Isolation of a laccase-coding gene from the lignin-degrading fungus *Phlebia brevispora* BAFC 633 and heterologous expression in *Pichia pastoris*

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**Keywords**
characterization, gene isolation, heterologous expression, laccase, *Phlebia brevispora*.

**Abstract**

**Aims:** Isolate and characterize a laccase-encoding gene (*lac I*) of *Phlebia brevispora* BAFC 633, as well as cloning and expressing cDNA of *lac I* in *Pichia pastoris*. And to obtain a purified and characterized recombinant laccase to analyse the biotechnological application potential.

**Methods and Results:** *Lac I* was cloned and sequenced, it contains 2447 pb obtained by PCR and long-distance inverse PCR. Upstream of the structural region of the laccase gene, response elements such as metals, antioxidants, copper, nitrogen and heat shock were found. The coding region consisted of a 1563-pb ORF encoding 521 amino acids. *Lac I* was functionally expressed in *P. pastoris* and it was shown that the gene cloned using the α-factor signal peptide was more efficient than the native signal sequence, in directing the secretion of the recombinant protein. *Km* and highest *kcat/Km* values towards ABTS, followed by 2,6-dimethylphenol, were similar to other laccases. *Lac I* showed tolerance to NaCl and solvents, and nine synthetic dyes could be degraded to different degrees.

**Conclusions:** *Lac I*-encoding gene could be successfully sequenced having cis-acting elements located at the regulatory region. It was found that *lac I* cDNA expressed in *P. pastoris* using the α-factor signal peptide was more efficient than the native signal sequence. The purified *Lac I* exhibited high tolerance towards NaCl and various solvents and degraded some recalcitrant synthetic dyes.

**Significance and Impact of the Study:** The cis-acting elements may be involved in the transcriptional regulation of laccase gene expression. These results may provide a further insight into potential ways of optimizing fermentation process and also open new frontiers for engineering strong promoters for laccase production. The *Lac I* stability in chloride and solvents and broad decolorization of synthetic dyes are important for its use in organic synthesis work and degradation of dyes from textile effluents respectively.

**Introduction**

Laccases (benzenediol: oxygen oxidoreductases (EC1.10.3.2)) are copper-containing enzymes capable of oxidizing a broad spectrum of phenolic compounds and nonphenolic substrates using molecular oxygen as the electron acceptor. In fungi, laccases probably play critical roles in several physiological functions, such as morphogenesis, fungal plant–pathogen/host interaction, degradation of lignocellulosic material and pigment production (Baldrian 2006). The low substrate specificity makes this enzyme interesting for biotechnology purposes in various industries such as pulp
and paper and textiles, and bioremediation of industrial pollutants (Mayer and Staples 2002). Several authors have reported laccases with interesting properties for their biotechnological application (Moredo et al. 2003; Wang et al. 2008; Fonseca et al. 2010; Preussler et al. 2009; Shimizu et al. 2009; Giorgio et al. 2013; Fonseca et al. 2015). The white rot fungus Phlebia brevispora Nakasone BAFC 633 produces a main laccase of 60 kDa that is constitutively expressed and another laccase of 75 KDa that is induced by the presence of CuSO4 (Fonseca et al. 2010). Both enzymes have been purified and thoroughly characterized (Fonseca et al. 2015). The presence of isoforms with similar biochemical properties makes it difficult to purify individual enzymes for analysis, a problem that can be overcome by expressing the corresponding gene in a heterologous host (Colao et al. 2006). The methylotrophic yeast Pichia pastoris is frequently used for heterologous expression and can be grown in methanol as the only source of carbon and energy (Cereghino and Cregg 2000). Pichia pastoris has the potential to express high levels of protein, with efficient secretion of extracellular proteins, post-translational modifications, such as glycosylation, and the ability to grow at high cell densities over a defined minimum medium. Another possible advantage with P. pastoris compared with many filamentous fungi is that it does not produce cellulolytic enzymes and laccase produced in this host could, therefore, potentially be applied directly in the pulp and paper industry without any purification. In addition, molecular genetics methods for P. pastoris are rapid and well developed, and the organism can be easily cultivated on a large scale (Hong et al. 2002). Laccase genes of Laccaria bicolor (Wang et al. 2016), and Ganoderma lucidum (You et al. 2014) were expressed in P. pastoris, indicating the suitability of this system for laccase production and also revealing that the system seems to be the most cost effective to use in ecological strategies.

In this work, we describe the isolation and the characterization of the chromosomal lac I from the white rot fungi (WRF) P. brevispora BAFC 633. The lac I cDNA was successfully expressed in P. pastoris. Nucleotidic as well as the in silico-deduced Lac aminoacidic sequences were compared with other well-known Lac sequences available throughout databases and the corresponding analysis is herein discussed. The laccase was then purified and the biochemical properties and decolorization potentials were analysed.

Materials and methods

Chemicals

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,6-dimethoxyphenol (DMP, catalogue number D135550) and sodium dodecyl sulphate (SDS, catalogue number L3771) were purchased by Sigma-Aldrich (St. Louis, MO, USA).

Micro-organism and culture conditions

The WRF isolated from the Misiones rainforest (Argentina) identified as P. brevispora BAFC 633, is deposited in the Filamentous Fungi Culture Collection at the Biological Sciences Department, Faculty of Exact and Natural Sciences, UBA, Argentina. Stock cultures were maintained at 4°C by periodic subculturing on malt extract agar (MEA: malt extract, 12.7 g l⁻¹; agar, 20 g l⁻¹). To prepare the liquid inocula, 4-cm² agar plugs from 5–7-day-old MEA plates were cut and transferred to 100-ml Erlenmeyer flasks containing 10 ml of malt extract liquid medium (ME: malt extract, 12.7 g l⁻¹) and incubated at 29°C under static conditions.

