pPIC3.5-*lac4* and pPIC9-*lac4*-SS (without the native 23 aa putative signal peptide) constructs were linearized with *Stu*I and transformed into electro-competent *Pic. pastoris* GS115 and KM71 cells. Green coloured zones on MM plates were observed with the pPIC3.5-*lac4* transformants after 24 h, while colour was observed around the pPIC9-*lac4*-SS transformants after 7 days. When both transformants were subsequently grown in liquid cultures, intracellular laccase activity was noted for pPIC9-*lac4*-SS but not for pPIC3.5-*lac4*, indicating that the native signal sequence is required for effective secretion of the recombinant laccase (data not shown). Subsequent experiments were then conducted with the pPIC3.5-*lac4* construct. No visible difference in colony growth rates was observed between the protease-positive GS115 and the protease-negative KM71 strains transformed with pPIC3.5-*lac4* on MM plates when methanol was used as the carbon source. This was confirmed in liquid cultures where the specific activity of laccase was almost identical for both strains after 120 h (Fig. 1). A *Pic. pastoris* GS115 strain transformed with pPIC3.5-*lac4* was used in all subsequent growth experiments.

A time-course analysis was performed with samples being taken after 0, 48, 96 and 144 h to examine protein production in *Pic. pastoris* GS115 (pPIC3.5-*lac4*) by SDS-PAGE (Fig. 2a). A band corresponding to approximately 59 kDa was observed after 48 h and became more pronounced at the later time points tested. Confirmation that this band corresponded to Psc Lac4
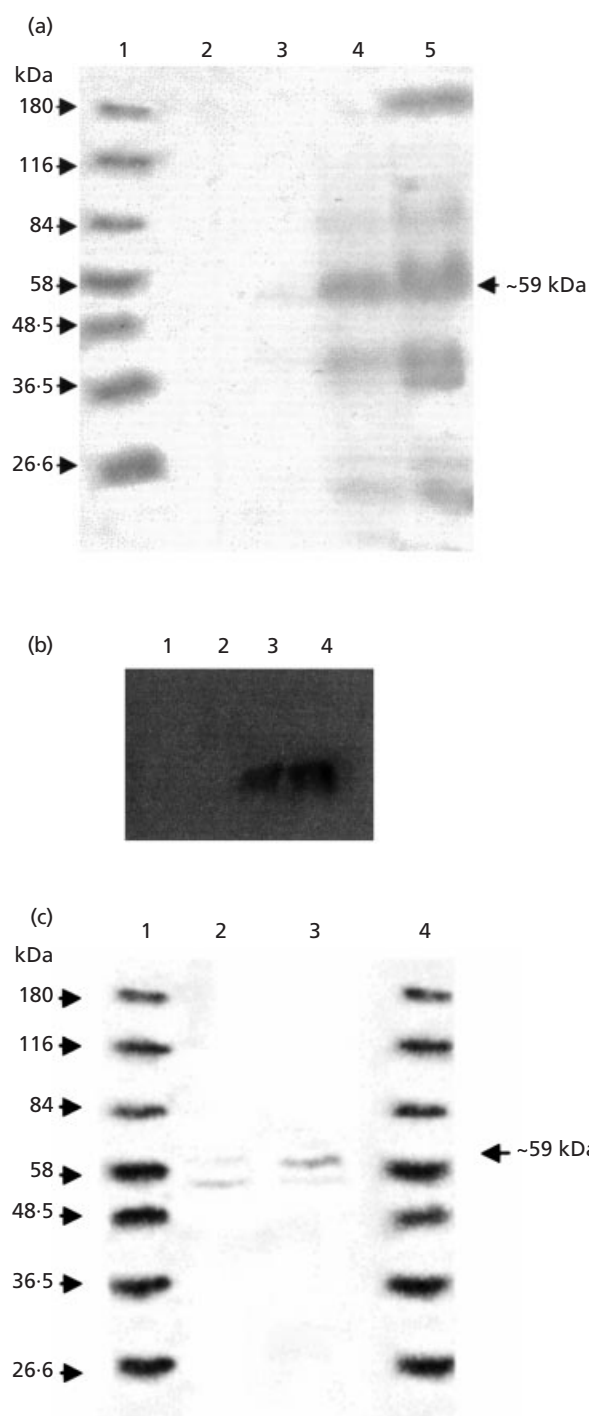
Fig. 2. (a) SDS-PAGE of a time-course analysis of laccase production by *Pic. pastoris* GS115 (pPIC3.5-*lac4*) in buffered minimal medium with 0·8 % alanine. Lane 1 contains molecular mass markers. Lanes 2, 3, 4 and 5 represent protein samples collected after 0, 2, 4 and 6 days respectively. The arrow indicates the molecular mass of Psc Lac4 (~59 kDa). (b) Immunoblot analysis of the extracellular proteins (10 μg per lane) in (a) above. Lanes 1–4 represent samples taken after 0, 2, 4 and 6 days respectively. (c) SDS-PAGE analysis of native and deglycosylated Psc Lac4 laccase from *Pic. pastoris* GS115 (pPIC3.5-*lac4*). Lanes 1 and 4 contain molecular mass markers, lane 2 contains 10 μg deglycosylated laccase (~55 kDa), lane 3 contains 10 μg purified native laccase (~59 kDa).

