

# Modeling the 3-D Structure of a Recombinant Laccase from *Trametes trogii* Active at a pH Close to Neutrality

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**Abstract** A cDNA encoding a novel laccase from the white-rot fungus *Trametes trogii* was cloned and expressed in *Pichia pastoris*. The recombinant protein (Lcc2) exhibited kinetic parameters for both phenolic and non phenolic substrates that were different from the previously described Lcc1, the main laccase isoform expressed by *T. trogii*; in addition, the pH/activity profiles for phenolic substrates of Lcc2 were shifted upward by 1–1.5 pH units towards neutrality as compared to Lcc1. Comparative modeling of the two laccases (69.2% identity) showed that the overall fold of Lcc2 is very similar to Lcc1 and other laccases. The substrate cavity of Lcc2 contains the Asp residue which is thought to mediate the laccase activity at acidic pHs, whereas two hydrophobic residues (Phe, Ile) on the cavity orifice of Lcc2 replace the two polar residues (Thr, Ser) of Lcc1. These structural differences may be responsible for the unique kinetic performances of Lcc2.

**Keywords** Laccase · *Trametes trogii* ·  
Heterologous expression · Comparative modeling ·  
*Pichia pastoris*

## Abbreviations

ABTS 2,2'-Azino-bis-(3-ethylbenz-  
thiazolinesulphonate)  
DMP 2,6-Dimethoxyphenol  
Lcc1 Predominant laccase isoform of *Trametes trogii*  
Lcc2 Recombinant laccase of *Trametes trogii*

ORF Open reading frame  
RACE Rapid amplification of cDNA ends

## 1 Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are extremely versatile enzymes, as they are able to oxidize an extensive list of aromatic compounds containing hydroxy or amino groups, including pesticides, polycyclic aromatic hydrocarbons and dyes. These properties make laccases good candidates for applications in the pulp and paper industry, textile industry, biosensor development, bioremediation of polluted water and soil, organic synthesis [2, 13, 17, 25, 27, 34, 36]. One of the primary drawbacks for the industrial use of laccases is that an acidic pH is required for their optimal activity; even though for the present laccase has a relatively small market, the industrial use of this enzyme may grow rapidly if its performance is improved.

Laccases were first discovered in plants and have been subsequently identified in fungi, insects and, more recently, in prokaryotes; laccases are thought to be nearly ubiquitous among fungi, and are predominantly found in the wood-rotting basidiomycetes where they are usually produced in multiple isoforms as extracellular proteins [2]. The culture broth of laccase-producing fungi generally contains a predominant laccase and several additional isoforms, which can be structurally and/or catalytically similar or diverse. The expression of different isoforms in fungi is due to the occurrence of multiple laccase genes; some of these genes are known to be upregulated in the presence of specific inducers such as copper, ferulic acid or 2,5-xylydine in the culture medium [26, 30, 33].

Most fungal laccases are monomeric glycoproteins with molecular masses ranging between 60,000 and 70,000 Da

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with an extent of glycosylation ranging between 10 and 25%; they belong to the multi-copper oxidase family and catalyze the one-electron oxidation of a multitude of substrates (usually diphenols or aromatic amines) coupled with the reduction of dioxygen into two molecules of water. In the blue laccases, the redox process is mediated by four copper ions arranged in three different centres [29]. One type-1 (T1) copper ion is characterized by a strong absorption nearly 600 nm which is responsible for the intense blue colour of these proteins. The T1 copper shows a trigonal coordination, with two histidine and a cysteine residues as conserved ligands; in fungal laccases, either a leucine or a phenylalanine residue occupies the fourth ligand position. The coordination geometry and ligand nature of T1 copper might be responsible for the high redox potential of fungal laccases, as compared to plant laccases and other blue copper oxidases. A second copper ion, coded as type-2 (T2), has a weak absorption in the visible region, is electron paramagnetic resonance (EPR)-active and is coordinated by two histidine residues. The last two copper ions [type-3, (T3)] form a binuclear centre characterized by an absorption at 330 nm; they are EPR-silent due to an antiferromagnetic coupling mediated by a hydroxyl bridge. The two T3 copper ions are coordinated by six histidine residues and are positioned close to the T2 copper ion to form a trinuclear cluster. The T1 copper is the site where substrate oxidation occurs; the extracted electrons are transferred, most likely through a strongly conserved HisCysHis tripeptide motif, to the T2T3 trinuclear cluster, where dioxygen is reduced to water.

Comparative analysis of a large number of fungal laccase sequences suggested the presence of several conserved or semi-conserved residues in addition to the copper-ligating residues. Kumar et al. [16] identified at least four ungapped semi-conserved sequences (coded L1–L4) in more than 60 examined fungal laccases; of these four regions, L2 and L4 were found in multi-copper oxidases too, whereas L1 and L3 are unique to laccases. The 12 amino acid residues involved in copper coordination are housed within these conserved regions. Intra-protein homology between sequences L1/L3 and L2/L4 suggested that the overall structure of laccases may be derived from a gene duplication event. Conformational analysis supported this hypothesis and suggested that the presence of the same residues or amino acid residues with similar conformational propensity and/or hydrophobic index in strategic positions within the conserved regions may be due to an evolutionary pressure aimed at maintaining the unique three-dimensional structure in proximity to the copper sites.