The Pichia pastoris strain GS115 (his4) was purchased from Thermo Fisher Scientific (Waltham, MA, USA) (Pichia Expression Kit, original kit, Invitrogen, catalogue number K1710-01, Carlsbad, CA, USA) and the yeast media and agar plates were prepared as described by the manufacturer.

Genomic DNA isolation

Mycelia from 6-day-old cultures of P. brevispora BAFC 633 grown in 10 ml ME at 29°C under static conditions were harvested by filtration. Filtrated mycelium was washed with 0·1 mol l⁻¹ Tris-HCl pH 8, 0·02 mol l⁻¹ EDTA. DNA extraction was carried out with a lysis buffer solution (100 mmol l⁻¹, Tris-HCl pH 8, 1·5 mol l⁻¹ NaCl, 50 mmol l⁻¹, EDTA pH 8) at 60°C containing 0·1 mg ml⁻¹ proteinase K, 10 mmol l⁻¹ β-mercaptoethanol and 2% (w/v) SDS. DNA was purified with chloroform: isoamyl alcohol (24 : 1, v/v) and 3 mol l⁻¹ potassium acetate, and finally precipitated with isopropyl alcohol (Fonseca et al. 2015).

Laccase gene fragments cloning and sequencing

The cloning strategy to amplify the Lac I gene (lac I) is summarized in Fig. 1.

Amplification of lac I was performed by PCR using P. brevispora BAFC 633 genomic DNA (gDNA) as template. Primer sequences used in this work are listed in Table 1. The accurate size PCR-amplified products were cut out from the agarose gel, purified, cloned into pGEM-T Easy Vector (pGEM®-T Easy Vector System II, catalogue number A1380; Promega, Madison, WI, USA) and sequenced (Macrogen, Seoul, South Korea). Plasmids were isolated by standard molecular biology methods (Sambrook et al. 1989).

PCR amplifications were carried out in a 20 µl final volume containing 1X KCl buffer, 2.5 mmol l⁻¹ MgCl₂, 200 µmol l⁻¹ dNTPs, 10 pmol of each primer, 0·5 U of
PFU DNA polymerase, and 25 ng DNA. PCR cycling consisted in 4 min at 94°C, 35× (40 s 94°C, 40 s 50°C, 40 s 72°C) and a final extension for 10 min at 72°C. *Escherichia coli* JM109 cloning host was obtained from Promega and competent cells were prepared by means of the classical CaCl₂ method. An A-tailing procedure for blunt-ended PCR was carried out and ligated into the pGEM-T vectors (pGEM-T Easy Vector System II, catalogue number A1380; Promega) following the manufacturer’s instructions. All reagents were of analytical grade and specific PCR products were purified using the DNA gel extraction kit (Wizard® SV Gel and PCR Clean-Up System, catalogue number A9280; Promega). Twelve clones with target fragments were analysed by sequencing.

### Amplification of laccase gene flanking sequences

Long-distance inverse PCR (LD-IPCR) was used to amplify the flanking sequences of *lac I*. To perform the reaction, *Phlebia brevispora* BAFC 633 gDNA was digested with *Bam*HI endonuclease (with no target sites in *lac I*). The product from each restriction (0.3 µg) was self-ligated in 1 ml reaction system using T4 DNA ligase. After phenol/chloroform purification steps and ethanol precipitation, the self-ligated products were used as templates for LD-IPCR using the relevant inverse primer pairs Lac-inv-S (P6) and Lac-inv-AS (P5) (d). [Colour figure can be viewed at wileyonlinelibrary.com]

Thus, the flanking sequences adjacent to the known *lac I* gDNA fragments were amplified. The LD-IPCR products with A-tailing were inserted into pGEM-T vector, and 12 clones of each product were sequenced. To assemble the final sequence, new primers hybridizing within the promoter and at the 3’ end of the gene (Lac-Es-S and Lac-Es-AS) were designed, and the PCR-amplified product was cloned and sequenced.

### Nucleotide sequence accession number

The *P. brevispora* BAFC 633 *lac I* sequence is currently deposited at the EMBL Nucleotide Sequence Data Bank under the accession number JQ728448.
Table 1 Oligonucleotide primers used in this study

| Primer name | Oligonucleotide sequence 5'-3' | Purpose | Reference |
|-------------|-------------------------------|---------|-----------|
| LacI-S      | ACN TTT TGG TAY CAY AGY CA     | Used for cloning a fragment of II and III copper-binding regions | This work |
| LacIII-AS   | KCC GTG KAG GTG GAA KGG RTG    | Used for cloning a fragment of II and III copper-binding regions | This work |
| Lac-S       | CAK TGG CAK GGN TTK TTK CA     | Used for cloning a fragment of I and IV copper-binding regions | D'Souza et al. (1996) |
| LacIV-AS    | TGR AAR TCD ATR TGR CAR TG     | Used for cloning a fragment of I and IV copper-binding regions | This work |
| Lac-Inv-S   | TGRAARAANCCRTGCCARTG           | Used for LD-IPCR | This work |
| Lac-Inv-AS  | CAYTGYCAYATHGAYTTYCA           | Used for LD-IPCR | This work |
| Lac-Es-S    | TATACTGGTGTTGTCACCT            | Used for cloning and confirmation of lacI sequence | This work |
| Lac-Es-AS   | TATGGGACAGAGYTGSTCCAAAGC       | Used for cloning and confirmation of lacI sequence | This work |
| Nlacs/ps-S  | CTCGAGGAATCTCATCACAGGACCTTTGG | Used for cloning cDNA lacI sequence without the signal peptide | This work |
| Nlcl        | CTCGAGGAATCTCGAGATGCTCTCCTAGTTCTGTGCA | Used for cloning cDNA lacI sequence with the signal peptide | This work |
| Nlackl-AS1  | GGTCTAGCCGGCCGGTTAGTTGTTCGCCAGAACCTTCTG | Used for cloning cDNA lacI sequence | This work |

