

Heterologous expression and structural characterization of two low pH laccases from a biopulping white-rot fungus *Physisporinus rivulosus*

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Abstract The lignin-degrading, biopulping white-rot fungus *Physisporinus rivulosus* secretes several laccases of distinct features such as thermostability, extremely low pH optima and thermal activation for oxidation of phenolic substrates. Here we describe the cloning, heterologous expression and structural and enzymatic characterisation of two previously undescribed *P. rivulosus* laccases. The laccase cDNAs were expressed in the methylotrophic yeast *Pichia pastoris* either with the native or with *Saccharomyces cerevisiae* α -factor signal peptide. The specific activity of rLac1 and rLac2 was 5 and 0.3 μ kat/ μ g, respectively. However, mutation of the last amino acid in the rLac2 increased the specific laccase activity by over 50-fold. The recombinant rLac1 and rLac2 enzymes demonstrated low pH optima with both 2,6-dimethoxyphenol (2,6-DMP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate). Both recombinant laccases showed moderate thermostolerance and thermal activation at +60 °C was detected with rLac1. By homology modelling, it was deduced that Lac1 and Lac2 enzymes demonstrate structural similarity with the *Trametes versicolor* and *Trametes trogii* laccase crystal structures.

Comparison of the protein architecture at the reducing substrate-binding pocket near the T1-Cu site indicated the presence of five amino acid substitutions in the structural models of Lac1 and Lac2. These data add up to our previous reports on laccase production by *P. rivulosus* during biopulping and growth on Norway spruce. Heterologous expression of the novel Lac1 and Lac2 isoenzymes in *P. pastoris* enables the detailed study of their properties and the evaluation of their potential as oxidative biocatalysts for conversion of wood lignin, lignin-like compounds and soil-polluting xenobiotics.

Keywords Laccase · Phenol oxidase · Thermostolerance · White-rot fungi · *Physisporinus rivulosus* · Basidiomycete · 3-D homology modelling · Wood decay · Lignin degradation

Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyse the one-electron oxidation of a variety of phenolic compounds, aromatic amines and low-redox potential substrates with the concomitant reduction of O₂ to H₂O. Laccases are widely distributed in nature, occurring in fungi as well as in plants, insects, bacteria and archaea (Hoegger et al. 2006; Lundell et al. 2010; Giardina et al. 2010; Uthandi et al. 2010). Although fungal laccases have been repeatedly associated with the depolymerisation of lignin, their actual role in lignin and wood biodegradation is uncertain. When fungal laccases are combined with oxidation mediator substrate compounds, their enzymatic capacities are improved (Bourbonnais et al. 1997; Call and Mücke 1997; Koroleva et al. 2001; Martinez et al. 2004; Hatakka and Hammel 2011).

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Multiple laccase genes are recognized in the genomes of lignocellulose-decaying basidiomycetes as well as in mycorrhizal and plant-pathogenic fungi and in ascomycetous filamentous fungi (Kilaru et al. 2006; Courty et al. 2009; Martinez et al. 2008).

One of the most studied lignin-degrading white-rot fungi, *Phanerochaete chrysosporium*, was reported to be deficient in laccase-encoding genes (Martinez et al. 2004; Kersten and Cullen 2007), while one or several laccase isoenzymes are usually expressed by wood-decaying fungi (Hakala et al. 2005; Mäkelä et al. 2006; Saparrat et al. 2010). Due to the broad substrate specificity and oxidation capacity, there is potential to use laccases in industrial and environmental applications (Hildén et al. 2009; Tuomela and Hatakka 2011). For these applications, the research focus is on the molecular characterization of fungal laccases as well as on the improvement of the production levels by recombinant expression.

Physisporinus rivulosus (Berk. & Curt.) Ryvardeen (synonym *Ceriporiopsis rivulosa*) is a selectively lignin-degrading, white-rot basidiomycete species, which is able to grow in a wide temperature range and efficiently decompose softwood lignin (Hakala et al. 2004). Pre-treatment of Norway spruce chips by cultivation of *P. rivulosus* strain T241i resulted in a 20 % decrease in the consumption of energy in mechanical pulping of wood (Hatakka et al. 2003). Under these biopulping conditions and with growth on spruce wood, *P. rivulosus* secreted, in addition to manganese peroxidases (MnPs) and oxalic acid, several laccases as multiple isoenzymes with low *pI* values (*pI* 3.5 to 4.8) (Hakala et al. 2005). Two of the isoenzymes demonstrated extremely low pH optima (below pH 3.5) for oxidation of phenolic substrate compounds with simultaneous thermostability and activation at elevated temperatures (Hildén et al. 2007). In defined liquid media, however, production of laccases by *P. rivulosus* is limited without lignocellulose, such as spruce wood sawdust as the sole carbon source (Hildén et al. 2007).

In the current study, we describe gene characteristics and heterologous expression in *Pichia pastoris* of two laccase-encoding genes (*lac1* and *lac2*) of *P. rivulosus* T241i. The 3-D protein homology models of the predicted Lac1 and Lac2 enzymes illustrate a typical four copper laccase fold but indicate differences in protein topology at the T1-Cu and substrate-binding site (Bertrand et al. 2002; Piontek et al. 2002). The recombinant laccases demonstrate low pH optima (pH 3.5) and moderate thermotolerance for recombinant Lac1 with the laccase substrates 2,6-dimethoxyphenol (2,6-DMP) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate). Due to their unique biochemical properties, the recombinant *P. rivulosus* laccases may be utilized in biocatalytic applications, leading to conversion of lignin-like phenolic compounds, dyes or environmentally hazardous

xenobiotics, under increased acidity or higher process temperature conditions. Via successful heterologous expression in *P. pastoris*, prominence for detailed molecular and functional characterisation of the low pH and thermotolerant laccases can be envisioned.

Materials and methods

Microbial strains

The white-rot fungus *P. rivulosus* (synonym *C. rivulosa*) strain T241i (FBCC949, DSM14618) is derived from a fruiting body found on a burned spruce wood log in a forest fire area in southern Finland (Hakala et al. 2004). The fungus is maintained on 2 % (wt/vol) malt extract agar (1.5 % wt/vol, Biokar, Beauvais, France) and deposited in the Fungal Biotechnology Culture Collection, University of Helsinki, Finland (fbcc@helsinki.fi). *P. pastoris* strain GS115 was purchased from Invitrogen (Gibco-BRL, Gaithersburg, USA).