In recent years, the three-dimensional structure of laccases from four fungi has been reported: *Coprinus cinereus* (in a T2 copper depleted form) [6], *Trametes versicolor* [3, 28], *Melanocarpus albomyces* [11] and *Rigidoporus*

*lignosus* [8]; in addition, the three-dimensional structure of the laccase from the endospore coat of *Bacillus subtilis*, crystallized in the presence of ABTS, has been published [7]. Recently, the crystal structure of the main laccase isoform from *T. trogii* (Lcc1) has been reported by Matera et al. [24].

Data from X-ray studies of laccase crystals reveal a common molecular architecture amongst these proteins. The overall fold consists of three sequentially arranged domains (coded A–C) that are organized into a globular structure; each domain exhibits a cupredoxin-like  $\beta$ -barrel topology closely related to that of other blue copper proteins such as azurin, plastocyanin and ascorbate oxidase monomer. The T1 copper is located in domain A (N-terminal portion), whereas the T2T3 trinuclear copper cluster lies at the interface between domains A and C (C-terminal portion). Two disulfide bridges connecting domains A with B and A with C, respectively, stabilize the structure. The T1 copper ion occupies a shallow depression spacious enough to account for the low degree of substrate specificity for laccases. Conversely, one or two narrow channels provide access to dioxygen and enable the release of water from the T2T3 copper cluster. It appears that, at least in ascomycete laccases, a C-terminal peptide may block the dioxygen channel so that a conformational change is necessary to open dioxygen access [11].

The detailed analysis of the substrate binding site indicated that a histidine and an aspartate residue, highly conserved among fungal laccases, exist in close proximity in the inner part of the cavity; these two residues appear to play a role in subtracting an electron from and forming a hydrogen bond with the reducing substrate, respectively, whereas a number of hydrophobic residues contribute to the positioning of the substrate [3]. The unique polar nature of the aspartate in the substrate cavity and the observation that the optimal pH for reducing phenolic substrates is in the range of the aspartate side chain deprotonation, led to the hypothesis that this residue could play a key role in determining the optimal pH for laccases activity.

We have expressed in *Pichia pastoris* a new laccase from *T. trogii* (coded as Lcc2), which shares 69.2% identity with the main isoform coded as Lcc1 [5]. Lcc2 conserves all the amino acid residues which are thought to be essential for laccase activity, including the aspartate located in the inner part of the substrate cavity, and shows an overall fold very similar to that of most laccases with a typical acidic optimal pH. Lcc2 has minor structural differences located on the substrate cavity orifice where a couple of polar residues in Lcc1 have been replaced by hydrophobic residues. A significant difference is that Lcc2 presents an optimal pH in reducing phenolic substrates that is significantly higher than Lcc1 and it conserves about 40% of its activity at pH 6.0. These properties of Lcc2

could be useful for application in biotechnological processes; moreover, the structure of Lcc2 may provide insight for the synthesis of modified laccases with the desired properties for industrial applications. In this study, the catalytic properties of Lcc2 are discussed in relation to the structural information obtained through homology modeling.

## 2 Materials and Methods

### 2.1 Microorganisms

The white-rot fungus *Trametes trogii* 201 (DSM11919) was maintained and propagated as previously described [9]. Liquid cultures (100 ml) in a 2% malt extract and 0.05% tween 80 medium were inoculated with 5 mL of a mycelial suspension obtained from a 5-day preculture and incubated at 28 °C under agitation (180 rpm). To stimulate laccase production, 64 µM CuSO<sub>4</sub> was added to fungal cultures 3 days after inoculation. The mycelium was harvested after 6 days of cultivation, and genomic and total RNA were extracted as previously described [4]. The yeast *Pichia pastoris* GS115, used as host for the expression of *lcc2* cDNA, was purchased from Invitrogen Corporation (Carlsbad, USA) and media and agar plates were prepared according to the manufacturer's protocol. DNA manipulation was carried out using standard procedures [31]. All other chemicals were obtained from Sigma–Aldrich (Weinheim, Germany).

### 2.2 Cloning of the Laccase Gene Fragments

Reverse transcription was carried out using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Degenerate oligonucleotide primers for PCR were synthesized based on the conserved sequences of the copper binding regions II and IV of fungal laccases. The oligonucleotide sense primer (5'-CARTAYTGTYGYGAYGGIYTIMGIGG-3') corresponds to the amino acid sequence QYC DGLRGP and the antisense primer (5'-TCDATRTGR-CARTGIARRAACC-3') corresponds to WFLHCHID. These primers were used to clone DNA fragments encoding novel laccase genes from *T. trogii* by PCR. For each amplification reaction, 2 µL cDNA was used as a template in 50 µL PCR mixtures containing 1 µM of each primer/1.25 U Taq DNA polymerase (Qiagen, Crawley, UK). Amplification was performed with initial denaturation (5 min at 94 °C), 30 cycles (45 s at 95 °C, 45 s at 56 °C, 90 s at 68 °C) and a final elongation step (7 min at 68 °C). Amplified products were visualized on a 1% agarose gel stained with ethidium bromide. After purification using the Wizard SV Kit, the PCR products were cloned using the

pGEM-T Easy Vector System according to the manufacturer's instructions (Promega, Madison, USA). Plasmids derived from cloning were verified by restriction digestion with *EcoRI* followed by sequencing, which was performed on an ABI 310 sequencer using BigDye Sequencing Chemistry (Applied Biosystems, Foster City, USA).