Y = C/T, N = A/G/C/T, R = A/G, D = A/G/T.

RNA isolation and amplification of Lac DNAc by PCR

Phlebia brevispora cultures were grown in ME liquid medium (Fonseca et al. 2010) and induced with 0.5 mmol l⁻¹ CuSO₄ (Fonseca et al. 2010). To isolate the total RNA, fungal mycelium was collected 14 days after induction by filtration and washed twice with sterile cold 0.05 mol l⁻¹ Tris, 0.02 mol l⁻¹ EDTA (Fonseca et al. 2014a). The samples were treated with DNases previous to retrotranscription step. The first strand of cDNA was synthesized using MuLV-Transcriptase (RevertAid Reverse Transcriptase, Carlsbad, CA, USA). The primers used for PCR technique are described in Table 1. The PCR reaction and conditions were the same as used by Fonseca et al. (2014a,b). The Signal P prediction (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the start of the mature laccase. A 1563 pb fragment corresponding to the laccase cDNA and a 1476 pb fragment without the signal peptide encoding the fragment were amplified using primer described in the Table 1. The PCR product was cloned into pGEMT easy vector in E. coli cells as per instructions (Promega). The presence of the desired PCR product was verified by restriction enzyme digestion, agarose gel electrophoresis and sequencing.

Cloning and expression of lacI gene from P. brevispora in P. pastoris through yeast shuttle vector

The CDNA of lclI from P. brevispora was cloned under control of the methanol-inducible alcohol oxidase (AOX1) promoter of P. pastoris into the expression vectors pPIC3.5K and pPIC9.K (catalogue numbers V17320 and V17520 respectively; Invitrogen). Two recombinant plasmids were obtained: pPIC3.5K/lacI, containing the lacI cDNA including the native signal sequence, and pPIC9.K/lacI, in which the cDNA sequence encoding the native LacI signal peptide was exchanged for that encoding the Saccharomyces cerevisiae z-mating factor signal peptide. Plasmids DNA were digested with Sac I (Thermo Scientific, catalogue number ER1131) prior to transformation for efficient integration into the P. pastoris genome. P. pastoris GS115 (his4) cells were transformed by EasyComp TM Transformation Kit (Pichia EasyComp™ Kit, catalogue number K1730-01; Invitrogen). Vectors without lacI cDNA were also used to prepare control strains. The cells were plated onto histidine-deficient RDB agar plates and incubated at 30°C for 72 h, after which His⁺ transformants were screened on minimal methanol (MM) agar plates and incubated at 30°C for 72 h, after which His⁺ transformants were screened on MM plates supplemented with 0.2 mmol l⁻¹ ABTS for development of green colour. One (pPIC3.5K/lacI and pPIC9.K/lacI) of the recombinants was selected on the basis of development of intense green colour in plate assay to continue in liquid media. Inocula were prepared by transferring cells from minimal dextrose agar plates into 500-ml Erlenmeyer flasks containing 50 ml of phosphate-buffered yeast nitrogen base supplemented with glycerol (2%) and biotin (400 μg l⁻¹). Cultures were grown at 30°C in an orbital shaker (200 rev min⁻¹) and cells harvested in log-phase growth were used as inoculum for shake-flask cultivations.
Shake-flask cultivations were performed at 30°C in phosphate buffered MM (BMM). Cells harvested from the inoculum were directly resuspended in BMM to an OD₆₀₀ of 1.0. After 48 h, the culture was centrifuged at 6000 g for 10 min; afterwards, the centrifuged sample was filtered using a filter Pierce Concentrator 20 ml/20 K (Pierce® Concentrator 20K MWCO 20Ml, catalogue number 89887A; Thermo Scientific). The resulting filtrate was used to prepare the protein precipitation with ammonium sulphate at 100% saturation. The protein was dissolved in 0.1 mol l⁻¹ sodium acetate buffer (pH 3-6) and dialysed 12 h against the same buffer.

The purity of the enzyme checked on SDS-PAGE was carried out according to the protocol of Laemmli (1970) with 4% stacking gel and 12% resolving gel. The molecular mass of the purified laccase was determined by calculating the relative mobility of molecular marker (Precision Plus Protein™ Standards Dual Color, catalogue number 161-0374; Bio-Rad, Hercules, CA, USA) running alongside. Proteins were stained with a silver reagent (Blum et al. 1987) and Coomassie Brilliant Blue (Wang et al. 2007). Laccase activity was confirmed by zymogram analysis on native PAGE performed as described by Fonseca et al. (2010). Staining was carried out after native PAGE by incubating the gel in 0.1 mol l⁻¹ sodium acetate buffer containing 5 mmol l⁻¹ of DMP or ABTS before detecting laccase activity. After incubating the gel for 5 min, the dye solution was discarded; the gel was immediately scanned using a scanner (HP Deskjet F300 All-in-One series).

