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Decolorization of Kraft liquor effluents and biochemical characterization of laccases from *Phlebia brevispora* BAFC 633

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ABSTRACT

In this work we evaluated the decolorization ability of fungus identified as *Phlebia brevispora* BAFC 633 on Kraft liquor effluents and we looked at the biochemical characterization of two novel laccases from the fungus. We conducted decolorization assays on diluted Kraft liquor with the enzyme supernatants. Ligninolytic activity was demonstrated by the 20% reduction of lignin peak absorbances between 215 and 280 nm after laccase supernatants treatment.

From these supernatants, two novel laccases were isolated and characterized: a constitutively expressed 60-kDa (Lac A), and a $CuSO_4$-induced 75 kDa (Lac B). Laccases were purified by anion-exchange and gel filltration chromatography and their biochemical properties were determined. The temperature and pH optima were 70–75 °C and 4.8 for Lac A, and 60–70 °C and 4.4 for Lac B; and both exhibited high stability at low pH and high temperature. Substrate specificity of the purified enzyme was tested and the highest oxidation was to 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) with a catalytic efficiency of 2.6×10^7 s^{-1} mol l^{-1} for Lac A and 9×10^8 s^{-1} mol l^{-1} for Lac B.

Enzyme supernatants can be used to remediate lignin-rich effluent. The isoenzymes tolerance to low pH and high temperature are significant features to optimize laccase production toward potential biotechnological applications.

1. Introduction

Basidiomycetous wood- and litter-degrading white-rot fungi (WRF) are unique in their ability to decompose the heterogeneous plant polymer lignin by using an array of extracellular lignin-modifying enzymes: lignin, manganese and versatile peroxidases (EC 1.11.1.13–16), and laccases (EC 1.10.3.2) (Hildén et al., 2008).

Laccases (benzenediol oxygen oxidoreductase) are polyphenol oxidases that catalyze the oxidation of phenolic compounds and aromatic amines using molecular oxygen as the electron acceptor. They are typically multicopper blue oxidases containing 2–4 copper atoms per molecule and are widely distributed in many plants and fungi (Min et al., 2001).

Fungal laccases usually contain four copper ions, which are distributed at three distinct sites (types 1, 2, and 3). Because of a continuous emergence of new applications of the enzymes, there is a renewed interest in the potential applications of WRF and their respective enzymes because of their varied functions and broad substrate specificity which make them suitable for many industrial processes such as biopulping, textile-dye removal, biobleaching, phenolics removal, effluent detoxification and other processes (Nyanhongo et al., 2002; Shimizu et al., 2009; Fonseca et al., 2010, 2013, 2014a, 2014b).

Under these considerations, our group has selected and analyzed enzyme systems of different WRF isolated from the Misiones rainforest that showed to be powerful laccase (Lac) producers with a promising biotechnological potential (Shimizu et al., 2009; Fonseca et al., 2010, 2013, 2014a, 2014b). From this selection, one of the most competent strains was *Phlebia brevispora* BAFC 633 because it showed the highest enzyme activity both in basal (Shimizu et al., 2009; Fonseca et al., 2010, 2013, 2014a) and induced

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conditions with significant improvement in the presence $CuSO_4$ (up to 50 times laccase activity that in basal condition) (Fonseca et al., 2010). *P. brevispora* BAFC 633 produced a major laccase of 60 kDa expressed constitutively and another of 75 kDa induced by the presence of $CuSO_4$ (Fonseca et al., 2010). *P. brevispora* showed high detoxification capacity in solid media supplemented with black liquor at 14 days of cultivation, indicating potential use for bioremediation of pulping industry effluents (Fonseca et al., 2010). Degradation studies of bromophenol blue and malachite green in solid cultures revealed the occurrence of oxidative enzymes secreted, showing the decolorization potential of industrial dyes (Fonseca et al., 2014b). Another significant application is the pulp and paper industry, where *P. brevispora* BAFC 633 caused a selective removal of lignin during biotreatment of pine wood chips and reduction in Kappa number and weight loss, creating a more open structure and better access to the pulping liquor, which would lead to lower chemical consumption to reach the same delignification degree (Fonseca et al., 2014a).

Concerning the enzymatic profile, several Lacs have been purified and biochemically characterized up to date (Farnet et al., 2000; Min et al., 2001; Baldrian, 2004; Moon-Jeong et al., 2005; Kalyani et al., 2012; Chairin et al., 2014) and some of the encoding genes have also been cloned and genetically characterized (Saloheimo et al., 1991; Mäkelä et al., 2006; Xiao et al., 2006). The enzymes secreted by basidiomycetes depend on the kind of fungus, the strain and the culture conditions. Therefore, additional strains need to be screened for their ability to degrade recalcitrant aromatic compounds, including dyes present in industrial effluents causing environmental problems (Mechichi et al., 2006).

In this work we evaluated the decolorization ability of *Phlebia brevispora* BAFC 633 on Kraft liquor effluents and we looked at the biochemical characterization of two novel laccases from the fungus.

2. Materials and methods

2.1. Microorganism and culture conditions

The WRF isolated from dead logs of the Misiones rainforest and previously misidentified by classical taxonomy methods as *Peniophora* sp. BAFC 633 (currently catalogued as *Phlebia brevispora* BAFC 633), is deposited in the Filamentous Fungi Culture Collection at the Biological Sciences Department, Faculty of Exact and Natural Sciences, University of Buenos Aires, 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 liquid inocula, 4 cm^2-agar plugs from 5-7-day-old MEA plates were cut and transferred to 100 ml Erlenmeyer flasks containing 10 ml of ME liquid medium (malt extract, 12.7 g l^{-1}; corn steep liquor, 5 g l^{-1}) and incubated at 29 °C under static conditions.

2.2. Molecular identification based on rDNA phylogenetic analysis

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 100 mM Tris–HCl pH 8, 20 mM EDTA. DNA extraction was carried out with a lysis buffer solution (100 mM, Tris–HCl pH 8, 1.5 M NaCl, 50 mM, EDTA pH 8) at 60 °C containing 0.1 mg ml^{-1} proteinase K, 10 mM β-mercaptoethanol and 2% (wt/vol) SDS. DNA was purified with chloroform: isoamylic alcohol (24:1, v/v) and 3 M potassium acetate, and finally precipitated with isopropyl alcohol.