### 2.3 Amplification of the Full-Length *lcc2* Gene

To obtain the 3'-end of the *lcc2* cDNA, 3'-RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, USA). A specific primer (5'-GAGAAGGTGAAGTTGGGGTCGCAAG-3') for RACE-PCR was designed based on the gene fragment sequence previously obtained. RACE-PCR products were amplified on the cDNA according to the manufacturer's instructions and then sequenced. For isolation of the 5'-end, a primer designed to match the ATG region (5'-GAG-CAAGTTCAGTCCCTCGCTGCC-3') was designed based on sequences in the database with a high degree of homology with the known 3'-sequence of *lcc2*. The full-length cDNA was generated using the sense primer (5'-ATGAGCAAGTTCAGTCCCTCGC-3') and the antisense primer (5'-AGTCGGGCAAGCTGTTCACCGG-3'), which were designed based on the sequence of the extreme 5' and 3' ends, respectively. Genomic DNA (20 ng) or cDNA (2 µL) were used as a template for each amplification in 50 µL PCR mixtures containing 1 µM of each primer/1.25 U Taq DNA polymerase. Amplification was performed with an initial denaturation (5 min at 94 °C), 30 cycles (30 s at 95 °C; 90 s at 68 °C) and a final elongation step (7 min at 68 °C). The ORF cDNA of *T. trogii* laccase *lcc2* was cloned and sequenced. The sequence of the *T. trogii* laccase gene *lcc2* has been assigned GenBank data library accession number AM292415.

### 2.4 Phylogenetic Relationships

Phylogenetic analyses were performed using the PHYLIP Package version 3.66. An initial set of fungal sequences was retrieved from the protein sequence databases using the *lcc2* sequence as a probe. The suitable format was generated using the ClustalX program [19]. A distance matrix was calculated from the multiple sequence alignment using the ProtDist program. The matrix was then transformed into a tree using the Neighbor joining method and a rooted tree was constructed using the Drawgram program.

### 2.5 Gene Expression in *P. pastoris*

The coding region of the *lcc2* gene was amplified by PCR with and without the predicted signal peptide, using the following oligonucleotide primers: 5'-GAATTCATGAG

CAAGTTCAGTCCC-3', sense primer including the native signal peptide; 5'-GAATTCGCCATCGGTCCCACCGCCG-3', sense primer without the signal peptide; 5'-AAGCTTTCACCGGCGCGCGTTCGCCG-3', antisense primer. Amplification was performed as previously described. The ORF cDNA of *T. trogii* laccase *lcc2* was cloned into pGEM-T Easy, confirmed by DNA sequencing and cloned into the expression vectors pHIL-D2 and pPIC9 (Invitrogen) under the control of the methanol-inducible alcohol oxidase promoter of *P. pastoris*. The recombinant plasmids, pHIL-D2/*lcc2* and pPIC9/*lcc2*, were digested with *StuI* and *NcoI*, respectively, prior to transformation. *P. pastoris* GS115 (*his4*) cells were transformed by electroporation using the GenePulser II apparatus (Bio-Rad Laboratories, Richmond, USA). Vectors without *lcc2* cDNA were also used to generate control strains. The electroporated cells were plated onto histidine-deficient minimal dextrose (MD, Invitrogen) agar plates and incubated at 30 °C for 72 h, then His<sup>+</sup> transformants were screened on minimal methanol (MM, Invitrogen) agar plates containing the chromogenic compound ABTS which served as reducing substrate for the laccase. The positive activity of the colonies was evaluated by the appearance of a green colour due to the oxidation of ABTS. More than 90% of the transformants were positive for laccase activity, while no positive clones were detected when GS115 cells were transformed with linearized vectors as a control. The transformed yeast colonies were cultured in a 2-L fermentor under the conditions described by Colao et al. [5].

## 2.6 Enzyme Purification and Analyses

The cultural broth was clarified by filtration on 0.45 µm cut-off filters, concentrated by ultrafiltration at 4 °C on a 10 kDa cut-off cellulose membrane (Millipore, Bedford, USA), and equilibrated in 10 mM imidazole.Cl buffer at pH 6.0. The protein concentrate was fractionated by anionic exchange chromatography on a Q-Sepharose Fast Flow matrix (Pharmacia, Sweden); the column (1.5 × 30 cm) was equilibrated at a flow rate of 2 mL min<sup>-1</sup> with 10 mM of the imidazole buffer and the proteins were eluted using a NaCl gradient from 0 to 0.4 M in 350 mL, and from 0.4 to 2 M in 50 mL. The eluate was monitored for absorbance at 280 nm and laccase activity. The active fractions were pooled, concentrated by ultrafiltration and equilibrated in 100 mM sodium phosphate buffer at pH 6.0.

Native-PAGE was carried out on 12% polyacrylamide gels at pH 8.8 under non-denaturing conditions as described by Laemmli [18]; the laccase activity was visualized in the gel with 10 mM *p*-phenyldiamine as substrate in 0.1 M acetate buffer at pH 5.0. The protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, USA), with bovine serum

albumin as a standard and according to the manufacturer's instructions.