Fungal culture conditions

The production of *P. rivulosus* laccase activity was followed in yeast extract–peptone (YEP) complex liquid medium and in wheat or rye bran-containing media. YEP medium contained 0.2 % (wt/vol) yeast extract (Oxoid Ltd, Basingstoke, UK), 0.5 % peptone (Oxoid Ltd) and 0.5 % (wt/vol) glucose (Fisher Scientific Ltd, Loughborough, UK) in distilled water. Bran media contained 2 % (wt/vol) wheat bran (Neal's Yard, Burton upon Trent, UK) or 2 % (wt/vol) rye bran (Raisio, Finland) in distilled water. The pH was adjusted to 4.5 prior autoclaving in all media used. Three parallel flasks, each containing 100 ml of cultivation medium, were inoculated with three agar plugs (10 mm in diameter) covered with 14-day grown *P. rivulosus* mycelium on 2 % (wt/vol) malt extract agar plates. The cultures were incubated stationary at 28 °C for 21 days. Aliquots of 1 ml were taken twice a week for laccase activity measurement.

Assay of laccase activity and steady-state kinetic measurements

Laccase activity was determined spectrophotometrically at 420 and 476 nm by following the oxidation of ABTS (Sigma, St. Louis, USA) and 2,6-DMP (Aldrich, St. Louis, USA), respectively, at pH 3.0 in 50 mM sodium malonate buffer. A Shimadzu PharmaSpec UV-1700 spectrophotometer was used in all the measurements. Laccase activity is expressed as $\mu\text{kat l}^{-1}$ ($10^{-6} \text{ mol s}^{-1} \text{ l}^{-1}$) of the specific product formed upon oxidation of each substrate. Steady-state kinetic parameters (K_m and k_{cat}) were determined by

using 2,6-DMP as reducing substrate. Triplicate reactions were initiated by the addition of 0.001–2.0 mM of 2,6-DMP. The K_m and V_{max} values were calculated based on Lineweaver–Burk plots and the k_{cat} values were calculated as $V_{max}/rLac$ protein concentration. The protein concentrations were measured by BCA Protein Assay Reagent (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) using bovine serum albumin as standard to determine specific laccase activities.

Isolation of nucleic acids and cDNA synthesis

Mycelia from the 7-, 14- and 21-day cultures were filtered through Miracloth (Calbiochem, Darmstadt, Germany), frozen to $-80\text{ }^\circ\text{C}$ and ground with a mortar and pestle in liquid N_2 . DNA was extracted by using a *N*-cetyl-*N,N,N*-trimethylammonium bromide-based buffer and purified with chloroform (Hildén et al. 2005). Total RNA was extracted by Trizol (Invitrogen) following the manufacturer's instructions. RNA samples were treated with RNase-free DNase (1 U/ μg ; Fermentas, Vilnius, Lithuania) at $37\text{ }^\circ\text{C}$ for 90 min.

Prior to cDNA synthesis, the total RNA was used as a template for PCR to ensure the absence of genomic DNA in the RNA samples. Synthesis of cDNA from 1 μg of total RNA and rapid amplification of cDNA ends (RACE) and genome-walking PCR were performed as described previously (Hildén et al. 2005; Mäkelä et al. 2006).

Amplification and cloning of laccase cDNAs and genes

P. rivulosus DNA was used as a template for the amplification of a 230- and 231-bp fragment of *lac1* and *lac2* genes, respectively, with degenerate laccase primers (D'Souza et al. 1996). The 25- μl PCR reaction contained 0.5 μl DNA template, 0.3 mM dNTP mixture (Fermentas), 0.5 μM 5' and 3' primers, 1 \times Phusion HF buffer (Finnzymes, Espoo, Finland), 3 % DMSO and 0.6 U Phusion Hot Start DNA polymerase (Finnzymes). PCR was performed with initial denaturation at $98\text{ }^\circ\text{C}$ for 30 s, then 40 cycles of denaturation at $98\text{ }^\circ\text{C}$ for 10 s, annealing at $57\text{ }^\circ\text{C}$ for 30 s, elongation at $72\text{ }^\circ\text{C}$ for 30 s and final extension at $72\text{ }^\circ\text{C}$ for 5 min.

A Smart Race cDNA Amplification kit (Clontech, Palo Alto, CA, USA) was used for amplification of the *lac1* cDNA ends. The 5' end of the transcript was amplified with the universal primer mix (UPM, Clontech) and a degenerate antisense laccase primer (Mäkelä et al. 2006) with cDNA extracted from *P. rivulosus* which was cultivated on wheat bran. In the nested PCR, an inner gene-specific antisense primer of *lac1* (CAGAAGGTACCGGCTTGGT) was used with a nested universal primer (NUP, Clontech). The 3' end of *lac1* was amplified with a gene-specific sense primer (TTTGTGAACCAATGTCCGA) and UPM. In the second

amplification round, nested gene-specific sense primer (CCCGAACTGGGTATTTTCGATCG) and NUP were used (Hildén et al. 2005). The full-length open reading frame (ORF) was amplified with the primer pair: sense CAAGCATGGCTCGCTTCC and antisense CACGTATCAGAAGGGTCAAGC.

The 5' and 3' ends of *lac2* gene were amplified by the inverse PCR method (Halaoui et al. 2006). One microgram of DNA from *P. rivulosus* was digested with *EcoRI* or *HindIII* (New England Biolabs, Hitchin, UK). The digested DNA was circularised with 5 U of T4 ligase (New England Biolabs) and used as PCR template. The gene-specific primer pair (sense: GACTTCAGCGTCCCGGACCA, antisense: GACAAATGCGGGACCA) was used in the first round of PCR. In the second PCR round, the nested gene-specific primer pair (sense: AACTCGCTGATATTGCGCACGA, antisense: CGATTGTCGTAGACTCGAGCA) was used. The full-length ORF was amplified with the primer pair: sense ATGCCTAACTTCCTGTGCG and antisense TCA CAGCTCATCGGAGGG. PCR reactions were conducted as described earlier.

The full-length ORF of laccase cDNA and gene fragments were cloned into the pJET1.2/blunt vector (Fermentas) and sequenced.

Protein characteristics and 3-D homology modelling

Protein sequence properties were predicted from the translated ORF sequences using Swissprot and EMBL web tools, the N-glycosylation software (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and secretion and targeting signal peptide prediction (<http://www.cbs.dtu.dk/services/SignalP/> and <http://www.cbs.dtu.dk/services/TargetP/>). Modelling of protein structures was performed with Modeller v9.4 software (Sali et al. 1995). Deduced crystal structure models of laccase from *Trametes versicolor* (PDB accession 1KYA and 1GYC) that showed the best homology to *P. rivulosus* Lac1 (70 % identity) were used as templates for homology modelling. Laccase 3-D structures from *Trametes trogii* (PDB accession 2HRH and 2HRG) were used as templates for *P. rivulosus* Lac2, showing 68 % identity. Verification of the structural models was performed with ProCheck v.3.4 (Laskowski et al. 1993) and ProSa2003 v.4.0 (Sippl 1993). The root mean square deviation between the main chain atoms of the modelled structures and templates was calculated to estimate the reliability of the 3-D models. Substrate-free models for Lac1 and Lac2 were overlaid onto the crystal structure of 1KYA containing one molecule of 2,5-xylydine, and 2HRG containing one molecule of *p*-methylbenzoate as a ligand in the reducing substrate-binding site, and using GRASP2 (Petrey and Honig 2003) and PyMol v.0.99 (DeLano 2002) software, which was also used for the calculation of molecular electrostatic potentials and visualization of the 3-D structural models.