Table 2. Purification of Psc Lac4 expressed in *Pic. pastoris*

Purification step	Vol. of fraction (ml)	Protein concn (mg ml^{-1})	Enzyme concn (U ml^{-1})	Specific activity (U mg^{-1})	Total amount (U)	Yield (%)	Purification factor (fold)
Crude cell-free extract	1800	0·11	10·2	92·7	23·9	100	1·00
Concentration Amicon (10 kDa)	3	26·14	5180	198·2	17·5	84·6	2·14
Gel filtration: Sephadex G-75	10	1·2	635	529·1	13·2	35·4	5·70
Ion exchange: Q-Sepharose	5	0·5	1050	2100	9·8	28·6	22·65

Table 3. Substrate specificity tests

	K_m (mM)	$10^4 \times k_{cat}$ (min^{-1})	$10^4 \times k_{cat}/K_m$ (min^{-1} mM^{-1})
ABTS	2·5	7·4	2·96
DMP	0·12	5·8	48·33
Guaiacol	0·066	0·68	10·3
Syringaldazine	0·28	3·5	12·5

came from a Western blot that was performed on samples taken from the time-course analysis, using anti-*Ple. eryngii* laccase antibodies (Muñoz *et al.*, 1997). Immunoblot analysis revealed that Psc Lac4 cross-reacted immunologically with the *Ple. eryngii* laccase antibody, with the strongest reaction corresponding to day 6, the day of maximal laccase activity (Fig. 2b).

### Purification of laccase from *Pic. pastoris* culture broth

The recombinant Psc Lac4 protein was purified from 1·8 l culture broth following 120 h growth in BMM medium. During purification ultrafiltration with a membrane with a 10 kDa molecular mass cutoff enriched for laccase by 2·14-fold. Subsequent purification steps involved Sephadex G-75 chromatography at pH 5, resulting in a 5·7-fold purification, and finally Q-Sepharose chromatography at pH 8·5, with one active peak being eluted. The purified protein had an activity of 1050 U ml^{-1}, with a specific activity of 2100 units mg^{-1}. A summary of the purification steps is shown in Table 2.

### Characterization of the purified laccase

Molecular mass. The calculated molecular mass of 54431 Da from the deduced amino acid sequence for Psc Lac4, correlates well with the apparent molecular mass of approximately 59 kDa determined by SDS-PAGE (Fig. 2c, lane 3). Psc Lac4 treated with *N*-glycosidase F migrated to a position on the SDS-PAGE gel that corresponded to an apparent molecular mass of approximately 55 kDa, indicating a carbohydrate content of around 7% (Fig. 2c, lane 3). Potential *N*-glycosylation sites (Asn-Xxx-Ser/Thr) (Gavel & Von Heijne, 1990) can be recognized at positions 241 and 467 of the amino acid sequence. The calculated isoelectric point (pI) is 4·38.