Spectrophotometric assays of laccase activity were carried out at 25 °C with 2 mM ABTS, 5 mM DMP or 5 mM 2-methoxyphenol (guaiacol) as substrates, in 0.1 M citric acid, 0.2 M K<sub>2</sub>HPO<sub>4</sub> (McIlvaine) buffer at pH 3.4, in a final assay volume of 1 ml. ABTS oxidation was monitored at 420 nm ( $\epsilon_{\text{mM}} = 36.0 \text{ mM}^{-1} \text{ cm}^{-1}$ ), DMP oxidation at 468 nm ( $\epsilon_{\text{mM}} = 27.5 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and guaiacol oxidation at 470 nm ( $\epsilon_{\text{mM}} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ); the enzymatic activity was expressed as international units (U).

## 2.7 Kinetic Studies

The Michaelis constants ( $K_M$ ) were obtained by assaying the laccase activity at a range of substrate concentrations from  $K_M/4$  to  $4 K_M$ ;  $K_M$  and  $V_{\text{max}}$  values were calculated using the nonlinear regression WinCurveFit (Kevin Raner Software, version 1.1.8); then  $k_{\text{cat}}$  ( $V_{\text{max}}/[E]_{\text{tot}}$ ) and  $K_A$  ( $k_{\text{cat}}/K_M$ ) were derived.

The activity/pH profiles of *lcc2* were determined in McIlvaine buffer with different ratios of 0.1 M citric acid and 0.2 M K<sub>2</sub>HPO<sub>4</sub> to obtain pH values in the range 2.5–7.0. The influence of halides on laccase activity was determined using the spectrophotometric assay with 20 mM DMP as substrate. The halide concentrations producing 50% inhibition ( $I_{50}$ ) were calculated by varying the inhibitor concentration in the assay up to 100 µM F<sup>-</sup>, 80 mM Cl<sup>-</sup>, 600 mM Br<sup>-</sup>.

## 2.8 Modeling of Lcc2

The 3-D model of Lcc2 was based on the three-dimensional co-ordinates of the highly similar *T. trogii* Lcc1 [24] (Protein Data Bank code 2hrq). The Swiss Model program was used to build the protein model according to comparative methodology [10]. The secondary structure was assigned by the DSSP program [14]. The stereo chemical quality was evaluated by the PROCHECK program [20]. Figures were constructed using the Swiss PDB Viewer [10] and Pymol (Delano scientific, <http://pymol.sourceforge.net>) programs. ABTS 3-D co-ordinates were downloaded from the Protein Data Bank as a ".pdb" file. The ABTS docking simulation was performed using the Molegro Virtual Docker program (<http://www.molegro.com>).

## 3 Results

### 3.1 Cloning of the *T. trogii* 201 *lcc2* cDNA

A PCR-based approach was used to amplify the laccase-encoding sequences, using primer sets based on conserved

copper-binding domains of previously cloned basidiomycete genes. A PCR product of approximately 1,000 bp was amplified from cDNA generated from RNA transcripts collected from mycelium grown on malt extract liquid medium supplemented with copper. Sixty clones were subjected to restriction analysis and fragments of four putative laccase isoforms were identified and assigned the names *lcc1*, *lcc2*, *lcc3* and *lcc4*; one of them, *lcc1*, corresponded to the previously isolated gene from *T. trogii* that encodes the predominant laccase Lcc1 [4]. Using a RACE protocol, a second laccase gene, *lcc2*, was cloned and sequenced. A 1,855 bp cDNA fragment was analysed and the obtained sequence contained an ORF of 1,587 bp, which corresponded to a precursor protein of 528 amino acid residues with a 22 residues leader sequence. A molecular mass of 56.8 kDa was calculated for the protein encoded from the translated DNA sequence. The characteristic laccase signature sequences, comprising of one cysteine and ten histidine residues involved in the binding of four copper ions, were conserved in the *lcc2* ORF and six potential N-glycosylation sites were present. The overall identity at the protein level between Lcc1 and Lcc2 was 69%, while the similarity was 87%. In comparison to Lcc1, Lcc2 contained 11 extra amino acid residues, six of which constituted the carboxy terminus. A data bank search determined that the nearest *lcc2* cDNA neighbours were: Lac2 from *Trametes* C30 (94% identity, 99% similarity), Lac 2 from *Polyporus brumalis* (82% identity, 94% similarity), Lcc 3–2 from *Polyporus ciliatus* (81% identity, 93% similarity), Lac3 from *Trametes* C30 (75% identity, 91% similarity).

### 3.2 Expression of Lcc2 in *P. pastoris*

To further characterize Lcc2, the corresponding cDNA was expressed in the yeast *P. pastoris*, under the control of the tightly regulated alcohol oxidase promoter induced by methanol, by either the native signal sequence or the  $\alpha$ -factor signal peptide from *Saccharomyces cerevisiae* to direct the secretion of the recombinant protein. The enzymatic activity was detected on minimal methanol plates containing ABTS: a green halo surrounding the colonies, revealing the production of active recombinant enzyme secreted in the medium, was only present when the  $\alpha$ -factor

prepro signal sequence was used as a signal peptide. Transformants exhibiting a deeper colour were used for the production of the recombinant protein under controlled conditions in a 2-L fermentor. The expression of the recombinant laccase was obtained using high cell density cultures by adjusting the methanol feed rate to enable full induction of the alcohol oxidase promoter-driven expression, as well as to avoid oxygen limitation at high cell density and prevent the methanol accumulation at levels toxic to the cells. Under the tested conditions, a laccase activity of 340 U L<sup>-1</sup> was reached after 6 days at a dry cell weight of 123 g L<sup>-1</sup> with a specific productivity of 2.7 U g<sup>-1</sup> biomass; the total soluble protein concentration after 6 days was 286 mg L<sup>-1</sup>.