The purified laccase enzyme was used for biochemical characterization.

Laccase (EC 1.10.3.2) activity was measured at 30°C using 5 mmol l⁻¹ 2,6-dimethoxyphenol (DMP) as substrate in 0.1 mol l⁻¹ sodium acetate buffer (pH 3-6) (Field et al. 1993). The absorbance increase in the reaction mixture was monitored at 469 nm (ε₄₆₉ = 7.5 mmol l⁻¹ cm⁻¹) in a Shimadzu UV-3600 spectrophotometer. Enzyme activity was expressed as International Units (U), defined as the amount of enzyme needed to produce 1 μmol of product min⁻¹ at 30°C.

The purified laccase activity towards DMP as a substrate was tested at a pH range of 3.6-5.6 in 50 mmol l⁻¹ sodium acetate buffer and was examined between 30 and 90°C at the determined optimal pH value. Laccase thermal stability was assessed by incubating the enzyme preparation at 30, 40, 50, 60 and 70°C and testing its residual activity at various time intervals during 7 h. The effect of pH on the stability of pure laccase was determined at pH 3-6, 4-8 and 5-6, and the remaining activity was determined at various periods of time for 6 h. Residual activity was calculated considering 100% the maximal enzymatic activity at optimal pH and temperature, and was expressed as per cent of the remaining activity.
The laccase substrate specificity of pure enzymes was tested using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS (ε_{460} = 29.3 mmol l⁻¹ cm⁻¹), 2,6-DMP (ε_{460} = 27.5 mmol l⁻¹ cm⁻¹). Rates of substrate oxidation were determined by measuring the absorbance increase in a given time interval, at the corresponding wavelengths (Ryan et al. 2003; Colao et al. 2006). \( K_m \) and \( V_{\text{max}} \) values of purified laccase were determined by measuring enzyme activity at various concentrations (0–0.1–5 mmol l⁻¹) of ABTS and 2,6-DMP as substrate, at optimal pH. Kinetic constants were calculated by the Michaelis–Menten method using nonlinear regression fit in the Graphpad Prism 5 software. Tolerance to chloride ions (NaCl) was determined by incubating laccase solution (0.5 IU) with varying concentrations of NaCl for 3 h in a total volume of 1.5 ml. The effect of EDTA and SDS at different concentrations (5–100 mmol l⁻¹) as some potential laccase inhibitors was monitored using 5 mmol l⁻¹ DMP as a substrate in sodium acetate buffer (pH 3.6). Activity in EDTA-free medium was defined as 100%. All measurements were carried out in triplicate.

**Dye decolorization**

Nine different dyes were used for this study. The reaction mixture (2 ml) contained 100 mmol l⁻¹ acetate buffer pH 3.6, individual dye (each 50 mg l⁻¹ in final concentration) and 0.5 IU laccase. The reaction was initiated by the addition of laccase and incubated at 30°C for 12 h. Decolorization was determined by monitoring the decrease in absorbance at the peak of maximum visible absorbance and expressed as percentage of decolorization. Decolorization was defined as: Decolorization (%) = 100 \times (A₀–Aₜ)/A₀. Where A₀ is the absorbance of the reaction mixture before incubation with the enzyme and Aₜ is absorbance after incubation. The heat-denatured laccase solutions were used as controls and the blanks contained all components of the reaction mixture except the dyes.

**Results**

**Isolation and characterization of a new Lac-coding gene from *P. brevispora***

Using *P. brevispora* BAFC 633 total gDNA as template, a 1800-bp partial fragment was obtained by means of degenerate primers able to hybrdize on I and IV copper-binding sites, whereas with primers hybridizing regions II and III, another fragment of 1600-bp could be amplified. The inverse PCR strategy yielded a fragment of 1200 bp.

The sequenced region from gDNA consisted of 2145 bp, including a gene structural region and 473 bp of the 5′-upstream region with several putative cis-acting elements (see Additional file 1). The coding region of lac I gene consisted of a 1563-bp ORF encoding 521 amino acids (aa). The region contained 12 introns with the relative positions of the splicing junctions and internal lariat formation sites, deduced on the basis of comparisons with other described fungal Lac genes, and conserved motifs found at the 5′ and 3′ introns ends. The consensus poly-adenylation signal sequence (AATAAAA) (Proudfoot 1991), was not found at the 3′ untranslated region.

The lac I 5′-uncoding region contained two TATA boxes found at positions 45 and 398, three CAAT boxes at positions 107, 142 and 169, and two putative CAAT inverted boxes at positions 328 and 352. Different transcription factor-binding sites were detected at position 284, corresponding to ACE1 adhering to the consensus sequences 5-THHNNNGTGDG-3 (Zhu and Thiele 1996), an inverted metal response element (MRE) 5-TGCRNC-3 (Thiele 1992) at position 288, a heat-responsive element (HSE) C-GAA- TTC- G (Pelham 1982) at 196, an antioxidant response element (ARE) TGACNNNGC (Rushmore et al. 1991) at position 207 and a nitrogen response element (NIT) TATCT (Marzluf 1997) at 217.