Heterologous expression of *P. rivulosus* laccases

To construct plasmids for the heterologous expression in *P. pastoris*, the *lac1* and *lac2* encoding cDNAs were cloned into pPICZB together with their native N-terminal signal peptide sequences and into pPICZ α A (Invitrogen) downstream of the α -mating factor signal peptide sequence from *Saccharomyces cerevisiae*. Primer pairs *lac1* sense: GTGCTGGAATTCTCAAGCATGGCTCGC and *lac1* antisense: TAAC TAGGCGGCCCGCCGACGTATCAG and *lac2* sense: AAC GAGGAATTCAATAATGCCTAACTTC and *lac2* antisense: GAAAGCTGGCGGCCGCGCACAGCTCATC were used to clone cDNAs with the native signal sequence. Sense primers *lac1*: GGCAGCAATTCGCCATCGGG and *lac2*: GCTGAAGCTGAATTCGCGATCGGCCCT with antisense primers as described above were used to clone cDNAs without the native signal sequence. An additional antisense primer (GAAAGCTGGCGGCCGCGCACACCTCATC) was designed to amplify a 3'-modified *lac2* cDNA encoding the variant Lac2-L497V with mutated C-terminus in order to avoid possible endoplasmic reticulum (ER) retention. The resulting expression constructs were confirmed by sequencing. Expression of inserts in both vectors is controlled by the methanol-inducible *AOX1* promoter. Both expression vectors contain C-terminal peptides containing the c-myc epitope and a polyhistidine (6 \times His) tag for detection and purification of recombinant fusion proteins. The plasmid constructs were linearized by *SacI* digestion and transformed into *P. pastoris* GS115-competent cells by electroporation. The transformants were selected on zeocin (100 μ g/ml) containing YEP plates. Several zeocin-resistant clones were randomly picked and spotted onto buffered minimal medium (BMM, Invitrogen; pH 6.0) 2 % (wt/vol) agar plates containing 0.004 % histidine, 4×10^{-5} % biotin, 0.5 % methanol, 0.3 mM CuSO₄ and 0.2 mM ABTS (Li et al. 2007). The plates were incubated at 28 °C for 4 days. Positive *P. pastoris* transformants were identified by the appearance of a green zone around laccase-expressing colonies.

According to the intensity of the green zone, ten of the positive transformant colonies were selected for laccase expression. The selected transformants were cultivated in liquid BMG medium (Invitrogen) containing 1 % glycerol at 28 °C in a rotary incubator until the OD₆₀₀ was approximately 6–8. The cultures were centrifuged (1,200g at 4 °C for 5 min) and washed with phosphate-buffered saline. The samples were re-centrifuged and the cell pellets were diluted with BMM medium supplemented with 0.3 mM CuSO₄ and 0.8 % (wt/vol) alanine to an OD₆₀₀ of 1 (Soden et al. 2002). Transformants were cultivated at 20 °C, with shaking at 200 rpm, and 0.5 % (vol/vol) methanol was added daily to maintain the induced production of the recombinant laccase. Cultivation time was 5–7 days and extracellular laccase activity was followed daily using the ABTS assay. The

highest laccase activity-producing transformants of rLac1 and rLac2 were selected for the scale-up cultivations.

Purification of recombinant laccases

The culture fluids of *P. pastoris* were harvested on days 5–6 and ca. 2-l batches were concentrated using Spin-X UF tubes (Corning, Loughborough, UK) with 30-kDa cutoff membrane filter at 4 °C. The extracellular protein concentrates were stored at –20 °C. The concentrates were purified by Ni-affinity chromatography using the ÄKTA Explorer apparatus (GE Healthcare, Uppsala, Sweden). The concentrates were dialyzed against 20 mM phosphate buffer (pH 7) containing 500 mM NaCl and 20 mM imidazole (Sigma-Aldrich) with Vivaspin 2 centrifugal concentrators (10 kDa cutoff, Sartorius Stedim Biotech GmbH, Göttingen, Germany). Samples were fed into HisTrap™ FF Crude column (GE Healthcare) and eluted with 500 mM NaCl and 500 mM imidazole in phosphate buffer with stepwise gradient. The absorbance of the effluent was followed at 280, 405 and 610 nm.

Characterization of recombinant laccases

The purity of the enzymes was analysed by SDS-PAGE (10 % MiniProtein TGX gels; Bio-Rad, Hercules, USA). Ten micrograms of purified proteins was used for deglycosylation treatment by using protein deglycosylation mix (New England BioLabs). The proteins were visualized by PageBlue (Fermentas) gel staining reagent. Color Plus Protein Ladder (New England BioLabs) was used for the determination of the molecular mass.

The pH optima of the rLac1 and rLac2–L497V were estimated by measuring the oxidation of 2,6-DMP and ABTS as described earlier and the oxidation of syringaldazine (SGZ; 4-hydroxy-3,5-dimethoxybenzaldehyde; Apin Chemicals Ltd, Oxon, UK) at 525 nm. SGZ was dissolved in ethanol and other substrates in distilled water. The pH in the reactions was adjusted with sodium malonate buffer (50 mM) from pH 2.0 to 6.0 and with MES buffer (50 mM) from pH 6.0 to 7.5.

The thermostolerance of the recombinant laccases was determined over the range from 40 to 80 °C at the optimum pH by incubating the enzymes in 100- μ l aliquots for 1 to 60 min. After incubation, the tubes were chilled on ice and the residual laccase activity was measured with 2,6-DMP as described earlier. Laccase activity at 25 °C was marked as 100 % for each substrate compound.

Nucleotide sequence accession numbers

The cDNA sequences are deposited at GenBank with accession numbers JQ027726 and JQ027727 for *lac1* and *lac2*,

respectively, and the internal transcribed spacer region with accession number JQ027728.