Ribosomal DNA regions including the D1/D2 domain of the large subunit, LSU (28S) rDNA, internal transcribed spacers (ITS1-5.8S-ITS2) region and small subunit, SSU (18S) rDNA, were amplified by PCR and sequenced by Macrogen, Korea. Amplifications were carried out with primers previously described (Table 1).

In all cases, PCR was carried out in a 20 μl final volume containing 1X KCl buffer, 2.5 mM, $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, 0.5 U Taq 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.

Ribosomal DNA sequences were deposited in GenBank database (18S: JX863666, ITS1-5,8S-ITS2: HM208154, D1/D2 domain: JX863667). All sequences were analyzed with Chromas Lite 2.01, BLASTn, BioEdit and CLUSTAL W before phylogenetic tree construction. First edition was performed with the obtained sequences, eliminating poor quality segments at both ends. When more than one sequence was available, CAP3 program (Huang and Madan, 1999) was used to construct a contig and possible cases with discordant positions were manually corrected, using the chromatograms as reference.

The contig was used to search for homologous sequences of the kingdom Fungi (excluding metagenomics and environmental sample sequences) using the BLASTn program with default parameters. From the first 500 sequences retrieved, redundant sequences were removed. The analysis included 149 sequences and the alignment was performed with the MUSCLE program in the *full processing* mode and other default parameters (Edgar, 2004). Then, an alignment cure was performed with the Gblocks program, eliminating low homology with positions that may interfere with the phylogenetic analysis (Castresana, 2000).

Phylogenetic analysis was performed using the PhyML program (http://atgc.lirmm.fr/phyml/) (Guindon and Gascuel, 2003) with a supported statistical approach likelihood-ratio test (aLRT) (Anisimova and Olivier, 2006). Trees were visualized with TreeDyn (Chevenet et al., 2006).

2.3. Kraft liquor decolorization assays

Decolorization assays were carried out with Kraft liquor obtained from loblolly pine wood chips cooked with white liquor (Na_2S, NaOH), pH 12. *P. brevispora* BAFC 633 was grown in Kraft liquor diluted 1:30 v/v in water supplemented with glucose 1% w/v and pH 4.7 (adjusted with H_2SO_4 4 N) for 10 days. Cell free supernatants were obtained by filtering in a Büchner funnel using fiberglass filters (GF/C) and spectral scan from 200 to 400 nm was performed using Techcomp 8500 II spectrophotometer. Absorbances were compared with media without fungus treatment.

2.4. Laccase purification

P. brevispora BAFC 633 was grown under the culture conditions already described for maximal enzymatic activity (Fonseca et al., 2010). Supernatants from submerged cultures were separated from mycelium by filtration through filter paper. Proteins were precipitated from cell-free supernatants with 80% (wt/v) ammonium sulfate. The precipitate was dissolved in 50 mM, Tris–HCl (pH 7.5) and dialyzed for 12 h against the same buffer at 4 °C. This crude extract was concentrated using an ultramembrane filter Pierce Concentrator 20 ml/20 K (MWCO: 20,000; Thermo Scientific).

Proteins were loaded into a Bio-Gel P 100 (1.5 × 21 cm; Bio-Rad) column and eluted with 7 column volumes of 50 mM, Tris–HCl (pH 7.5) at 1 ml min^{-1} constant flow. Fractions containing Lac activity were pooled and concentrated as mentioned above. The final concentrate was then applied to an UNOsphere Q column (40 × 12.6 mm; Bio-Rad) previously equilibrated with 50 mM, Tris–HCl buffer (pH 7.5) and eluted with 10 column volumes in a linear gradient from 50 mM to 1 M NaCl in the presence of the same buffer at a constant flow of 1 ml/min. Active fractions were pooled

Table 1
Primers used in this study.

Primer name	Oligonucleotide sequence 5′- 3′	Amplified region	Reference
ITS-1	TCC GTA GGT GAA CCT GCG G	ITS1-5.8S-ITS2	(White et al., 1990)
ITS-4	TCC TCC GCT TAT TGA TAT GC	ITS1-5.8S-ITS2	(White et al., 1990)
NL-1	GCA TAT CAA AAG CGG AGG AAA AG	D1/D2 domain	(Kurtzman and Robnett, 1997)
NL-4	GGT CCG TGT TTC AAG ACG G	D1/D2 domain	(Kurtzman and Robnett, 1997)
NS1	GTA GTC ATA TGC TTG TCT C	18S	(White et al., 1990)
NS8	TCC GCA GGT TCA CCT ACG GA	18S	(White et al., 1990)
NS20	CGT CCC TAT TAA TCA TTA CG	18S	(Gargas and Taylor, 1992)
NS21	GAA TAA TAG AAT AGG ACG	18S	(Gargas and Taylor, 1992)

and concentrated by ultrafiltration. Enzyme purity was confirmed by SDS-PAGE (see below).

2.5. Laccase quantification assay

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 5 mM 2,6-dimethoxyphenol (DMP) as substrate in 0.1 M sodium acetate buffer (pH 3.6) (Field et al., 1993). The absorbance increase of the reaction mixture was monitored at 469 nm (ε_{469} = 27.5 mM^{-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.

2.6. Polyacrylamide gel electrophoresis

The homogeneity of purified laccase preparation was confirmed on 12% (wt/v) SDS-PAGE (Laemmli, 1979) using a Bio-Rad mini VE vertical electrophoresis system (Bio-Rad, CA, USA). Protein was determined by micro-test using the Bradford technique (BioRad) following manufacturer's instructions with bovine serum albumin as the standard. Secreted proteins were expressed on μg $ml\ l^{-1}$. The samples were loaded with pH 6.8 loading buffer (Edens et al., 1999) with or without 75 mM SDS and 5% (vol/vol) β-mercaptoethanol (Edens et al., 1999). The electrophoretic run was carried out at 100 V for 120 min in 1.5 M Tris-glycine buffer (pH 8.3). Proteins were stained with a silver reagent (Blum et al., 1987).