### 3.3 Physico-Chemical Properties and Kinetic Parameters

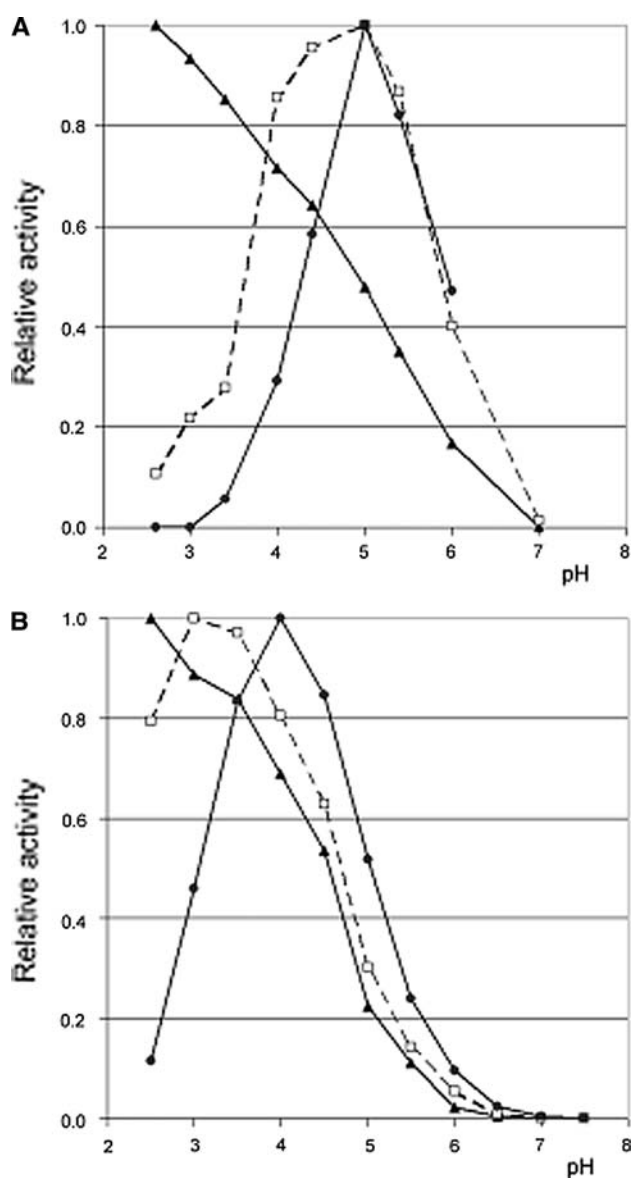
The recombinant Lcc2 was purified from the culture medium of *P. pastoris* by a single step anionic exchange chromatography that increased the specific activity of the enzyme two-fold. SDS–polyacrylamide gel electrophoresis showed that the recombinant enzyme had a molecular weight of 70,000, which was higher than the predicted probably because of glycosylation in *P. pastoris* (data not shown).

The kinetic parameters of the recombinant Lcc2 for three common laccase substrates in comparison to those of the previously characterized recombinant Lcc1 laccase from *T. trogii* [5] are shown in Table 1. Lcc2 exhibited a significantly different catalytic behaviour than Lcc1; in particular, Lcc2 had a lower efficiency ( $k_{\text{cat}}/K_{\text{M}}$ ) in oxidizing ABTS, due to an approximately 20-fold higher  $K_{\text{M}}$  value, whereas the efficiency towards the phenolic substrates was the same order of magnitude than that of Lcc1, since an increase in the affinity for the substrates (decrease in  $K_{\text{M}}$ ) compensates for the general decrease in the kinetics of electron transfer (decrease in  $k_{\text{cat}}$ ) exhibited by Lcc2.

The effect of the pH on the activity of Lcc2 with the substrates guaiacol, DMP and ABTS is shown in Fig. 1. Considering all the phenolic and non phenolic substrates, Lcc2 exhibits an activity over a wider pH range than Lcc1. Of particular interest are the pH/activity profiles of Lcc2

**Table 1** Kinetic constants of recombinant laccases

Substrate	Recombinant Lcc2			Recombinant Lcc1		
	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}}$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}}$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )
ABTS	218 $\pm$ 11	347 $\pm$ 7	1.6	9 $\pm$ 1	5899 $\pm$ 100	641
DMP	117 $\pm$ 22	107 $\pm$ 6	0.9	529 $\pm$ 27	3339 $\pm$ 55	6
Guaiacol	961 $\pm$ 38	30 $\pm$ 1	3 $\times$ 10 <sup>-2</sup>	4177 $\pm$ 70	305 $\pm$ 3	7 $\times$ 10 <sup>-2</sup>



**Fig. 1** a pH/activity profiles of Lcc2, with the substrates ABTS (filled triangle), DMP (empty square) and guaiacol (filled square), determined at 25 °C in McIlvaine buffer in the pH range 2.5–7.0. The pH/activity profiles of recombinant Lcc1 are provided in (b) for comparison

with phenolic substrates, as they are 1–1.5 pH units closer to neutrality, as compared to those obtained for Lcc1.