Results

Characterization of Lac1 and Lac2 polypeptides and respective genes

Two laccase-encoding genes and cDNAs of *P. rivulosus* were cloned and sequenced. As predicted from the full-length cDNA clones, *lac1* and *lac2* encode 519- and 518-aa-long polypeptides, respectively. The ORF of *lac1* gene is interrupted by 11 introns of 51–93 nt in length, whereas the *lac2* gene contains 12 introns of 50–68 nt in length. The exon–intron junctions of the laccase genes are shown in Fig. 1. The intron–exon organization of *P. rivulosus lac1* and *lac2* genes and the translated amino acid sequences of Lac1 and Lac2 showed the highest pairwise identity (86 and 91 % , respectively) to the Lcs-1 (gene *lcs-1*, AF053472) and the gene and protein model #137686 (<http://genome.jgi-psf.org/Cersu1/Cersu1.home.html>), respectively, from the taxonomically nearest-related polypore species *Ceriporiopsis (Gelatoporia) subvermispora*.

The Lac1 and Lac2 protein sequences are preceded by 21-aa-long putative N-terminal leader peptides, and they show 74 % amino acid pairwise identity. The

calculated *pI* values of predicted mature Lac1 and Lac2 are 4.5 and 4.1, respectively. The four conserved laccase copper-binding regions are conserved in both *P. rivulosus* laccases. Lac1 contains six potential N-glycosylation sites, while eight may be deduced in Lac2. Interestingly, the tripeptide (-DEL-) present in the C-terminal end of Lac2 may be an indication of a signal for protein retention in the ER.

Homology modelling of Lac1 and Lac2

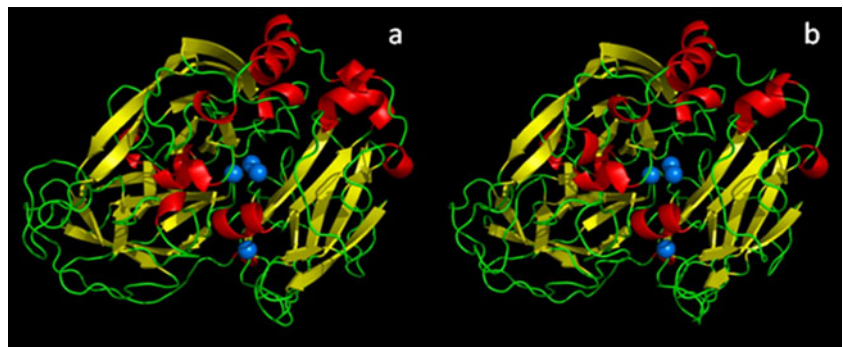
The theoretical structural models for Lac1 and Lac2 were constructed by protein homology modelling. The 3-D models predict a typical protein fold for laccases and consist of three β-barrel cupredoxin-like domains, which form a globular structure (Fig. 2). Both enzymes contain a cluster of four copper atoms, which form the laccase catalytic site. In both laccases, the copper atom of the T1 site is coordinated by two histidines, cysteine and isoleucine (His396, His457, Cys452, Ile454 for Lac1 and His395, His456, Cys451, Ile453 for Lac2) and the three copper atoms of the T2/T3 site by eight histidines (His66, His68, His111, His113, His399, His401, His451, His453 for Lac1 and His64, His66, His109, His111, His398, His400, His450, His452 for Lac2). The T1 copper is linked with the T2/T3 copper cluster by the conserved His–Cys–His tripeptide (His451–

		L1
Priv-Lac1	AIGPVTDLVITDAFVTPDGPPLGREAFLAGGTFPGPLIQGNKGDNFQINVVNLTNHTMLKTTSS	HHWHLGFQHGTTNADGPAFV
Cs-lcs1	AIGPVTDLVITDAFVSPDGPGLGREAFLAGGTFPGPLIQGNKGDNFQINVVNLTNHTMLKTTSS	HHWHLGFQHGTTNADGPAFV
Priv-Lac2	AIGPVTDLTITDASISPDG--FERAAVLAGGTFPGPLITGNIGDNFQINVVNQLSNETMLESTT	HHWHLGFQKGTAWADGPAFV
Cs-137686	ATGPVANLAITDTNISPDG--YERAAVLAGGTFPGPLIQGNIGDNFQINVVNQLTNETMLESTT	HHWHLGFQGGTTNADGAAVF
	L2	I
Priv-Lac1	NQCPITASGNSFLYNFNVPDQAGTFWYHSHLATQYCDGLRGP	VVYDPNDPYADLYDVDDDESTVITLSDWYHAAAST-LTFPT-F
Cs-lcs1	SQCPITASGNSFLYNFNVPDQAGTFWYHSHLATQYCDGLRGP	VVYDPNDPHADLYDVDDDESTVITLSDWYHAAAST-LTFPT-F
Priv-Lac2	SQCPITATGNSFLYDFSVDPDQAGTFWYHSHLATQYCDGLRGP	VVYDPNDAYLSDYDVDDDTTVITLADWYHTAATLGRPFPTQA
Cs-137686	SQCPITATGNSFLYDFTVDPDQAGTFWYHSHLATQYCDGLRGP	VVYDPNDAYLSDYDVDDNETTVITLADWYHTAATLGRPFPTVQA
	L3	II
Priv-Lac1	DTTLINGLGRFAGTGGSDSLAVITVEQGRYRFRLLAISCDPNWFVSI	IDEHVMTIIEADAVNTLPLTVDAIQIFAAQRYSFVL
Cs-lcs1	DTTLINGLGRFAGTGGSDSLAVITVEQGRYRFRLLAISCDPNWFVSI	IQHELTVEVDGVNAVPLTVDAIQIFAAQRYSFVL
Priv-Lac2	DATLINGLGRYS--GGPESNLTVITVESGKRYRFRLLAISCDENYVFS	IDDHMIVIEADGVETQPVTVDSIQIFAAQRYSFIL
Cs-137686	DATLINGLGRYS--GGPASNLTVITVESGKRYRFRLLAISCDENYVFS	IDGHMIVIEADGVETQPVTVDSIQIFAAQRYSFIL
	L4	III
Priv-Lac1	NANQTVGNYWIRANPNNGNMGFANGINSAILRYSGATIEDPTTNST	IVNQLNEADLSPLTNPQAPGAPSDPGAVDVMNLAIFS
Cs-lcs1	NANQTVGNYWIRANPNNGNMGFANGINSAILRYVYVADVEPTSTGT	TANLLNEADLSPLVPAAPGAPNQDFDAVDVPMNLTFT
Priv-Lac2	NATEAVDNYWIRATPDLGTTGPTDGINSAILRYVYVADNDTPTAS	TNLLSELDLHPLTNAAPGAASQDPGSDVSMNLEIG
Cs-137686	NATEAVDNYWIRAQPNEGNTSFTGGINSAILRYVYVADNDPTTST	ASNLLSEVDLHPLTNAAPGAASQDPGSDVSMNLDLIG
	L5	IV
Priv-Lac1	FNGTRLFINGVSVFPPSPVLLQILSGSMTAQDLLPTGSVYTLPRN	ATVQISMPGNI IAG-FHPFHLHGHTFSVIRSAGESDYN
Cs-lcs1	FNGTNLFINGATFVPPSPVLLQILSGAMTAQELLPAGSVYTLPRN	ATVQLSIPGNI IAG-FHPFHLHGHTFSVIRSAGQSDYN
Priv-Lac2	FDGTNFTINGATFVPPPTVPLLQILSGAQSATDLLPSGSVFTLP	SNSTVQVTIAAGTVAGGHPFHLHGHTFSVVRSGSDTYN
Cs-137686	FDGTNFTINGATFVPPPTVPLLQILSGAQSATDLLPSGSVFTLP	ANSTVQVTIAASTVAGGHPFHLHGHTFSVVRSGSDTYN
	L6	V
Priv-Lac1	YDNPVQRDVTISIGGATDNVTIRFTTNNEGPWFHLCHIDWHLQAG	FAIVFAEDTPDIASANPVPQAWSDLCPYDALDPSDT
Cs-lcs1	YVDPIQRDVTISIGGATDNVTIRFTTNNEGPWFFHCHIDWHLQAG	FAIVFAEETADVASANPVPADWSALCPYDALSDADH
Priv-Lac2	YDNPVQRDVTISIGADGNVTIRFDTNNEGPWFHLCHIDWHLNAG	FAIVFAEDTADIASANPVPDWSDLCPYDALPSDEL
Cs-137686	YVNPVQRDVTISIGADGNVTIRFDTNNEGPWFHLCHIDWHLNAG	FAIVFAEDTADIASANPVPDWSNLCPTYDALPSDEL