Laccase molecular weight was evaluated by 7.5% SDS-PAGE and compared to a molecular weight marker (Kaleidoscope, Bio-Rad). After gel incubation in 50 mM sodium acetate with 0.2% (w/v) Triton X-100 to remove SDS, the sample was stained with 5 mM DMP to detect laccase activity by zymography (Fonseca et al., 2010; Murugesan et al., 2007).

2.7. Laccase activity and stability

The purified laccase activity towards DMP as substrate was tested at a pH range of 3.6–5.6 in 50 mM sodium-acetate buffer and was examined between 40 and 80 °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 5 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 time for 18 h. Residual activity was calculated considering as 100% the maximal enzymatic activity at optimal pH and temperature, and was expressed as percent of remaining activity.

2.8. Substrate specificity, enzyme kinetics and effects of EDTA on laccase activity

The laccase substrate specificity of pure enzymes was tested using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (ε_{436} = 29.3 mM cm^{-1}), 2,6-DMP (ε_{469} = 27.5 mM cm^{-1}) and guaiacol (ε_{470} = 26.6 mM 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_{max} values of purified laccase were determined by measuring enzyme activity at various concentrations (0.01–5 mM) 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. The effect of EDTA at different concentrations (0.1–10 mM) as a potential laccase inhibitor was monitored using 5 mM DMP as substrate in sodium acetate buffer (pH 3.6). Activity in EDTA-free medium was defined as 100%. All measurements were carried out in triplicate.

3. Results

3.1. Molecular identification and rDNA-based phylogenetic analysis of the selected laccase producing fungus

The basidiomycetous fungus selected because of its outstanding potential for laccase production, *P. brevispora* BAFC 633, isolated from the Misiones rainforest and incorporated to the Microbiological Culture Collection of the Department of Science Biology, Faculty of Natural Sciences, and University of Buenos Aires, Argentina was studied at the molecular level for the first time.

The sets of primers NS1-NS8, ITS1-ITS4 and NL1-NL4 produced amplicons subsequently sequenced leading to 1734 bp to 18S rDNA (Genbank Accession JX863666.1), 598 bp to ITS1-5,8S-ITS2 rDNA (Genbank Accession HM208154.1), and 636 bp to 28S rDNA (Genbank Accession JX863667.1). All sequences were subsequently analyzed using BLASTn. The 18S rDNA phylogenetic tree revealed that the fungus under study belongs to the same monophyletic clade as *Phlebia brevispora* (AB084600) (99% identity). Species of the genus *Phlebia* clustered together, except for the presence of *Tyromyces subgiganteus* (AY219401), *Tyromyces fumidiceps* (AY219400), *Spongipellis pachyodon* (DQ457638) and *Climacodon septentrionales* (AY705964) (Fig. 1).

The ITS (ITS1-5, 8S-ITS2) rDNA phylogenetic tree also revealed that the fungus belongs to the same monophyletic clade as *Phlebia brevispora* (AB084616, AB084615, AB084614, AB519182), in all cases with a 99% identity (Fig. 2). It is part of a cluster within the genus *Phlebia*, except for the inclusion of *Tyromyces fissilis* (HQ728292) and *Aurantiopileus mayanensis* (HM772140).

Based on the 28S rDNA D1/D2 domain sequencing, the corresponding phylogenetic analysis revealed a close positioning between *Phlebia radiata* and *Phlebia tremellosa* (Fig. 3), however, representative sequences of this region for *P. brevispora* could not be retrieved from the database, which would have resulted from the observed position of *P. brevispora* BAFC 633 among other *Phlebia* species. According to the 18S, ITS and 28S D1/D2 domain sequence