Catalytic properties

(a) Substrate range and specificity. The purified Psc Lac4 oxidized the common laccase substrates ABTS, DMP, syringaldazine and guaiacol with K_m values ranging from 2·5 to 0·066 mM for ABTS and guaiacol respectively. Similarly, ABTS exhibited the highest k_{cat} values (7·4 × 10^4 min^{-1}) whereas DMP demonstrated the highest k_{cat}/K_m value (4·83 × 10^5 min^{-1} mM^{-1}) (Table 3). The kinetic parameters for guaiacol were determined in the presence of 10% acetone, due to its low solubility
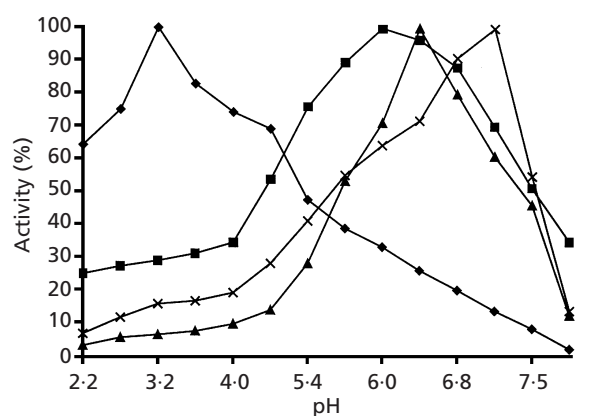
Fig. 3. Effect of pH on laccase activity with different substrates ABTS (♦), DMP (■), syringaldazine (▲) and guaiacol (×). Values represent the mean of three separate determinations. The standard deviation was less than 10%.
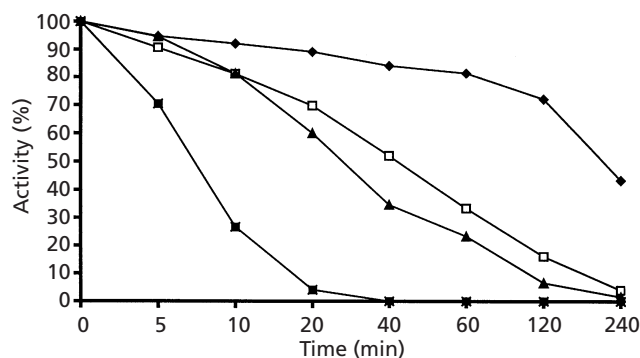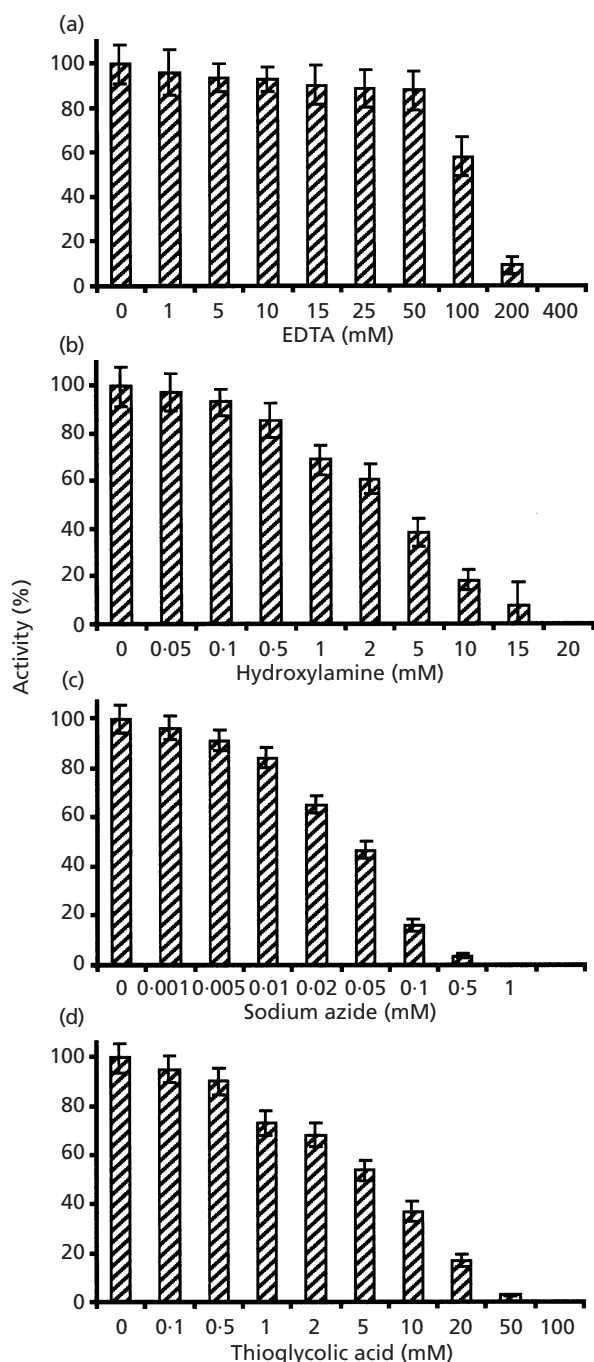

Fig. 4. Effect of temperature on the stability of the recombinant Psc Lac4, with assays being conducted at pH 7 using ABTS as substrate. Values represent the mean of three separate determinations. The standard deviation was less than 8%. ♦, 35 °C; □, 45 °C; ▲, 55 °C; ■, 65 °C.

in dH$_2$O. DL-Dopa did not act as a substrate for the recombinant laccase (Table 3).