Fig. 1 *P. rivulosus* laccases. Alignment of the predicted mature translated Lac1 and Lac2 amino acid sequences with their homologues from *Ceriporiopsis subvermispora* (Cs-lcs1 sequence accession AAC97074 and Cs-137686 protein model from the genome site at <http://genome.jgi-psf.org/Cersu1/Cersu1.home.html>, respectively). Boxed regions: L1–L4, laccase signature sequences containing the

conserved copper atom ligand amino acid residues (Kumar et al. 2003); I–IV, variable loop sequence sites (Larrondo et al. 2003a, b). Exon–intron junctions in corresponding positions in the laccase genes are shaded and potential asparagines for N-glycosylation are underlined

Fig. 2 Overall topology and folding of the proposed 3-D homology models of Lac1 (a) and Lac2 (b). α -Helixes (red), β -sheets (yellow), loops (green) and copper atoms (blue) are depicted. T1-Cu site and reducing substrate-binding cleft is facing downward



Cys452–His453 for Lac1 and His450–Cys451–His452 for Lac2).

The modelled 3-D structures of Lac1 and Lac2 were superimposed near the vicinity of the T1 copper on the corresponding protein sites of the structurally characterized laccases from *T. versicolor* (PDB accession 1KYA) and *T. trogii* (PDB accession 2HRG) for determination of the amino acid residues playing a role in binding of the reducing organic substrates in these enzymes. The 3-D crystal structures 1KYA and 2HRG both contain a substrate-like ligand molecule (1KYA, 2,5-xylydine (2,5-dimethylaniline); 2HRG, *p*-methylbenzoate) (Fig. 3). At this substrate-binding site, the amino acid residues are in close proximity (maximum 5 Å distance) to the substrate molecules in 1KYA and 2HRG. The respective sites in the Lac1 and Lac2 models demonstrate four amino acid residues (Phe, His, Ile and Asp) that are highly conserved for all the four laccases.

The reducing substrate-binding cavity is comprised of 17 and 18 amino acid residues in Lac1 and Lac2, respectively (Fig. 4). A comparison of the cavities showed that 12 amino acids were conserved, whereas the other five residues were substituted as follows: Pro209/Glu207, Asn266/Asp264, Asn267/Leu265, Leu340/Phe338 and Ile392/Val390 for Lac1/Lac2, respectively. Furthermore, an insertion of Gly392 was present in the substrate-binding site region of Lac2. The shape and volume of the substrate-binding cavity as well as the surface electrostatic charge vary between Lac1 and Lac2 (Fig. 4). The cavity of Lac2 is less bulky and more negatively charged than is observed in Lac1. In both laccases, the catalytically essential and conserved histidine (His457/His456 for Lac1/Lac2) and aspartic acid (Asp208/Asp206 for Lac1/Lac2) residues are located at the bottom of the cavity, with their side chain groups exposed to the surface of the enzymes.

Physico-chemical properties and kinetic parameters of the recombinant laccases

The Lac1 and Lac2 encoding genes were expressed in *P. pastoris* under the control of the tightly regulated *AOX1* promoter, which was induced for expression by addition of

methanol. Among the positive transformants, extracellular ABTS oxidation was observed with recombinant laccase constructs, harnessing either their native N-terminal signal peptides (rLac1/pPICZ and rLac2/pPICZ) or the α -factor

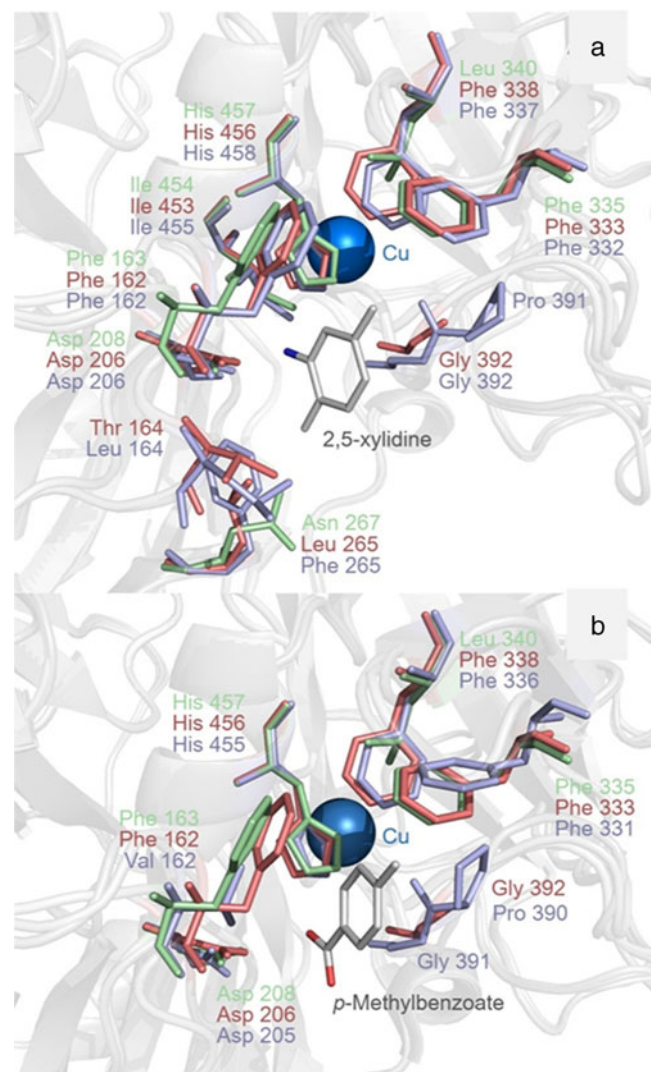
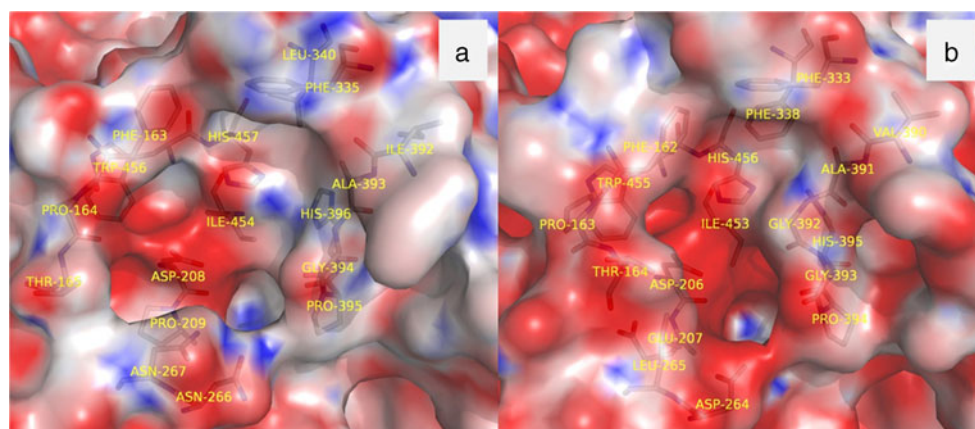


Fig. 3 Superimposition of active site amino acid residues of Lac1 (green) and Lac2 (red) onto the (a) 1KYA (light purple) and (b) 2HRG (light purple). The co-crystallized ligand (substrate analogues) 2,5-xylydine and *p*-methylbenzoate are illustrated (carbon skeleton in silver, nitrogen atoms in blue, oxygen atoms in red) in the figures

Fig. 4 Electrostatic surface representation and amino acid residues in the substrate-binding site cavity of the laccases Lac1 (**a**) and Lac2 (**b**). Negative charge is coloured red and positive charge is coloured blue



signal peptide from *S. cerevisiae* (rLac1/pPIC α Z and rLac2/pPIC α Z). However, the latter constructs resulted in faster ABTS oxidation rates and demonstrated slightly higher oxidative activities within liquid cultures and were thereby selected for the scale-up studies (Fig. 5). For the rLac1/pPICZ, the specific laccase activity reached a maximum of 3.14 μ kat/ μ g after 7 days of liquid expression cultures, whereas for rLac1/pPICZ α the maximum of 4.9 μ kat/ μ g was reached already after 2 days. For rLac2, both signal peptide variants, rLac2/pPICZ and rLac2/pPICZ α , showed negligible laccase activity of 0.4 and 0.3 μ kat/ μ g, respectively. In the liquid cultures, the transformant rLac2–L497V, which produced the one codon mutated variant, showed higher extracellular ABTS oxidation activities (16.5 μ kat/ μ g) than either of the wild-type rLac2 variants and was thus selected for the enzyme characterisation studies (Fig. 5).

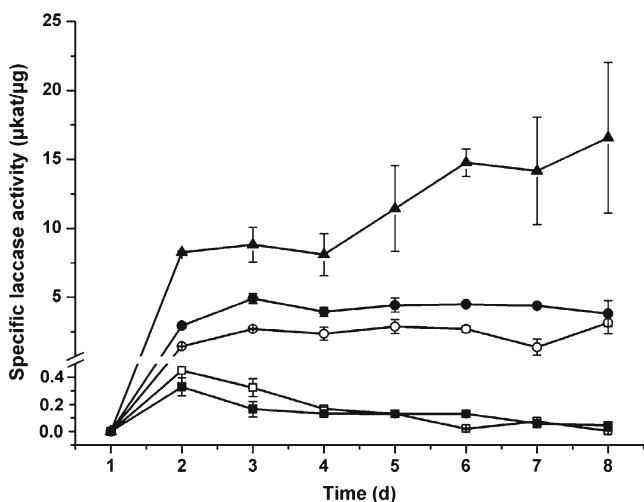


Fig. 5 Time course of specific laccase activity in the extracellular medium of *P. pastoris* cultures producing recombinant laccases. Comparisons of rLac1 and rLac2 expression using vector with native (pPICZ) and α -factor secretion signal sequence (pPICZ α). rLac1/pPICZ (open circle), rLac1/pPICZ α (filled circle), rLac2/pPICZ (open square) and rLac2–L497V /pPICZ α (filled triangle). Activity was determined by ABTS oxidation. The mean values represent the specific laccase activities of three transformants of each type

The molecular masses of the purified rLac1 and rLac2–L497V were 71 kDa, which was higher than the predicted masses of 54 and 53 kDa (Fig. 6). However, treatment of protein deglycosylation mix (PNGaseF, O-glycosidase, neuraminidase, β 1–4 galactosidase, β -N-acetylglucosaminidase) showed that the molecular masses were not affected by deglycosylation.

The rLac1 and rLac2–L497V recombinant laccases both showed acidic pH optima (around pH 3.5) for the oxidation of ABTS and 2,6-DMP (Fig. 7). With SGZ as reducing substrate, no oxidation activity was detected. The recombinant laccases were stable at temperatures below 50 °C (Fig. 8). The half-life of rLac1 for 2,6-DMP oxidation at pH 3.5 and at 60 °C was 60 min, whereas for rLac2–L497V a half-life of only 25 min at 60 °C was observed. Interestingly, rLac1 showed thermal activation since the 2,6-DMP oxidation rate after incubation for 10 min at 60 °C was increased up to 30 % of the original activity at room temperature prior to incubation. On the contrary, no such thermal activation was observed with rLac2–L497V enzyme.

The kinetic parameters (K_m and k_{cat}) were determined with 2,6-DMP as reducing substrate at pH 3.0. The V_{max} of rLac1 and rLac2–L497V were 7 ± 0.01 and 57 ± 0.17 μ kat/l, respectively. The K_m values showed that rLac1 (39 ± 0.07 μ M) has higher affinity towards 2,6-DMP than rLac2–L497V (206 ± 0.6 μ M). The K_{cat} values of rLac1 and rLac2–L497V were $5 \pm 0.01/s$ and $34 \pm 0.1/s$, respectively. The catalytic efficiencies expressed as k_{cat}/K_m were 1.3×10^5 μ M $^{-1}$ s $^{-1}$ for rLac1 and 1.6×10^5 μ M $^{-1}$ s $^{-1}$ for rLac2–L497V.

Discussion

In this study, we cloned and heterologously expressed two laccases of the biopulping white-rot basidiomycete *P. rivulosus*. Biochemical characteristics showed that these novel laccases are acidic, thermotolerant and thermoactivated. The structural features were analysed by homology modelling.

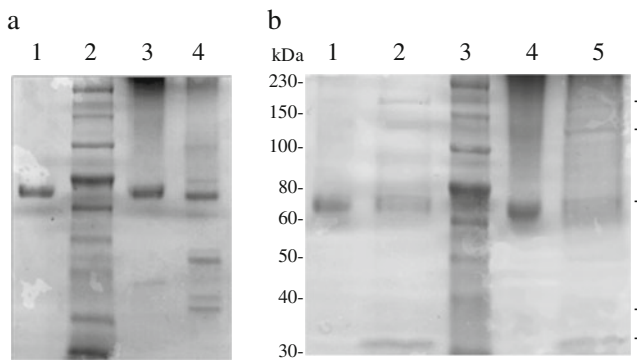


Fig. 6 SDS electrophoresis of purified laccases rLac1 and rLac2–L497V (**a**). Lane 1, rLac1; lane 2, protein standard; lane 3, rLac2–L497V; lane 4, *P. pastoris* culture extract. Deglycosylation of recombinant rLac1 and rLac2–L497V (**b**). Lane 1, rLac1; lane 2, rLac1 treated with deglycosylation mix; lane 3, protein mass marker; lane 4, rLac2–L497V; lane 5, rLac2–L497V treated with deglycosylation mix. The bands corresponding deglycosylation enzymes are marked. Approximately 10 μ g of recombinant protein was applied to each lane

Based on our earlier work on cultivating *P. rivulosus* on spruce (*Picea abies*) wood chips, our hypothesis is that the acidic and thermotolerant laccases, together with the MnP isoenzymes (Hakala et al. 2005), provide *P. rivulosus* with an advantage in the decomposition of lignin (over cellulose) in coniferous wood.

In order to characterize the acidic laccases, the recombinant enzymes (rLac1 and rLac2–L497V) were expressed in the methylotrophic yeast *P. pastoris*, which has previously been applied as a host organism for a few basidiomycetous laccases, i.e. from *T. versicolor*, *Rigidoporus microporus* and *Polyporus gramocephalus* (Jönsson et al. 1997; Liu et al. 2003; Huang et al. 2011). In our study, increased

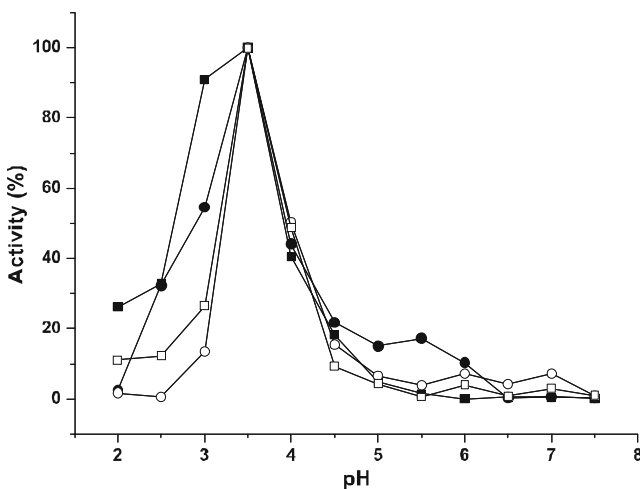


Fig. 7 Effect of pH on the activity of purified recombinant *P. rivulosus* laccases (rLac1 ABTS (filled square), rLac1 2,6-DMP (filled circle), rLac2 ABTS (open circle), rLac2 2,6-DMP (open square)). The measurements were carried out as duplicates and averages are plotted. The standard deviation did not exceed 1 %

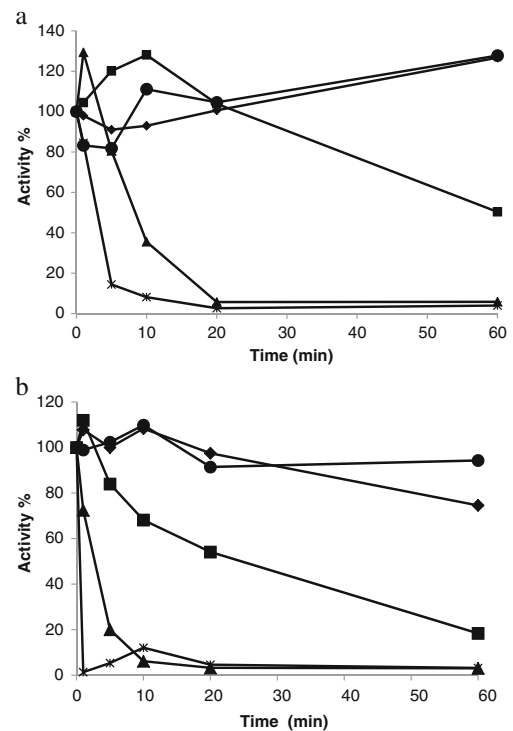


Fig. 8 Thermotolerance of the recombinant *P. rivulosus* rLac1 (**a**) and rLac2 (**b**) 40 °C (filled circle), 50 °C (filled diamond), 60 °C (filled square), 70 °C (filled triangle) and 80 °C (multiplication symbol) using 2,6-DMP as the substrate at pH 3.0. The measurements were carried out as duplicates and averages are plotted. The standard deviation did not exceed 1 %

specific activity levels of the recombinant *P. rivulosus* laccases were achieved with the *S. cerevisiae* α -factor signal sequence fusions when compared to the expression constructs harnessing the native laccase N-terminal signal peptides.