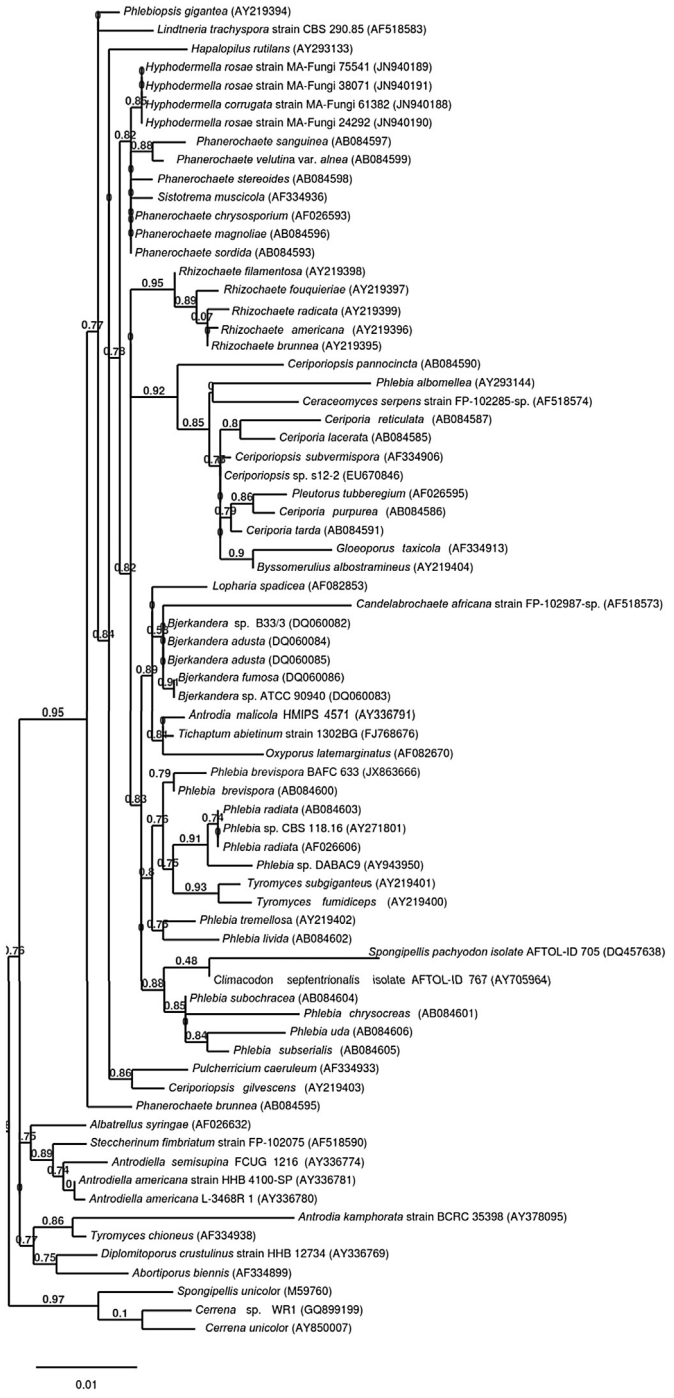

Fig. 1. Phylogenetic tree performed using maximum likelihood methods based on the sequence alignment of the 18S rDNA of the fungus under study with homologous sequences obtained from the NCBI GenBank. The tree was constructed with PhyML program, using the aLRT statistical support. aLRT statistical values are indicated at each node. The bar indicates 0.01 substitutions per site. Only the closest sequences are included.

phylogeny, the fungus was molecularly identified as *P. brevispora* BAFC 633.

3.2. Decolorization of Kraft liquor assays

The results of the spectral scanning of the filtered black liquor broth after 10 days of culture show a significant decrease of the

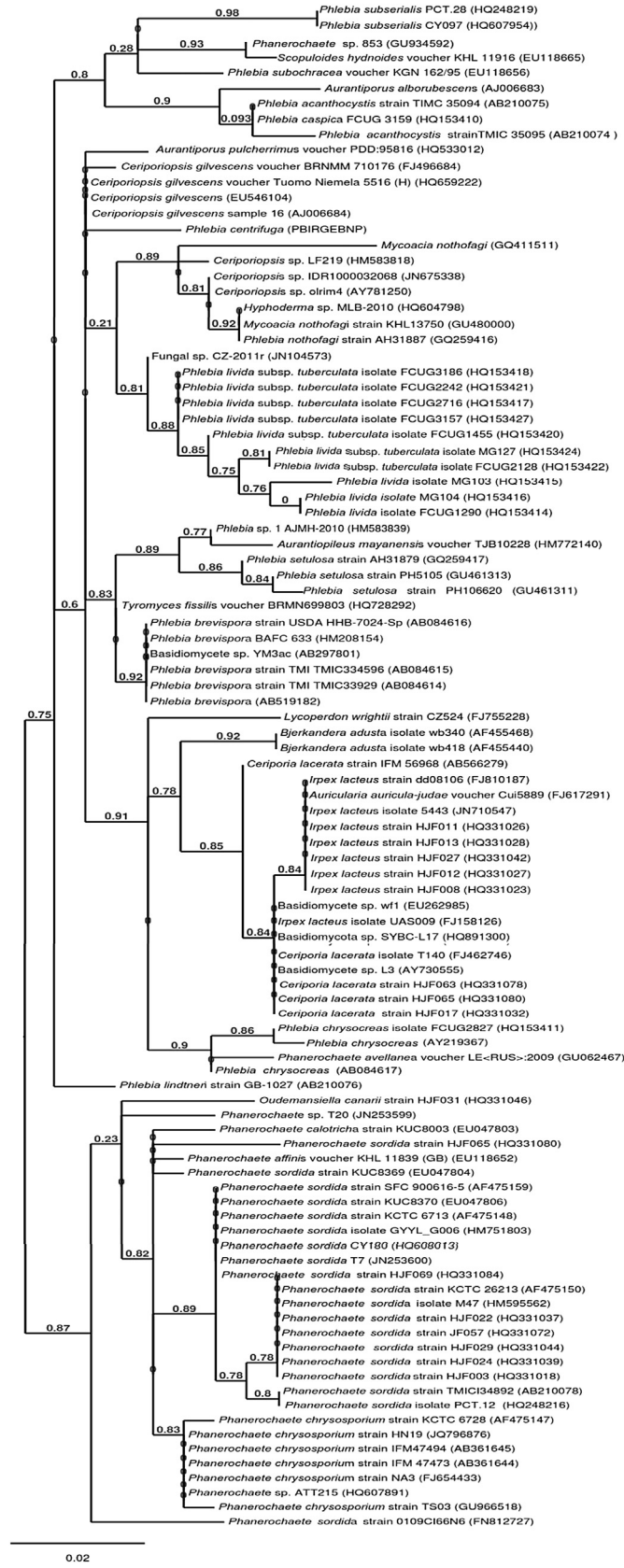

Fig. 2. Phylogenetic tree performed using maximum likelihood methods based on the sequence alignment of the ITS of the fungus under study with homologous sequences obtained from the NCBI GenBank. The tree was constructed with PhyML program, using the aLRT statistical support. aLRT statistical values are indicated at each node. The bar indicates 0.02 substitutions per site. Only the closest sequences are included.