(b) pH optimum and stability. The activity of purified Psc Lac4 as a function of pH was determined. The optimum pH differs for each individual substrate assayed from 3·3, 6·0, 6·5 and 7 for ABTS, DMP, syringaldazine and guaiacol respectively, with all substrates demonstrating a broad activity over a wide pH range (Fig. 3). At 4 °C,

Fig. 5. Effect of various enzyme inhibitors on Psc Lac4 laccase activity. All experiments were conducted in triplicate with ABTS as the enzyme substrate.

2% and 50% of the original value when the enzyme was incubated at pH 5, pH 3 and pH 9, respectively.

(c) Effects of temperature on activity. The optimal temperature for Psc Lac4 activity was 35 °C with ABTS as substrate. Thermal stability studies ranging from 35 °C to 65 °C were carried out at pH 7 (Fig. 4). Psc Lac4 was relatively stable after 1 h at 35 °C; however, over the same period of time residual activity decreased to approximately 35% and 25% of the original value when incubated at 35 °C and 45 °C respectively, while no detectable activity was observed after 1 h at 65 °C.

(d) Inhibitor studies. The effect of various enzyme inhibitors on laccase activity was assessed. Oxidation of ABTS was unaffected by low levels of EDTA (<50 mM); however, concentrations above 100 mM were inhibitory (Fig. 5a). Total inhibition was observed with 20 mM hydroxylamine (Fig. 5b), 0·5 mM sodium azide (Fig. 5c) and 100 µM thioglycolic acid (Fig. 5d).

(e) Copper content. The absorption spectrum of Psc Lac4 is characterized by the lack of a peak around 600 nm (data not shown). This peak is representative of the type 1 copper site, which gives purified laccase its characteristic blue colour, which may explain the colourless nature of the purified Psc Lac4.

DISCUSSION

We have previously reported on the cloning of four laccase isozyme genes from the edible fungus *Ple. sajorcaju* (Soden & Dobson, 2001). Here we report on the cloning of the full-length gene for one of these isozyme genes, namely Psc *lac4*, and on its subsequent expression in the heterologous host *Pic. pastoris*. Primers based on the previously cloned *Pox1* laccase gene (Giardina *et al.*, 1995) were used to clone the Psc *lac4* gene of 1695 bp, with an open reading frame of 1603 bp. The deduced pLac4 amino acid sequence encodes a protein of 533 amino acids, which contains the 10 highly conserved histidine residues which act as copper ligands (Thurston, 1994), and also exhibits a high degree of similarity to other laccases and ascorbate oxidases, from basidiomycete and ascomycete fungi, and plants, particularly in the carboxy-terminus copper-binding site. This similarity is reflected in the fact that Western blot analysis revealed Psc Lac4 to be immunologically related to *Ple. eryngii* laccase I (Fig. 2b). Analysis of the predicted 23 aa signal peptide of Psc Lac4 reveals a potential peptidase recognition site of Thr, His, Ala, Ala with the most likely cleavage site being between the two Ala residues. This follows the −3, −1 rule of Von Heijne (1986), which predicts that there is a small uncharged amino acid residue (Ala) at position −1 relative to the cleavage site and the absence of an aromatic charged or large and polar residue in position −3.

It has been proposed that for the type 1 copper ligand, the amino acid residue located 10 amino acids downstream of the conserved cysteine affects the redox potential of the type 1 copper in the active site (Canters & Gilardi, 1993), thus providing a basis for assigning

Psc Lac 4 was stable for at least 5 h between pH 6 and pH 8. Over the same period of time, the activity decreased to approximately 80%, 70% and 64% of the original value when the enzyme was incubated at pH 5, pH 3 and pH 9, respectively. At 25 °C, the pH stability of Psc Lac 4 was reduced. While the activity was still stable for at least 5 h at pH 7, over the same period of time residual activity decreased to approximately 30%,

laccases to class 1 (Met), 2 (Leu) or 3 (Phe) (Eggert et al., 1998). Psc Lac4 is a class 2 laccase based on this classification. Subsequent analysis of the purified recombinant Psc Lac4 by means of a UV-visible wavelength scan revealed the absence of a peak around 600 nm, the presence of which represents a T1 copper site and is believed to be responsible for the blue colour of the enzyme. To examine whether the absence of this peak might be due to copper depletion during the purification process, the scan was also conducted in the presence of 0·2 mM CuSO$_4$, without altering the result. The purified Psc Lac4 protein was indeed white/yellow in colour and in this regard is similar to previously characterized 'white laccases' from Agaricus bisporus (Wood, 1980) and Ple. ostreatus (Palmieri et al., 1997).

Heterologous expression

We chose the yeast Pic. pastoris as the heterologous host to express Psc Lac4 as it has been previously used in the expression of a wide variety of proteins (Cereghino & Cregg, 2000). Pic. pastoris is known to secrete foreign protein in the presence of low levels of native proteins, most importantly proteinases, thus helping simplify the purification procedure. In addition, the inducibility of the expression system is particularly important where the strong regulated alcohol oxidase 1 (AOX1) promoter, which is repressed by glucose and most other carbon sources, is induced > 1000-fold when cells are shifted to methanol as the sole carbon source (Cereghino & Cregg, 1999). Previous work on laccase expression in Pic. pastoris has identified temperature, pH and oxygen as factors that affect laccase production. Cassland & Jönsson (1999) have reported that production of active enzyme is favoured by lower cultivation temperatures, with laccase activity being 16-fold higher after 3 days at 19 °C when compared to 28 °C, with the same being true for laccase production in Sac. cerevisiae.

In this work cultures were first grown in BGM media at 28 °C and then transferred to BMM media at 20 °C. At 20 °C an OD$_{600}$ of approximately 20 (initially inoculated at an OD$_{600}$ of 1) after 96 h was obtained, with a laccase activity of 64 units (mg protein)$^{-1}$ (Fig. 1). Previous work has also reported that the pH of unbuffered growth media decreases from 5 to approximately 3 within the first day of growth and this has a detrimental effect on recombinant laccase levels (Gelo-Pujic et al., 1999; Jönsson et al., 1997). This may be due at least in part by the activation of acidic proteases and the loss of enzyme stability at a lower pH. The use of buffered BMM media only maintains the pH for short periods of time and has been shown to be inadequate unless the pH is raised during growth of Pichia (Jönsson et al., 1997). However O'Callaghan et al. (2002) have reported the successful use of alanine as an additional buffer component to BMM. In this work we used 0·8 % alanine in all BMM growth media and the pH remained at the initial pH of 6 for up to 144 h post-inoculation. In addition, all cultures were grown in baffled flasks with shaking to ensure an adequate supply of oxygen, which

is necessary for the oxidation of methanol to formaldehyde by alcohol oxidase.

The native signal peptide of Psc Lac4 was effective for directing both the secretion and proper proteolytic maturation of lac4 expressed in Pic. pastoris. In contrast, use of the Sac. cerevisiae α-factor secretion signal peptide resulted in intracellular production of active laccase, as indicated by the presence of a dark green colour (ABTS$^+$ radical) localized within the colonies transformed with pPIC9-lac4-SS. This was confirmed by assaying cell pellets of liquid cultures that had been washed and ruptured with acid-washed glass beads (Invitrogen, 1998). This intracellular production of laccase may be due to the inappropriate processing of the fusion protein.

Other studies which compare the Sac. cerevisiae α-factor signal peptide to native fungal signal peptides for secretion of recombinant laccase have reported differing findings.

While the amount of laccase produced was seven-fold higher when the native secretion signal was used instead of the α-factor secretion signal of pPIC9 for the T. versicolor lccI gene product (Jönsson et al., 1997), similar levels of laccase activity were observed for the Lac1 isoform from Pyc. cinnabarinus when either the native or yeast leader sequence was employed (Otterbein et al., 2000). Thus the use of native laccase signal sequences from fungi such as Ple. sajor-caju or T. versicolor may be a viable alternative to the α-factor signal peptide for the expression and subsequent secretion of other recombinant proteins in Pic. pastoris. This would be of relevance to the commercial production of numerous recombinant proteins in the Pichia system, given that the use of the α-mating factor signal peptide is subject to proprietary rights. Due to the generally regarded as safe (GRAS) status of Ple. sajor-caju, the use of native laccase signal sequences from this fungus may be particularly relevant to recombinant proteins with therapeutic applications.