The enzymatic properties of the recombinant laccases demonstrated low pH optima (pH 3.5) for oxidation of ABTS and the phenolic compound 2,6-DMP. Previously characterized native laccases (Lac-3.5 and Lac-4.8) of *P. rivulosus* showed similar properties (Hildén et al. 2007). However, the correlation between recombinant and native ones (Lac-3.5 and Lac-4.8) has not been determined yet since *P. rivulosus* produces at least five different laccases (unpublished data). In contrast to the native *P. rivulosus* laccases, the recombinant enzymes were unable to oxidize the typical fungal laccase substrate SGZ. For the native *P. rivulosus* laccases, oxidation of SGZ occurred at a slow rate, with the highest activity peaking at pH 3.0 (Hildén et al. 2007). This is an unusually low pH for optimal activity of basidiomycetous laccases as the pH optima for oxidation of SGZ is commonly observed to be between pH 5 and 6 (Garzillo et al. 2001; Baldrian 2006) and implies significant differences at the organic substrate-binding cavity in the *P. rivulosus* laccases. It may be that the

recombinant *P. rivulosus* laccases were even more restricted at the T1-Cu site.

Of the two acidic laccases of *P. rivulosus*, Lac1 demonstrates the highest protein sequence level identity with the only characterized laccase of *C. subvermispota* (Lcs-1; Karahanian et al. 1998; Larrondo et al. 2003a, b), while Lac2 shares the highest degree of sequence identity with one of the seven laccase protein and gene models (model #137686) recognized in the genome of *C. subvermispota* (<http://genome.jgi-psf.org/Cersu1/Cersu1.home.html>). The molecular mass of rLac1 corresponds to *C. subvermispota* laccase L1 of which N-terminus corresponds to the translated amino acid sequence of the *lcs-1* gene (Fukushima and Kirk 1995; Karahanian et al. 1998). However, a deglycosylation assay did not alter the molecular mass of rLac1, indicating differences in glycosylation between rLac1 and L1. In Lac2 and gene model #137686, the C-terminus ends with SDEL, which we propose to potentially act as a remnant of the eukaryotic ER-retention signal (Pagny et al. 1999; Tonkin et al. 2006). This is supported by the fact that by changing the last amino acid of the C-terminal tripeptide (L497V), extracellular production of the recombinant rLac2 enzyme was facilitated by *P. pastoris*. In the phylogenetic tree of basidiomycetous laccases, the laccases of *P. rivulosus* and *C. subvermispota* cluster in the same branch (data not shown), which confirms the close phylogenetic relationship of the two species inferred from the nuclear and mitochondrial ribosomal RNA encoding gene and ITS sequences (Tomšovský et al. 2010; this work).

Analysis of the 3-D models for Lac1 and Lac2 showed their overall protein structures to be typical for fungal laccases consisting of three protein domains. As is conserved in the laccase architecture, the T1 copper site is in domain 3, and the T2/T3 trinuclear copper site is between domains 1 and 3 (Bertrand et al. 2002; Piontek et al. 2002). The reducing substrate-binding pocket is at the boundary between domains 2 and 3. The coordination sphere of the four copper ions is conserved in the fungal laccases and consists of ten histidine, one cysteine and one isoleucine or methionine residues (Giardina et al. 2010).

Superimposition of the substrate-binding pocket, occurring around the substrate ligands in the laccase 3-D structures of *T. versicolor* (Bertrand et al. 2002) and *T. trogii* (Matera et al. 2008), on the corresponding residues showed the presence of four conserved amino acids in both Lac1 and Lac2. Among them, the His residue (H457 in Lac1 and H456 in Lac2, respectively) coordinates the copper ion of the T1-Cu site and is exposed to the surface of the substrate-binding cavity. This histidine is assumed to play an important role in electron transfer from the reducing substrate molecule to the T1-Cu (Garzillo et al. 2001; Bertrand et al. 2002; Matera et al. 2008). The conserved acidic aspartate (Asp206 in *T. versicolor* LacIIIb) residue, which is normally deprotonated and thereby carrying a negative charge, is

possibly involved in the binding and correct orientation of the substrate ligand molecule in the catalytic pocket (Garzillo et al. 2001; Bertrand et al. 2002).

Comparison of the substrate-binding pockets in the structural models of Lac1 and Lac2 indicated the presence of five amino acid substitutions. As a result of these substitutions, the size of the substrate-binding cavity in Lac2 is smaller compared with the Lac1 3-D protein model. Also, Lac2 contains the insertion of Gly392, which may affect the topology of the enzymatic cavity. The differences in the shape and volume of the substrate-binding site can be assumed to cause a distinction in the catalytic activity of the two *P. rivulosus* laccases. This is supported by the kinetic constant values of rLac1 and rLac2-S497V obtained with the phenolic substrate 2,6-DMP. Alteration of the oxidation ability with respect to bulky phenolic substrates was demonstrated for the structurally characterized laccase LacIIIb from *T. versicolor* using a series of site-directed mutations on the phenylalanines located in the enzymatic cavity, thus altering the shape of the substrate-binding pocket (Galli et al. 2011).

Considering the oxidation of the phenolic substrate 2,6-DMP, the rLac1 and rLac2-L497V showed acidic pH optima, which is in accordance with the properties of the native acidic laccases of *P. rivulosus* (Hildén et al. 2007). The recombinant laccases were stable at temperatures below 50 °C. The half-life of rLac1 at 60 °C was 60 min at pH 3.0 whereas rLac2-L497V showed a half-life of only 25 min at 60 °C. The native laccases of *P. rivulosus* tolerated higher temperatures than rLac1 and rLac2-L497V (Hildén et al. 2007).

Temperature activation for oxidation of phenolic substrates or ABTS has earlier been described only for a few laccases of fungal and bacterial origin (Hildén et al. 2009). Interestingly, rLac1 presented thermal activation, since the 2,6-DMP oxidation was increased up to 30 % (of the original activity) after 10 min incubation at 60 °C, which is in line with the thermal activation of the native Lac-4.8 of *P. rivulosus* at 50 °C (Hildén et al. 2007). In contrast, rLac2-L497V demonstrated no such thermal activation at any of the tested temperatures. These data imply that the rLac1 shares properties with the previously recognized Lac-4.8 isoenzyme. It may be proposed that the unusual thermal stability and activation for oxidation of the phenolic substrate (2,6-DMP) by rLac1 could be due to the more open architecture of the substrate-binding cavity in Lac1 than in Lac2, thus giving more flexibility for Lac1 for binding and oxidation of substrate molecules of variable sizes.

We present two novel recombinant laccases originating from the selective lignin-degrading and biopulping fungus *P. rivulosus*. By 3-D homology modelling, differences in the substrate-binding cavities near the T1-Cu site could be demonstrated in Lac1 and Lac2. The enzymatic properties described (acidity, thermotolerance and thermal activation)

indicate that the novel recombinant Lac1 may be applied as a robust enzyme for oxidation and conversion of phenolic compounds under acidic conditions and at elevated temperatures. Heterologous expression of the novel Lac1 and Lac2 will facilitate the development of their use as oxidative biocatalysts for conversion of wood lignin, lignin-like compounds and soil-polluting xenobiotics.

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