The putative translated Lac I aa sequence included a 20-aa signal peptide with an A-I cleavage site (see Fig. S1). The mature Lac I would be 501 aa residues in length with a calculated molar mass of 54-14 kDa, and an estimated pI value of 5-53. Eight putative N-glycosylation sites could be deduced from the consensus sequence (N-X-T/S, in which X is not P). The deduced Lac I aa sequence of *P. brevispora* BAFC 633 shared 63–73% identity with other basidiomycetous laccases, including those from *Coriolopsis gallica* (AAF70119.2), *Trametes* sp. AH18-2 laccase B (AAW31597.1), *Trametes* sp. 420 (AAW28938.1), *Phlebia radiata* LAC2 (CAI56705.1) and *Steccherinum murashkinskyi* lac 1 (AFI41888), especially in the copper-binding region, with all the His and Cys residues conserved.

All the expected Lac Cu (II) ligands could be identified in the deduced Lac I sequence: eight His residues in the highly conserved motif of four His-X-His repeats that coordinate the trinuclear Type 2/Type 3 copper (red boxes); an additional four Cys and His were also found to be conserved, and these would be likely involved in binding to the Type 1 copper site (Garg et al. 2012). The LEL sequence adjacent to the last conserved His is conserved in laccases of high redox potential, Ala being the most replaceable aa at this position, in contrast with low-potential redox laccases which harbour VSG replacing LEA tripeptide. The finding of the LNA tripeptide in Lac I (see Fig. S2, green box), in association with the presence of Leu at the T1 copper-binding position, led us to
suggest that *P. brevispora* BAFC 633 Lac I would have a higher potential redox.

Likewise, according to the analysis of the deduced protein sequence, Lac I from *P. brevispora* BAFC 633 showed to be closely related to Lac2 of *P. radiata* (Fig. 2).

**Heterologous expression of Lac I in *P. pastoris***

To express the Lac I in *P. pastoris*, four different expression plasmids were monitored under the control of the tightly regulated AOX1 promoter, in frame with the native signal sequence or the *α*-factor signal peptide from *S. cerevisiae* to direct the secretion of the recombinant protein with or without polyhistidine tag. By the plate detection, the dark green zones appeared around the transformant, both constructs pPIC9/lac I and pPIC3.5K/lac I implied that bioactive lac I was expressed and secreted into the extracellular medium in both transformants without polyhistidine tag. Transformants showing a deeper green colour were used for the production of the recombinant protein using liquid cultures. The laccase-positive transformants with constructs pPIC9.K/lac I and pPIC3.5K/lac I, were then fermented in BMM liquid medium at 30 °C and induced by the addition of 0.5% methanol daily and different copper concentrations. After 6 days of growth, the laccase activities reached 30 and 500 U l⁻¹ for pPIC9.K/lac I and pPIC3.5K/lac I respectively (Fig. 3).

Figure 2 Phylogenetic tree of the evolutionary relationships based on the sequence alignment of the Lac I of the *Phlebia brevispora* BAFC 633 with homologous amino acids sequences obtained from the NCBI GenBank. Group support, assessed with 1000 Bootstrapping and Parsimony Jackknifing replicates. Numbers above branches correspond to Jackknife support. Bootstrap supports are given in parentheses. Phylogenetic tree performed using maximum likelihood methods.
The laccase activity was found in the culture medium and no intracellular activity was detected at any time during the growth in minimal medium with methanol as a carbon source.

Purification and characterization of recombinant Lac I

The recombinant Lac I was purified with two steps procedure. SDS-PAGE analysis revealed that the molecular masses of purified recombinant lack were about 110 kDa (Fig. 4), a value higher than the predicted masses of 54-14 kDa. The zymogram shows the active laccase with DMP and ABTS. (Fig. 4).

The optimal pH values of Lac I was 3-6 and the optimal temperature value was 50°C for DMP (Fig. 5a,b respectively). The recombinant laccases were stable at a temperature of 40 and 50°C for 4 h maintaining above 50% of the activity while at 30°C it was for 6 h (Fig. 5c). Recombinant laccase enzyme showed high pH stability, maintaining a constant activity after 6 h of incubation at pH 3-6 and 4-6, and decreasing by only 20% in the case of pH 5-8 after 5 h (Fig. 5d). It was very sensitive to SDS and EDTA (Table 2). The metal ions such as Ca²⁺, K⁺, NH₄⁺, Mn²⁺ increased the Lac I activity while Zn²⁺ and Cu²⁺ decreased the activity (Table 2).

Kinetic parameters of the laccase were determined by using ABTS and DMP as substrates and summarized in Table 3. The oxidizing power and catalytic coefficient \( k_{cat}/K_m \) of purified Lac I was higher for ABTS than DMP.

The effect of various water-miscible organic solvents (acetone, ethanol, dimethyl sulfoxide or DMSO), was investigated on laccase activity and the results are shown in Fig. 6a. Lac I was stable in ethanol up to 3 h at 70% (v/v) concentration retaining 90% activity. Lac I in the presence of acetone and DMSO retained up to 40 and 20% of activity, respectively, to the highest concentration used. With chloride ions, the stability was monitored for 3 h and Lac I retained more than 50% of activity until 300 mmol l⁻¹ of concentration Fig. 6b.

Dye decolorization by recombinant Lac I

Nine synthetic dyes were used to evaluate the decolorization ability of the recombinant Lac I. All the dyes were discoloured with different efficiency, thymol blue was the most discoloured with 90% (Table S1). The broad decolorization specificity of Lac I rendered great potential in industrial applications, such as degradation of dyes from textile effluents.