Several fungal laccase genes have previously been cloned and heterologously expressed in Trichoderma reesei (Saloheimo & Niku-Paavola, 1991), Sac. cerevisiae (Cassland & Jönsson, 1999), A. niger (Record et al., 2002) and A. oryzae (Yaver et al., 1996; Berka et al., 1997). The level of Psc Lac4 laccase produced here in Pic. pastoris is 4·85 mg l^{-1} and is comparable to levels previously reported in yeast which were around 5 mg l^{-1}, while fungal hosts resulted in levels of 10–20 mg l^{-1}. The highest levels obtained to date were those with a Pyc. cinnabarinus laccase expressed in A. oryzae where levels of 8–135 mg l^{-1} were obtained (Yaver et al., 1999). While the laccase levels obtained here are not comparable with those obtained in A. oryzae, they are nonetheless sufficient to allow the biochemical characterization of the enzyme.

Biochemical characterization

The level of Psc Lac4 expression reported here (4·85 mg l^{-1}) enabled the purification and the first biochemical characterization of a fungal laccase isozyme

expressed in *Pic. pastoris*. It appears to be a monomeric protein of molecular mass of approximately 59 kDa, with a calculated pI of 4·38. The purified Psc Lac4 oxidized typical laccase substrates such as ABTS and syringaldazine very efficiently as is evident from the high values determined for the catalytic constants k_{cat} and the catalytic efficiencies k_{cat}/K_m for some selected substrates (Table 3). The introduction of a second methoxyl group as found in DMP resulted in an almost 11-fold increase in k_{cat}/K_m as compared to the monomethoxylated substrate guaiacol. A likely explanation for this is the strong electron-donating effect of the two methoxyl substituents and the favourable redox potential of the substrate DMP (Xu *et al.*, 1996).

In terms of activity, the optimal pH was 3·3, 6, 6·5 and 7 with ABTS, DMP, syringaldazine and guaiacol as substrates respectively. Psc Lac4 displays certain similarities to Pox2, a previously characterized laccase isozyme from *Ple. ostreatus* (Palmeri *et al.*, 1997) to which it shows a high degree of similarity at the deduced amino acid level. These similarities include molecular mass ($\sim$59 kDa) and pH optimum with ABTS as substrate; however, Pox2 has a higher temperature optimum than Psc Lac4. Indeed the stability of Psc Lac4 as a function of temperature was low relative to other laccases. One possible explanation is the level of glycosylation of Psc Lac4 following expression in *Pic. pastoris* ($\sim$7%), which may be lower than when the protein is expressed in *Pleurotus* given that proteins secreted by *Pic. pastoris* are known to be less extensively glycosylated and that carbohydrate content has been proposed to protect laccases from *Botrytis cinerea* (Slomczynski *et al.*, 1995) and *T. versicolor* from high-temperature denaturation. Furthermore, it has been proposed that the expression of an *A. niger* phytase gene in *Sac. cerevisiae* resulted in an increased level of glycosylation for the phytase protein and a corresponding increase in temperature stability (Han *et al.*, 1999). Other similarities between Pox2 and Psc Lac4 include similar patterns of pH stability at 25 °C with both remaining unaltered at pH 7 but decreasing in stability as the pH decreased. Psc Lac4 was strongly inhibited by the copper chelating agents, sodium azide and thioglycolic acid, but not by EDTA, which is typical of other previously characterized laccases.

In conclusion, we have demonstrated the use of the heterologous *Pic. pastoris* expression system to produce sufficient quantities of recombinant Psc Lac4 protein to allow its biochemical characterization. While several laccases have been cloned, their biochemical characterization has been difficult due to the low levels of expression in their native systems, such as for example the Pox1 laccase in *Ple. ostreatus* (Giardina *et al.*, 1995). If we are to gain a fuller understanding of the structure–function relationships of fungal laccases with respect to substrate specificity, pH profile and redox potential, then experiments involving site-directed mutagenesis similar to that which has already been undertaken with a *T. versicolor* laccase (Gelo-Pujic *et al.*, 1999) need to be conducted. From the work presented here it appears that

Pic. pastoris would provide an ideal heterologous host in which to express these mutants, with the system providing sufficient quantities of recombinant enzyme to allow subsequent biochemical characterization of mutant proteins. In addition it is clear that given the (GRAS) status of both *Ple. sajor-caju* and *Pic. pastoris* the production of a 'food-grade' fungal laccase using this system would have potential utility in several industrial applications, particularly in the food biotechnology sector.

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