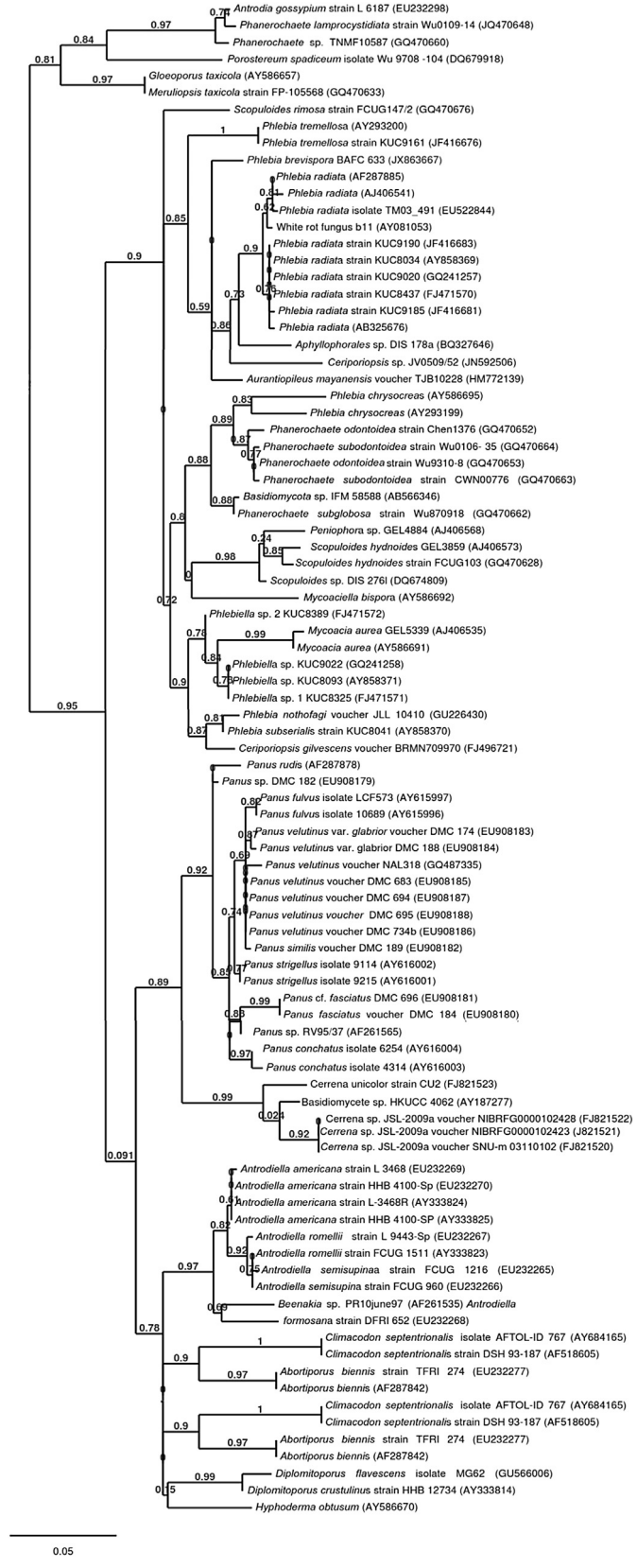

Fig. 3. Phylogenetic tree performed using maximum likelihood methods based on the sequence alignment of the rDNA 28S D1/D2 domain of the fungus under study with homologous sequences obtained from the NCBI GenBank. The tree was constructed with PhyML program, using the aLRT statistical support. aLRT statistical values are indicated at each node. The bar indicates 0.05 substitutions per site. Only the closest sequences are included.

absorbance of the treated samples comparing to the control samples ($p < 0.05$). Both spectrograms show the same typical shape of the lignin polymer, with a first maximum at 220–230 nm and a second at 270–280 nm originating from non-condensed lignin phenolic groups (Fig. 4).

The spectra show that the characteristic shape of basic structure of the lignin polymer is conserved and also the peak reduction of 20% in the 280-nm peak of the treated samples is an indication of the degrading action of *P. brevispora* BAFC 633 (Fig. 4).

3.3. Laccase purification and characterization

When *P. brevispora* BAFC 633 was grown under optimal conditions for laccase production [4], a maximal enzymatic activity of 4139.55 UI^{-1} was obtained after 10 days of cultivation. Laccase isoenzymes could be first partially purified from this culture supernatant through gel filtration chromatography, separating them from most major impurities, including brown pigments. Meanwhile, the last step involving the use of a high-efficiency anion exchange column (Q-Cartridge), led to two laccase peaks which were arbitrarily named Lac A and Lac B (Fig. 5). The molecular weights (MWs) of Lac A and Lac B were 60 and 75 kDa, respectively, as estimated by SDS-PAGE (Fig. 5).

The estimated optimal pH of both purified isoenzymes Lac A and Lac B were 4.8 and 4.5 respectively (Fig. 6A and B). The optimal incubation temperature lay between 70 and 75 °C for Lac A, and between 60 and 70 °C in the case of Lac B (Fig. 6C and D).

With regard to the enzymatic stability of purified laccases at different temperatures and incubation times, Lac B showed better stability than Lac A at all temperatures tested. An enzymatic half-life of 5 h was reached when both enzymes were incubated at 30, 40 and 50 °C and additionally at 60 °C for Lac B. The half-life for Lac A at 60 °C was reduced to 2.5 h. At 70 °C both isoenzymes rapidly lost their activity, with a 10 min and a 25 min half-life for Lac A and Lac B, respectively (Fig. 6E and F).

Both enzymes showed high pH stability, maintaining a constant activity after 18 h of incubation at all tested pHs for Lac B, and decreasing by only 20% in the case of Lac A at pH 5.8 (Fig. 6G and H).

The oxidizing power of purified isoenzymes for different substrates in descending order was: ABTS > DMP > guaiacol (Table 2). Kinetic properties were subsequently determined using ABTS and DMP and a superior catalytic coefficient (k_{cat} K_m^{-1}) was found for both enzymes with ABTS, being higher for Lac B (Table 2). In addition, no significant inhibition ($p > 0.05$) on laccase activity could be detected by EDTA at varied concentrations (0.1–10 mM) and optimum pH.

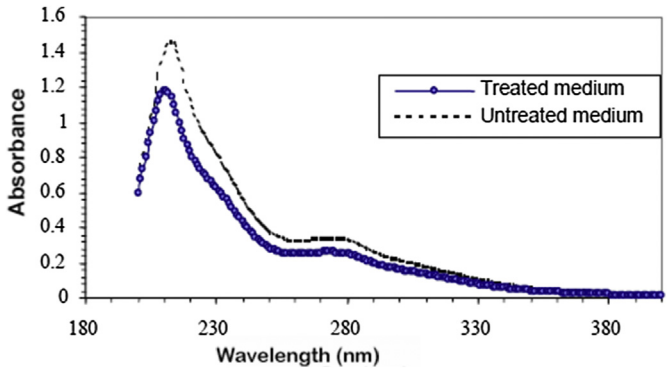

Fig. 4. Kraft liquor 1:30 v/v decolorization assays. Absorbance was compared with media without fungal treatment.