Discussion

Many fungal laccases are blue copper oxidases (Messerschmidt and Huber 1990; Desai and Nityanand 2011) and because nucleotide sequences of the copper-binding regions are highly conserved, they have been frequently used for designing PCR primers in order to clone laccase gene fragments in several basidiomycetes (D’Souza et al. 1996). Based on this knowledge, a laccase gene harbouring four copper-binding sites could be successfully cloned in this work. Within the regulatory region of lac I, characteristic eukaryotic regulatory elements such as TATA and CAAT motifs were identified. CAAT motifs play a pivotal role in determining the efficiency of the promoter. The spacing of these motifs is consistent with those seen for other fungal promoters (Padgett et al. 1984). Neither the TATAAA nor CAAT motifs are strictly conserved in filamentous fungal genes (Padgett et al. 1984). Several potential consensus transcriptional regulation elements which might affect the production of P. brevispora BAFC 633 laccase were also found. Such potential regulation sites specific for laccase were: an inverted MRE, an ARE, a NIT and an ACE-like element. Overall response elements are differentially distributed throughout the
promoter sequence and some of them are characteristic of laccase subfamilies, such as the presence of ARE and the absence of XRE (Piscitelli et al. 2011).

MRE elements have been identified in animals and plants as target sites for transcription factors when exposed to toxic concentrations of Cd, Cu and Zn (Whitelaw et al. 1997). Some authors (Karahanian et al. 1998; Mansur et al. 1998; Galhaup et al. 2001; Klonowska et al. 2001) found other promoters containing multiple putative MRE sites with consensus sequences 5-
TGCRCNC-3 (Thiele 1992). Although MREs are included in the promoters of pox and poxa 1b in *Pleurotus ostreatus* in both orientations (Faraco et al. 2003), only an inverted MRE could be detected in *P. brevispora* BAFC 633 lac I. Metal-regulated gene transcription plays an important role in homeostasis and metal detoxification (Kagi and Shaffer 1998) and is widely distributed in eukaryotes (Hagen et al. 1988; Greco et al. 1990; Hill et al. 1991).

We also found a putative ACE1 transcription factor-binding site. This latter was originally reported in metallothioneins (Furst et al. 1988) and superoxide dismutase promoters from *S. cerevisiae* as a recognition site for the ACE1 transcription factor which responds to Cu(I) and Ag (I), but not to Zn(II) (Grala et al. 1991). Metallothioneins have been suggested to be involved in several cellular processes including metal storage and detoxification (Marbach et al. 1989). Several heavy metals induce the expression of these genes, with regulation via a metal-regulatory protein that functions both as a metal receptor and as a trans-acting transcription factor (Mansur et al. 1998).

It has been shown that protein factors could bind MREs of the laccase gene promoters from *P. ostreatus* only when copper is absent (Faraco et al. 2003). Copper has been previously reported to increase laccase activity in *Neurospora crassa* (Huber and Lerch 1987), *Trametes pubescens* (Mansur et al. 1998) and *P. ostreatus* (Palmieri et al. 2000), and to enhance laccase gene transcription in *T. versicolor* (Collins and Dobson 1997) and *P. ostreatus* (Palmieri et al. 2000). We found that copper has an important effect on both the activity (Fonseca et al. 2010) and gene transcription of laccases in *P. brevispora* BAFC 633 (Fonseca et al. 2014a). Copper requirement for high expression of *P. brevispora* BAFC 633 laccase suggests that MREs and ACE in the promoters of lac I have important physiological functions. However, the mechanism of how copper ion at different concentrations is able to modulate differential expression of extracellular laccases remains unknown.

The HSE from lac I was also detected in a promoter gene coding for MnP in *Phanerochaete chrysosporium* and it showed to be heat shock regulated (Collins and Dobson 1997). Homologous to the HSE, consensus sequence (C—GAA—TTC—G) (Pelham 1982) was found three times in the gene promoter of *P. radiata* (Salohimeho et al. 1991) showing homologies of 6/8, 5/8 and 6/8 with the consensus sequence. However, heat-shock regulation should be investigated in the case of *Phlebia brevispora* BAFC 633 lac I gene.

Another element (TGACNNNNCG) also detected in the lac I gene of *P. brevispora* BAFC 633 was ARE. Although XRE (xenobiotic response element) was not found in the promoter fragment obtained in this study, XRE and ARE have been found in promoters of genes related to xenobiotic aromatic hydrocarbon degradation, such as in fungal laccase genes (Soden and Dobson 2003), CypP450 genes, glutathione-S-transferase and NAD(P)H: quinone oxidoreductase of eukaryotes (Kuramoto et al. 2002; Nguyen et al. 2003). XRE and ARE sequences increase (in cis) transcription of genes related to oxidative stress. Genes regulated by ARE and XRE encode proteins that help controlling redox state of cells and thus defend against oxidative damage (Kuramoto et al. 2002).

Moreover, regulatory elements, such as Mig and Nit2, involved in the regulation by carbon and nitrogen, respectively, during fungal genes expression, have been reported in the promoter regions of *P. sajor-caju* (Soden et al. 2003). Copper has been previously reported to increase laccase activity in *Neurospora crassa* (Huber and Lerch 1987), *Trametes pubescens* (Mansur et al. 1998) and *P. ostreatus* (Palmieri et al. 2000), and to enhance laccase gene transcription in *T. versicolor* (Collins and Dobson 1997) and *P. ostreatus* (Palmieri et al. 2000). We found that copper has an important effect on both the activity (Fonseca et al. 2010) and gene transcription of laccases in *P. brevispora* BAFC 633 (Fonseca et al. 2014a). Copper requirement for high expression of *P. brevispora* BAFC 633 laccase suggests that MREs and ACE in the promoters of lac I have important physiological functions. However, the mechanism of how copper ion at different concentrations is able to modulate differential expression of extracellular laccases remains unknown.