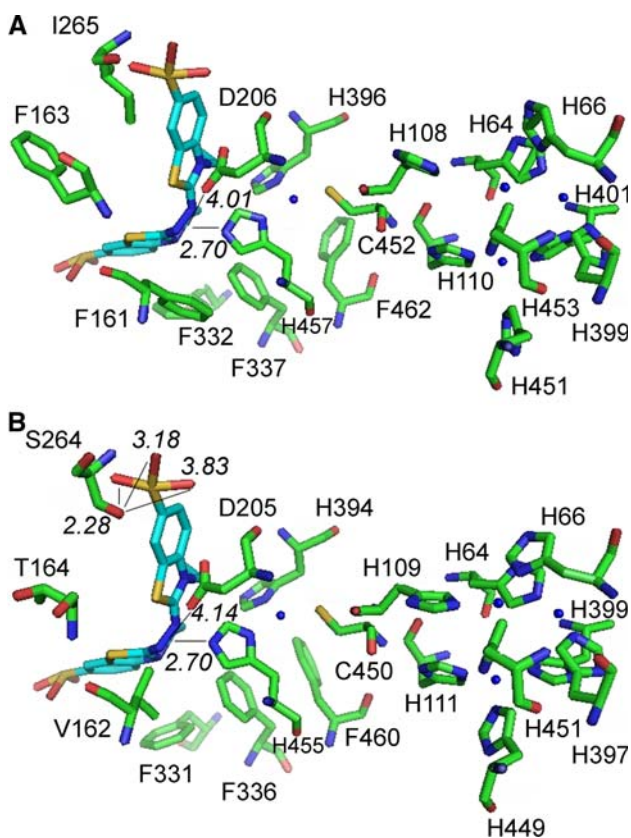
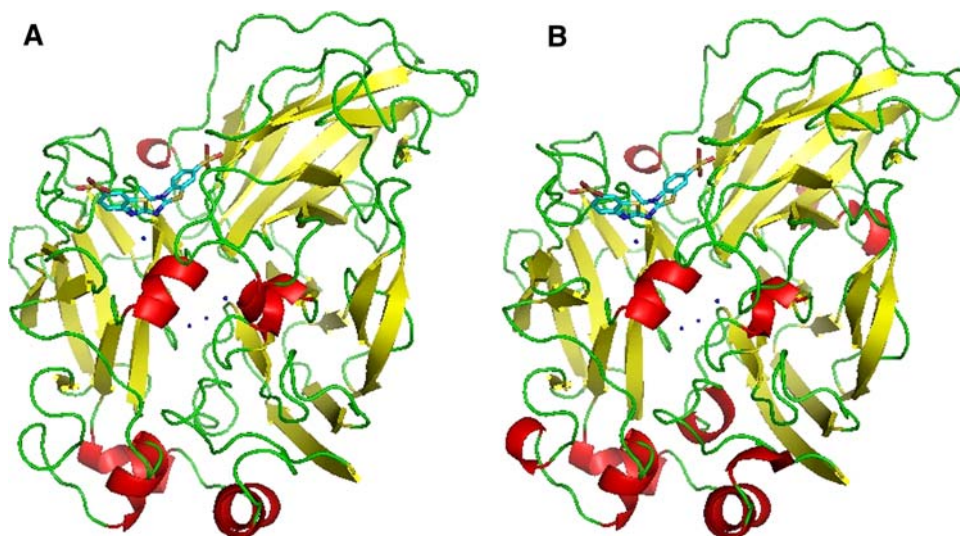
The effect of halides on the activity of the two recombinant laccases Lcc2 and Lcc1 with the substrate 2,6-dimethoxyphenol was analysed and, in accordance with our previous data [9], the inhibition order observed for both proteins was  $F^- > Cl^- > Br^-$ . The concentration of fluoride producing 50% inhibition ( $I_{50}$ ) for both laccases was comparable (0.02 mM); conversely, the bulkier halides  $Cl^-$  and  $Br^-$  were more efficient in inhibiting Lcc2 ( $I_{50}$  was 4 and 50 mM, respectively), as compared to Lcc1 ( $I_{50}$  was 18 and 340 mM, respectively).

### 3.4 Modeling the Lcc2 Three-dimensional Structure

The three-dimensional structure of Lcc1 laccase from *T. troglia* [24] was used as a template for the construction of the Lcc2 model. The best alignment of the two protein sequences (69.2% identity) was used to construct the three-dimensional model, and the stereo-chemical quality was assessed using the PROCHECK program [20]. The Ramachandran plot confirmed the model, with the percentage of residues in most favoured and additional allowed regions being higher than 98.2 (data not shown). The overall fold of Lcc2 is very similar to that of Lcc1, including the elements of secondary structure that mainly consist of antiparallel  $\beta$ -barrels; the differences are restricted mainly in loops where amino acid residue insertions or deletions occur (Fig. 2). Similarly to Lcc1 and most monomeric fungal laccases [11, 24, 28], the Lcc2 molecular architecture shows a globular protein formed from three sequentially arranged domains that could be approximately recognized in amino acid sequences 1–156 (domain A), 157–340 (domain B) and 341–506 (domain C). Lcc1 has two carbohydrate chains on Asn 54 and Asn 433, respectively; since the two consensus sequences Asn-XXX-Thr (54–56 and 435–437) are conserved in Lcc2, we propose that this protein is glycosylated at these positions. The carbohydrate chains account for the difference observed between the calculated molecular mass (56,808) and the value estimated by SDS-PAGE (approximately 70,000). According to the Lcc1 reference structure, it is conceivable that Lcc2 is also stabilized by two disulfide bridges connecting the A and B (Cys 117–Cys 205) and A and C (Cys 85–Cys 487) domains, respectively.

The basic architecture of Lcc2 copper and substrate sites is very similar to that of Lcc1 (Fig. 3) and the residues coordinating the four copper ions are conserved. The binding cavity for the reducing substrate is close to the T1 copper site, and is rather wide, which allows the accommodation of molecules of various sizes. In the interior of the cavity, His 457, one of the residues that coordinates T1 copper, and Asp 206 (corresponding to His 455 and Asp 205, respectively, of Lcc1) are located in close proximity to one other (about 5 Å). This histidine residue, which is highly conserved in the fungal laccases, is thought to initiate the catalytic cycle by subtracting an electron from the reducing substrate, while the negatively charged Asp residue can stabilize the radical cation that is formed [3, 28]. In addition to these polar contacts, a number of hydrophobic protein-ligand interactions take place between the aromatic ring(s) of the substrates and the lateral chains of Lcc2 apolar residues, such as Phe 161, Phe 332 and Phe 337, which correspond in Lcc1 to Val 152, Phe 331 and Phe 336, respectively, and delineate part of the substrate orifice. The most striking difference between Lcc1 and

**Fig. 2** Three-dimensional structures of *Trametes trogii* laccases Lcc2 (a) and Lcc1 (b). The Swiss Model program was used to build the Lcc2 model according to the three-dimensional co-ordinates of the highly similar Lcc1 laccase. Copper ions are represented by *small dots*. Docking of ABTS in the substrate cavity is also shown



**Fig. 3** Amino acid residues present at T1 and T2/T3 copper sites of Lcc2 (a) and Lcc1 (b) laccases. Copper ions are represented by *small dots*; docking of ABTS in the substrate cavity is also shown

Lcc2 substrate sites is observed on the opposite side of the cavity, where the polar residues Thr 164 and Ser 264 of Lcc1 are replaced by Phe 163 and Ile 265 in Lcc2, thus affecting the capacity of this protein to interact with polar groups present on the substrate.

#### 4 Discussion

The most widely studied laccases are those secreted by basidiomycete fungi where these enzymes are encoded by gene families. *T. trogii*, a typical basidiomycete, produces at least five laccase isoforms (Garzillo et al., unpublished observations). The predominant isoform, coded as Lcc1, has been purified and characterized [9]; the purification and characterization of the other isoforms is time-consuming since they account altogether for less than 10% of the total laccase activity. Thus, we amplified laccase-encoding genes from *T. trogii* cDNA using degenerate primers designed from conserved regions of basidiomycete laccases. We identified a new laccase gene, *lcc2*, that we cloned, sequenced and expressed in the heterologous host *P. pastoris*, a methylotrophic yeast that has been used to express several fungal laccases [5, 21, 32]. Since the minor *T. trogii* laccase isoforms have not yet been characterized, we were unable to establish a sequence-enzyme link between the product of *lcc2* and one of the native fungal laccases.

The predicted protein encoded by the *lcc2* gene displays a high degree of amino acid sequence similarity with the other basidiomycete laccases that have been characterized thus far. The phylogenetic analysis, based on sequence alignment of the predicted protein, indicated that Lcc2 from *T. trogii* grouped together with Lac2 from *Trametes* C30, which is the closest neighbour, while Lcc1, the other *T. trogii* sequence, formed an independent branch (data not shown). The two *T. trogii* laccase encoding genes are apparently paralogous and could have evolved through duplication-divergence events, as has been suggested for other laccases [23]. According to a detailed analysis of laccases from basidiomycetes, it has been suggested that clustering of sequences reflects at least partially the function of the respective enzymes [12]. The *T. trogii* laccases

Lcc1 and Lcc2 grouped together with other enzymes from typical wood decay species but they are separated on the Neighbor joining tree, which suggests that these two enzymes have different functions. In fact, the two laccase isoforms produced by *T. trogii* display different biochemical properties and the recombinant Lcc2 exerts a distinct activity towards aromatic substrates exhibiting an optimal pH closer to neutrality than Lcc1.