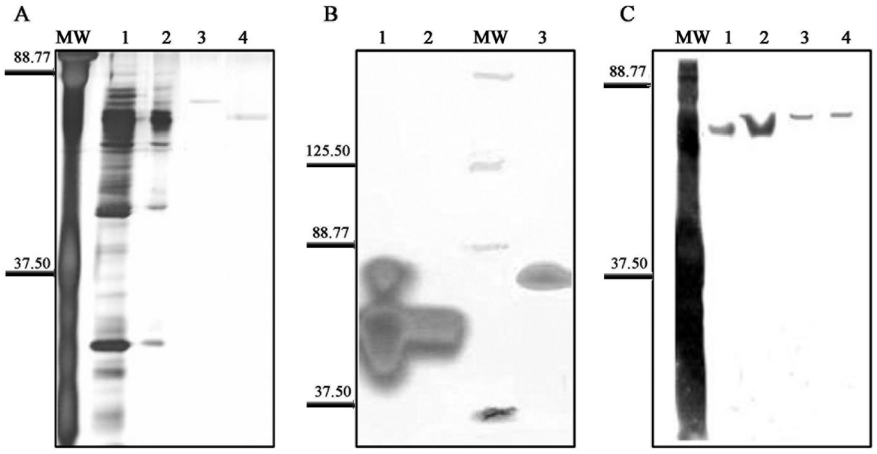

Fig. 5. Molecular weight estimation of Lac isoenzymes of *P. brevispora* BAFC 633 purified from 0.5 mM $CuSO_4$-amended ME culture supernatant after 10 days of incubation. A) SDS-PAGE (12% wt/v) after silver nitrate staining. (MW) molecular weight marker, (1) culture supernatant, (2) protein profile following gel filtration (Bio-Gel P100), (3) Lac fraction of 75 kDa (Lac B) purified by anion exchange (Q Cartridge), (4) Lac fraction of 60 kDa (Lac A) purified by anion exchange (Q Cartridge). B) SDS-PAGE (7.5% wt/v) revealed with DMP substrate. (1) culture supernatant, (2) purified Lac I, (MW) molecular weight marker, (3) purified Lac B. C) SDS-PAGE (12% w/v) after silver nitrate staining for (1) SDS- and (2) β-mercaptoethanol-treated Lac A, (3) SDS- and (4) β-mercaptoethanol-treated Lac B.

4. Discussion

The SSU and LSU rRNA-encoding genes are highly conserved and commonly present in all living organisms, allowing phylogenetic comparisons among distantly related species (Berbee and Taylor, 2001). The ITS1 and ITS2 regions, in contrast, can have more nucleotide variations since their transcripts are excised from the final rRNA fragments. Therefore, the ITS sequence including both ITS1 and ITS2, which are separated by the conserved short 5.8S rRNA, has been commonly used to infer phylogenetic relationships of closely related species as well as to assess the variability of a population, e.g. of geographically distant isolates (ecotypes) (Hildén et al., 2008).

In this paper we conducted a phylogenetic study based on 18S, ITS (ITS1-5, 8S-ITS2) and D1/D2 domain of 28S rDNA sequences. The fungus selected in this study could be classified as a representative of a species of Phlebia, specifically *Phlebia brevispora*, based on ITS and 18S sequences, and as *Phlebia* sp. based on D1/D2 domain 28S rDNA. This is due simply to a lack of sequences corresponding to the 28S rRNA gene for *P. brevispora* in the database. For these reasons, it was proposed to identify the fungus as *P. brevispora* BAFC 633.

Despite the known difficulty with the current taxonomic system (Hibbett and Donoghue, 1998), where a large number of ranks are necessary to classify complex phylogenetic trees, we were able to locate *P. brevispora* BAFC 633 in the Phlebia cluster in the 18S sequence phylogenetic tree. *Tyromyces subgiganteus* (AY219401), *Tyromyces fumidiceps* (AY219400), *Spongipellis pachyodon* (DQ457638) and *Climacodon septentrionales* (AY705964) are also part of the Phlebia cluster. These three fungi belong to the same order but different family. Consistent with our data, previous studies of phylogeny of 18S rDNA region also show that *Tyromyces fumidiceps* and *Tyromyces subgiganteus* are grouped within the Phlebia clade (Greslebin et al., 2004).

However, the characteristics of basidioma and culture morphology of Tyromyces do not bear a close relationship with Phlebia. Thus the location of Tyromyces in the Phlebia core may be a result of the peculiarities of the region that reflect the phylogenetic relationship of that particular region, but not of the species as a whole (De Koker et al., 2003).

Phlebia albomellea (AY293144) appears in a different clade in the 18S tree. In this regard, several studies have shown that Phlebia's molecular phylogeny is not a monophyletic group (De Koker et al., 2003; Parmasto and Hallenberg, 2000; Moncalvo et al., 2000). Also in the ITS and 28S trees, *Phlebia* species are associated in different clades. On the other hand, species of *Phanerochaete* and *Ceriporiopsis* sometimes form distant clades, in this sense there are studies that establish that Phanerochaete and Ceriporiopsis are not monophyletic groups (De Koker et al., 2003). *Ceriporiopsis* is recognized as a collection of taxa unrelated phylogenetic origin (Ryvarden and Gilbertson, 1993).

Since the basidiomycete morphologically identified as *Peniophora* sp. was originally isolated from the fruiting body grown on pine wood, it can be assumed that both *Peniophora* sp. and *Phlebia* sp. strain were present in the decaying log, but only the latter organism survived during the cultivation and preservation procedures. According to the molecular identification on ribosomal 18S rRNA and ITS sequences, we recommend designating the basidiomycete isolate as *Phlebia brevispora* BAFC 633 until a new systematic identification of the fungus were accomplished.

In this work we evaluated the decolorization ability of *Phlebia brevispora* BAFC 633 on lignin-rich effluents through the action of secreted enzyme in media containing Kraft liquor. Our group has been working in biotechnological applications of laccases secreted by *P. brevispora* BAFC 633 and other fungi. *P. brevispora* BAFC 633, *Ganoderma applanatum* (strain F), *Pycnoporus sanguineus* BAFC 2126 and *Coriolus antarcticus* BAFC 266 caused black liquor dephenolization at 14 days of cultivation indicating potential uses for bioremediation of pulping industries effluents (Fonseca et al., 2010). In the same way, Shimizu et al. (2009), reported black liquor degradation with *Ganoderma applanatum* (strain F) and *Pycnoporus sanguineus* BAFC 2126 confirmed by spectral scanning using UV/VIS technique. Recently, we reported an increased dissolution of lignin in pulps from wood chips treated with *P. brevispora* BAFC 633 previous to kraft pulping is consistent with results obtained by other authors. These results indicate that the fungus causes structural changes in the fiber that would facilitate the penetration of the cooking liquor, which in turn enhances and accelerates the delignification (Fonseca et al., 2014a). This treatment produces improved pulp brightness with fungal pretreatment with *P. brevispora* BAFC 633 (Fonseca et al., 2014a). The modification introduced by the biological action on the wood chips left a more open structure, which allowed better access of the pulping liquor to the cell wall components. The effectiveness of kraft pulping was

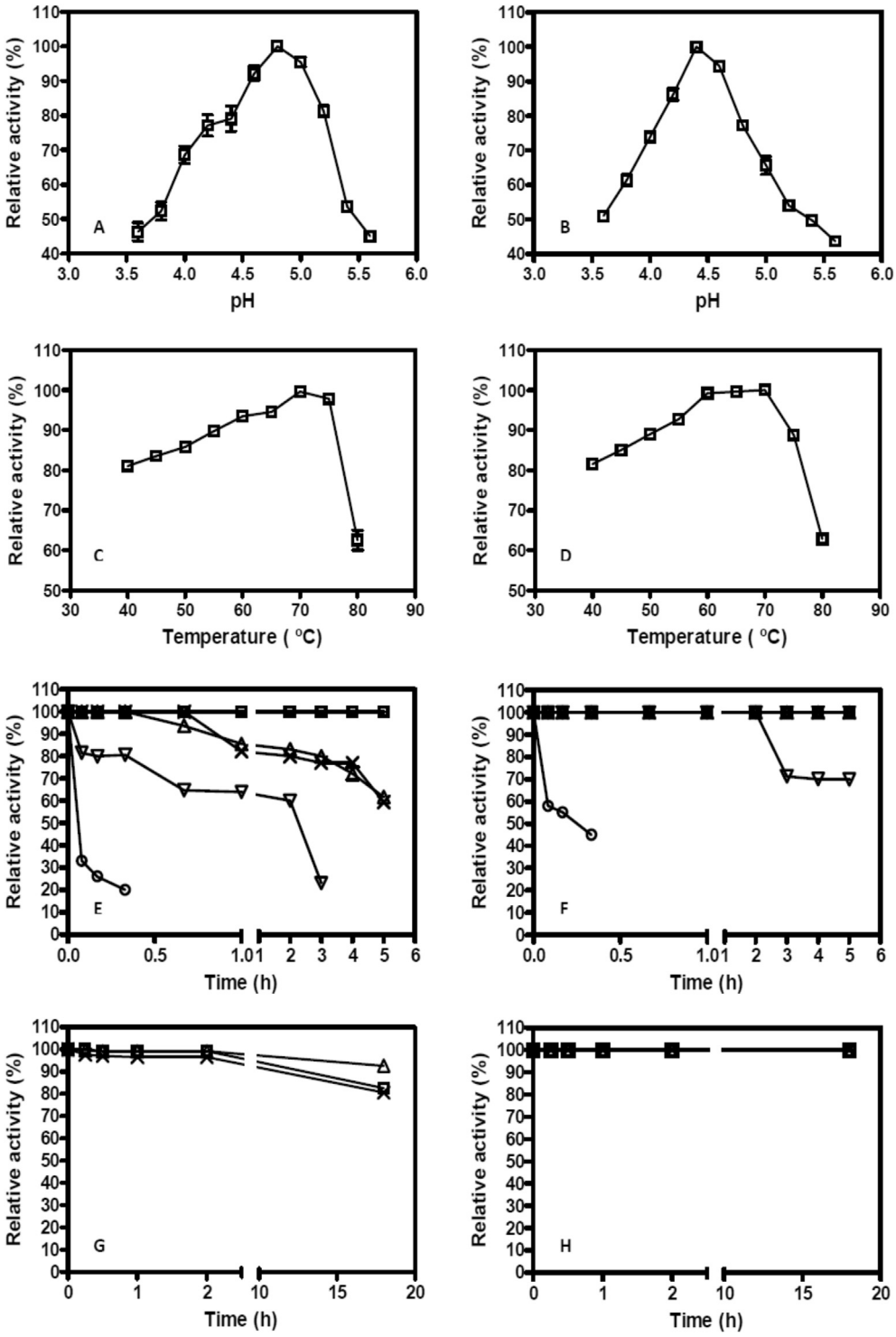

Fig. 6. Characterization in function of pH and temperature (A–D) and Enzymatic stability (E–H) of purified laccase isoenzymes from *P. brevispora* BAFC 633. Optimum pH for Lac A (A) and Lac B (B). Optimum temperature for Lac A(C) and Lac B (D). Enzymatic stability at different temperatures (E: Lac A, F: Lac B) and at different pH (G: Lac A, H: Lac B). The symbols represent: 30 °C (□), 40 °C (△) 50 °C, (×), 60 °C (○), 70 °C (○); y pH 3.6(×), pH 4.8(□), pH 5.8(△).

improved, as demonstrated by the increased dissolution of lignin in pulps from wood chips treated with *P. brevispora* BAFC 633 (Fonseca et al., 2014a). The chemically modified lignin was more readily solubilized by the cooking liquor. Considering that brightness is one of the most important parameters defining pulp, and that fungal pretreatment with *P. brevispora* BAFC 633 improved pulp brightness, contributing to end-product quality.