**Table 2** Effects of ions and inhibitors on Lac I activity

| Inhibitor | Concentration (mmol l⁻¹) | Relative activity (%) |
|-----------|--------------------------|----------------------|
| None      | –                        | 100                  |
| EDTA      | 5                        | 80                   |
|           | 25                       | 75                   |
|           | 50                       | 67                   |
|           | 75                       | 59                   |
|           | 100                      | 50                   |
| SDS       | 5                        | 15                   |
|           | 25                       | 10                   |
|           | 50                       | 5                    |
|           | 75                       | 0                    |
|           | 100                      | 0                    |
| Ca²⁺      | 1                        | 145                  |
| Cu²⁺      | 1                        | 38                   |
| K⁺        | 1                        | 137                  |
| Mn²⁺      | 1                        | 118                  |
| NH₄⁺      | 1                        | 110                  |
| Zn²⁺      | 1                        | 39                   |

Values are the mean of triplicate determinations and standard deviation in less than 5%.

**Table 3** Substrate specificity and kinetic constants of purified Lac I secreted by *P. pastoris*

| % of enzymatic activity | Kₘ (µmol l⁻¹) | Vₘₐₓ (µmol l⁻¹ min⁻¹) | kₗₘₐₓ (s⁻¹) | kₗₐₜ/Kₘ (µmol l⁻¹) |
|-------------------------|---------------|------------------------|--------------|--------------------|
| ABTS DMP                |               |                        |              |                    |
| Lac I                   | 100 ± 1       | 60 ± 1                 | 727          | 245                |

Values are the mean of triplicate determinations and standard deviation in less than 5%.

**Table 2** Effects of ions and inhibitors on Lac I activity

| Inhibitor | Concentration (mmol l⁻¹) | Relative activity (%) |
|-----------|--------------------------|----------------------|
| None      | –                        | 100                  |
| EDTA      | 5                        | 80                   |
|           | 25                       | 75                   |
|           | 50                       | 67                   |
|           | 75                       | 59                   |
|           | 100                      | 50                   |
| SDS       | 5                        | 15                   |
|           | 25                       | 10                   |
|           | 50                       | 5                    |
|           | 75                       | 0                    |
|           | 100                      | 0                    |
| Ca²⁺      | 1                        | 145                  |
| Cu²⁺      | 1                        | 38                   |
| K⁺        | 1                        | 137                  |
| Mn²⁺      | 1                        | 118                  |
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|-------------------------|---------------|------------------------|--------------|--------------------|
| ABTS DMP                |               |                        |              |                    |
| Lac I                   | 100 ± 1       | 60 ± 1                 | 727          | 245                |

Values are the mean of triplicate determinations and standard deviation in less than 5%.
changes in regulate expression (Marzluf 1997). Thus, NIT homologous may midе utilization showed to require NIT2 for protein amino acid metabolism, protein catabolism and acetate involved in the nitrate assimilation, purine metabolism, BAFC 633. In this regard, a number of structural genes and Dobson 2003) and other fungal species. In the present study, only one NIT could be found in P. brevispora BAFC 633. In this regard, a number of structural genes involved in the nitrate assimilation, purine metabolism, amino acid metabolism, protein catabolism and acetamide utilization showed to require NIT2 for protein expression (Marzluf 1997). Thus, NIT homologous may regulate lac I expression in response to cultural nitrogen changes in P. brevispora BAFC 633.

The identified P. brevispora BAFC 633 laccase gene (lac I) contains 12 introns with splicing junctions and internal lariat formation sites adhering to the GT-AG rule (Padgett et al. 1984). Intron positions were inferred from comparison with other genes and the consensus. The intron size obtained was conserved, which is typical for most fungal introns (Padgett et al. 1984).

The lac I-deduced protein product displays a high aminoacidic sequence similarity (62–75%) with other basidiomycetous laccases characterized so far. The in silico-predicted Lac I polypeptide shares 72% identity with Lac2 of P. radiata and both laccases carry the conserved copper-binding sites, laccase signature sequence regions L1, L2, L3 and L4 (Gurr et al. 1987), and four substrate-binding loops (Kumar et al. 2003). The aa residue located 10 aa downstream the conserved Cys would have an important effect on the redox potential of the T1-copper at the active site (Caneters and Gilardi 1993). Based on the difference of this residue, laccases are proposed to be classified into three types: class 1 (Met), class 2 (Leu), and class 3 (Phe) in increasing order of redox potential (Eggert et al. 1998). The phylogenetic analysis indicates that P. brevispora BAFC 633 and P. radiata laccases are closely related and belong to the same branch in the tree (Fig. 2). Both P. brevispora BAFC 633 and P. radiata laccase-encoding genes are apparently orthologous as a speciation event. As depicted in Fig. 2, P. brevispora BAFC 633 Lac I (calculated pI 5.53) and P. radiata Lac2 (calculated pI 5.7) (Mäkelä et al. 2006) are evolutionarily related fungi.