In recent years, laccase crystals have been obtained to determine the three dimensional structure of the molecule. Until now, the crystal structures of laccases from several basidiomycete fungi (*C. cinereus* [6], *T. versicolor* [3, 28], *Pycnoporus cinnabarinus* [1], *R. lignosus* [8], *T. trogii* [24]), from the ascomycete *M. albomyces* [11] and from the prokaryotic organism *B. subtilis* [7] have been described; one of these crystalline structures was found to be a T2 copper depleted form [6], whereas the others contained the full complement of four copper ions. In addition, three of the described crystalline laccases bind a ligand (2,5-xylidine, ABTS or p-methylbenzoate, respectively) in the substrate cavity [3, 7, 24].

Despite their different origins and the absence, in one case, of a metal ion essential for enzymatic activity, the overall structure of the crystalline laccases is very similar. The crystal analysis demonstrated that the substrate cavity is wide enough to accommodate molecules of various sizes. Small aromatic molecules (DMP, guaiacol, 2,5-xylidine, etc.) are completely embedded in the cavity where the hydrophobic interactions with apolar amino acid residues predominate [3]; larger ligands, such as ABTS, assume a non-linear structure with part of the molecule buried in the cavity, while the other part interacts with the amino acid residues surrounding the substrate binding cavity [7]. In the inner part of the hydrophobic substrate cavity of laccases a highly conserved aspartic acid residue is present, very close to the functionally active histidine residue, which appears to have an intriguing role [3]. The presence of this dissociable polar residue has been associated with the optimal acidic pH for substrate oxidation, as substitution of this residue with an uncharged residue, such as asparagine or alanine, reduces the laccase activity at low pH values [22].

Our model of Lcc2 from *T. trogii* has a substrate cavity very similar to those of previously crystallized laccases with conservation of the aspartic acid residue and its close proximity to the histidine residue. We observed a primary difference between Lcc1 and Lcc2 with regard to two spatially close residues surrounding the substrate cavity: Thr 164 and Ser 264 of Lcc1 are replaced by the two hydrophobic residues Phe 163 and Ile 265, respectively, in Lcc2. This variation affects the capacity of Lcc2 to interact with ligands. The catalytic efficiency of Lcc2 towards bulky ligands carrying polar groups (e.g., ABTS) significantly decreases due to both an increase in  $K_m$  and decrease

in  $k_{cat}$  (Table 1), indicating a concomitant decrease in the affinity for the substrate and kinetics of electron transfer. As suggested by the ABTS docking simulation (Fig. 3), the sulphonate group of the exposed part of ABTS is hydrogen bonded in Lcc1 to the hydroxyl group of Ser 264; the substitution of this residue with a polar one (Ile 265) in Lcc2 weakens the interaction with the bulky substrate and may explain the lower affinity of Lcc2 for ABTS as compared to Lcc1. Conversely, the catalytic efficiency of Lcc2 towards small hydrophobic substrates (e.g., guaiacol, DMP) moderately decreased as compared to Lcc1, due to an approximate 5-fold increase in the affinity for the substrates and to a more sensible decrease in  $k_{cat}$  (Table 1). The increase in the affinity of Lcc2 towards hydrophobic ligands which are embedded in the substrate cavity could be due to an induced-fit closing movement of the cavity mediated by the amino acid residues of the border loops closest to the ligand. Bertrand et al. [3] observed that the substrate cavity of the *T. versicolor* laccase LacIIIb binding to 2,5-xylidine was more closed than that of *C. cinereus* LacCc. The substrate cavity of LacIIIb was surrounded by a number of hydrophobic amino acid residues higher than LacCc; in particular, a Leu and Phe residue in LacIIIb replaced a Gln and Lys residue in the corresponding positions of LacCc. The decrease in  $k_{cat}$  that we observed in Lcc2, as compared to Lcc1, indicates that the electron transfer from the substrate to the T1 copper, which is the rate-limiting step in laccase kinetics, must have been affected by the structural differences between the two laccase isoforms. However, it cannot be excluded that the internal transfer of electrons from the T1 copper to the T2T3 trinuclear cluster, then to the dioxygen molecule, and the product release have been affected. It is widely accepted [15, 35] that anions such as hydroxyl and halides bind to the T2T3 trinuclear cluster and inhibit laccase activity. The significant increase in inhibition of Lcc2 activity by bulky halides, such as chloride and bromide, as compared to Lcc1 might be attributed to a structurally altered channel that connects the trinuclear site to the solvent and participate in the electron transfer to the substrate and product release.

In a recent study, Madzak et al. [22] observed that replacing the aspartic residue in the substrate cavity of *T. versicolor* laccase with less polar or apolar residues, such as asparagine or alanine, alters the optimal pH towards phenolic substrates; in particular, there is a shift in the optimal pH for DMP towards higher pH values of 0.5 and 1.4 units with Asp → Ala and Asp → Asn substitutions, respectively. In both cases, the observed shift in pH was attributed to a corresponding shift of the ascending part of the activity/pH profile. Lcc2 from *T. trogii* shows an optimal pH towards phenolic substrates of about 5 (Fig. 1) with a shift towards higher pH values of 1.6 and 1.0 units



for DMP and guaiacol, respectively, as compared to Lcc1. It is worthwhile to note that the whole pH/activity profile of Lcc2 with phenolic substrates appears shifted towards values closer to neutrality, so the enzyme conserves about 40% of its activity at pH 6.0. Finally, at the optimal pH, Lcc2 shows a higher affinity (lower  $K_m$  values) towards the phenolic substrates, as compared to Lcc1.

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