Considering these promissory finding and also that laccase is one of the main oxidative enzyme secreted by *P. brevispora* BAFC 633 (Fonseca et al., 2014b), we decided to look at the biochemistry of the laccases from the fungus. The two purified laccase isoenzymes showed a molecular mass in the range of those reported for other laccases which are generally monomeric with a molar mass of 60–80 kDa (Baldrian, 2006; Hildén et al., 2009; Piscitelli

Table 2
Substrate specificity and kinetic constants of purified laccases secreted by *P. brevispora* BAFC 633.

	% of enzymatic activity[a]			K_m (μM)		V_{max} (μM min^{-1})		k_{cat} (s^1)		k_{cat}/K_m (s^{-1} μmol l^{-1})	
	ABTS	DMP	Guaiacol	ABTS	DMP	ABTS	DMP	ABTS	DMP	ABTS	DMP
Lac A	100 ± 1	87 ± 1	63 ± 1	445	117	10.93	175.5	194	3118	4.3×10^5	2.6×10^7
Lac B	100 ± 1	75 ± 1	56 ± 1	6233	464	120.2	741.1	2.6×10^4	4.2×10^5	4.2×10^6	9×10^8

[a] 100% of Lac activity corresponds to 500 UI^{-1}.

et al., 2010). The biochemical parameters of various purified laccases were revealed in recent publications (Baldrian, 2006; Hildén et al., 2009; Piscitelli et al., 2010), showing some similar characteristics (pH, T and molecular mass), and other differences particularly regarding K_m values.

Many fungal laccases exhibit pH optima within the acid range depending on the substrate: pH 2–5 for ABTS, 3–8 for DMP, or between 3.5 and 7 (Guo et al., 2011; Irshad et al., 2011; More et al., 2011; Sun et al., 2013; Tian et al., 2012; Patel et al., 2013) for syringaldazine (Baldrian, 2006). Also, the biochemical properties of constitutive and induced expressions of laccase may be different. In this work, the optimal pH for both constitutive (Lac A) and induced (Lac B) *P. brevispora* BAFC 633 laccases were 4.8 and 4.4 respectively. These results were in agreement with those described in the literature for other fungal laccases with optimal activities at acidic pH (More et al., 2011; Sun et al., 2013; Tian et al. 2012; Patel et al., 2013). Enzymatic activity dropped sharply to an almost undetectable level as pH approached to neutral, possibly due to hydroxide ion binding to the enzyme's T2/T3 site (Sadhasivam et al., 2008). The ability for both herein studied laccases 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.

Regarding the optimal temperature for reported laccases, maximal activity usually ranges between 25 and 80 °C (Baldrian, 2004) and most of them exhibit optima between 50 and 60 °C.

In general terms, laccases have been reported to be stable between 50 and 70 °C (Farnet et al., 2000; Tian et al., 2012). Enzyme stability is a desired feature when producing enzymes at a commercial scale and it represents a relevant property for any industrial application (Farnet et al., 2000). In terms of stability, Lac B from *P. brevispora* BAFC 633 was more stable at 60 °C than Lac A (5 h *vs.* 2.5 h half-life, respectively). Moreover, they both have the desired ability to stand high temperatures (up to 25 min at 70 °C for Lac B).

Concerning laccase kinetics, the catalytic performance expressed as k_{cat} spans over several orders of magnitude for different substrates and is typically characteristic for a given enzyme (Baldrian, 2006). Kinetic analysis of the two laccase isoenzymes of *P. brevispora* BAFC 633 showed higher specificity (k_{cat} K_m^{-1}) towards the non-natural substrate ABTS. In accordance, most known laccases showed high affinity and catalytic constant for ABTS (Baldrian, 2006; Quaratino et al., 2007). The oxidation ability using guaiacol and DMP of herein purified laccases was considerably lower than with ABTS, as revealed by the respective higher K_m values. The removal of methoxy groups in positions 2 or 6 usually leads to a decrease in enzyme affinity. In this case, the presence of two *ortho*-methoxy groups in the substrate molecule (DMP) showed to be preferable than a single group (guaiacol). It is possible that such substituents may be involved in further interactions in the T1-copper surroundings of laccases, thus facilitating the electron transfer. Differences in affinity of this substrate could be related with the redox potential of these enzymes and its role in the degradation of recalcitrant compounds present in contaminated soils or residual wastewater (Jaouani et al., 2003). Chelating agents such as EDTA inhibit laccase activity by forming complexes with its copper ions, modifying the laccase active site (Lorenzo et al., 2005).

Regarding the influence of enzyme inhibitors, it is known that Cu^{2+} is part of the protein structure of laccase, and its selective removal by chelating agents such as EDTA may lead to a loss of activity by modifying the active site. Nevertheless, this effect was not observed with the EDTA concentrations tested in this study for any purified laccase from *P. brevispora* BAFC 633 and similar findings were previously reported (Kalyani et al., 2012).

The difference in molecular weight between laccase isoenzymes (60 and 75 kDa) could be explained not only by the expression from different genes (Chen et al., 2003) but also by translational and post-translational differences, such as premature termination, differential glycosylation or proteolysis, features already reported in other basidiomycetous laccases (Bertrand et al., 2002; Larrondo et al., 2003).

5. Conclusions

Two novel identified laccases referred as Lac A and Lac B were isolated from cultures of the selected ligninolytic basidiomycete *P. brevispora* BAFC 633 and were subsequently purified and characterized. Purified laccases exhibited better catalytic efficiency towards the non-natural substrate ABTS. The tolerance of both laccases towards rigorous conditions, including low pH and high temperature, demonstrated suitability for the potential use in harsh process conditions such as bioremediation, textile, paper and pulp industries. These results may provide a further insight into potential ways of optimizing fermentation for fungal Lac production. In this sense also the results of black liquor degradation support efforts to scale-up effluent treatments with *P. brevispora* BAFC 633.

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