The lac I cDNA sequence from P. brevispora identified in this study that encoding novel laccase enzyme in P. brevispora BAFC 633 was successfully expressed in P. pastoris under the control of the tightly regulated alcohol oxidase promoter (PaAox1) induced by methanol. There are several strategies used to increase the expression level of heterologous proteins in Pichia, such as the use of native promoters and multiple gene copies, codon optimization, altering of secretory signal sequences and optimization of culture conditions (Gu et al. 2014). In this study lac I cDNA cloned using the α-factor signal peptide from S. cerevisiae was more efficient to direct the secretion of the recombinant protein than the native signal sequence. However, in other species such as Pleurotus and Trametes the use of native laccase signal sequences proved to be more alternative to the α-factor signal peptide to drive the secretion of recombinant proteins in P. pastoris (Jönsson et al. 1997; Brown et al. 2002; Soden et al. 2002; Colao et al. 2006).

The high molecular weight of Lac I expressed in P. pastoris should be attributed to the presence of hyperglycosylation. The biochemical parameters of various purified recombinant laccases were revealed in recent publications (Mate et al. 2013; Gu et al. 2014), showing some similar characteristics, such as increased activity at acid pH (between 3 and 4) and at temperatures close to 50°C. The Lac I activity was highest with ABTS followed DMP which is typical for laccases and reflects the different oxidation mechanisms that depend on the substrate (Boa et al. 2012). The turnover rates (kcat) for Lac I was the highest kcat/Km value towards ABTS, followed DMP as reported for other recombinant laccase (Boa et al. 2012, Gu et al. 2014).

Figure 6 Residual activity of laccase after incubation of purified enzyme for 3 h. (a) Residual activity of laccase in the presence of different concentrations of organic solvents 40% (white bar), 50% (grey bar), 70% (black bar). (b) Residual activity of laccase in the presence of NaCl concentration. The values are the mean of triplicate experiment.
The Lac I was stable at temperature of 40 and 50°C for 4 h which is crucial during the biotechnological process (Fonseca et al. 2014b).

Lac I ability to maintain their activity at pH higher than the optimum, along with pH stability, represents an advantage from the biotechnological standpoint since enzymes stable near neutrality would allow minimizing the risk of equipment corrosion during industrial processing Fonseca et al. 2015).

The effects of metal ions and inhibitors on Lac I activity were tested. It was very sensitive to SDS and observing the same for the SDS to recombinant laccase of Coprinus comatus (Boa et al. 2012). Sodium dodecyl sulphate is a strong protein denaturant that inactivates most laccases even at a low concentration (Gu et al. 2014). The activity level of Lac I could be significantly influenced by the metal ions, such as Ca²⁺, K⁺, NH₄⁺, Mn²⁺, increased the Lac I activity while Zn²⁺ and Cu²⁺ decreased the activity.

In this sense recombinant laccase of Coprinus comatus was strongly inhibited by Fe²⁺, Mn²⁺, Zn²⁺, Fe³⁺ and Co²⁺ and activated in the presence of K⁺ (Boa et al. 2012).

Lac I exhibited higher tolerance towards various water-miscible organic solvents. Garg et al. (2012) reported a correlate the tolerance to organic solvents as a by-product of this altered glycosylation pattern and this property is important for its use in organic synthesis work. It has been observed that laccase structure, stability and activity are affected by water miscible solvents through direct interaction with enzyme and through its effect on water activity (Rodapiewicz-Novak 2000).

Higher resistance to chloride was observed for the Lac I. Laccases are generally inhibited by chloride ions, an important component in dye wastewaters, which limits its use in treatment plants (Garg et al. 2012).

The broad decolorization specificity of Lac I rendered great potential in industrial applications, such as degradation of dyes from textile effluents. Not all the dyes were oxidized by Lac I at the same extent. The differences in dye oxidation could be explained by the different electron donating properties of the substituents and their locations on the phenolic ring (Colao et al. 2006). So the different chemical structures of dyes might explain these differences in the decolorization efficiencies (Moldes et al. 2003). In this work it was not possible to establish a correlation between the type of dye and the degree of discoloration, as reported for a laccase of Coprinopsis cinerea cloned on P. Pastoris (Bao et al. 2013). This work provides evidence for the efficient role of laccase for the decolorization of dyes such as thymol blue, a triaryl-methane type dye, which is used in dye manufacturing.

In conclusion, the corresponding lac I-encoding gene was successfully sequenced allowing finding cis-acting elements located at the lac I regulatory region. These results may provide a further insight into potential ways of optimizing fermentation for fungal Lacs production, and also open new frontiers for engineering strong promoters for Lac production. Also in this study lac1 cDNA was expressed in P. pastoris using the α-factor signal peptide from S. cerevisiae which was more efficient to direct the secretion of the recombinant protein that the native signal sequence. Lac I exhibited higher tolerance towards various water-miscible organic solvents. This property is important for its use in organic synthesis work. Synthetic dyes could be degraded to different degrees, the broad decolorization specificity of Lac I indicates their great potentials in industrial applications, such as degradation of dyes from textile effluents.

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Conflict of Interest

No conflict of interest declared.

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Recombinant laccase from *P. brevispora* BAFC 633

M.I. Fonseca et al.

**Figure S1** Nucleotide and deduced amino acid sequences of *lac I* and the corresponding Lac product, respectively, from *P. brevispora* BAFC 633.

**Figure S2** *In silico*-deduced amino acid sequence alignment of laccase of *Phlebia brevispora* BAFC633 with other fungal laccases.

**Table S1** Decolorization of synthetic dyes with recombinant Lac 1 after incubation for 12 h

from *Ganoderma lucidum* and heterologous expression in *Pichia pastoris*. *J Basic Microbiol* **54**, 134–141.

Zhu, Z. and Thiele, D.J. (1996) A specialized nucleosome modulates transcription factor access to a *C. glabrata* metal responsive promoter. *Cell* **87**, 459–470.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article: