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

Running Head: Recombinant laccase from *P. brevispora* BAFC 633

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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 analyze 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 in a 1563-pb ORF encoding 521 amino acids. Lac I was functionally expressed in *Pichia 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 solvent 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 a NaCl and various solvents and degraded some recalcitrant synthetic dyes.

Significance and Impact of 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.

key words: *Phlebia brevispora*; Laccase; Characterization; Gene isolation; heterologous expression.

Introduction

Laccases [benzenediol: oxygen oxidoreductases (EC1.10.3.2)] are copper-containing enzymes capable of oxidizing a broad spectrum of phenolic compounds and non-phenolic 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.* 2010; Shimizu *et al.* 2010; 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 other induced by the presence of $CuSO_4$ of 75 KDa (Fonseca *et al.* 2010). Both enzymes have been purified and thoroughly characterized (Fonseca *et al.* 2015). The presence of isoforms with similar chemico-physical properties makes 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). *P. 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 growth at high cell densities over a defined minimum medium. Other possible advantage with

P. pastoris compared to 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) *Phlebia 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 analyzed.

Materials and methods

Chemicals

2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2.6-dimethoxyphenol (DMP, Catalog Number D135550), sodium dodecyl sulfate (SDS, Catalog Number L3771) were purchased by Sigma-Aldrich.

Microorganism and culture conditions

The WRF isolated from the Misiones rainforest (Argentina) identified as *Phlebia 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 cm^2-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 29ºC under static conditions.

The *Pichia pastoris* strain GS115 (*his4*) was purchased from Thermo Fisher Scientific (Pichia Expression Kit, original kit, Invitrogen, Catalog Number K1710-01, 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 29ºC under static conditions were harvested by filtration. Filtrated mycelium was washed with 0.1 M Tris-HCl pH 8, 0.02 M 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 60ºC 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 (Fonseca *et al.* 2015).

Laccase gene fragments cloning and sequencing

The cloning strategy to amplify the Lac I gene (*lac I*) is summarized in Figure 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, Promega, Catalog Number A1380, USA) and sequenced (Macrogen, 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 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primer, 0.5 U of Pfu DNA polymerase, and 25 ng DNA. PCR cycling consisted in 4 min at 94ºC, 35 × (40 sec 94ºC, 40 sec 50ºC, 40 sec 72ºC) and a final extension for 10 min at 72ºC. *Escherichia coli* JM109 cloning host was obtained from Promega and competent cells were prepared by means of the classical $CaCl_2$ 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, Promega, Catalog Number A1380, USA) following the manufacturer's instructions. All reagents were analytical grade and specific PCR products were purified using the DNA gel extraction kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Catalog Number A9280, USA). Twelve clones with target fragments were analyzed 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, *P. 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 Kit Pfu polimerasa (Kit Pfu polimerasa 100U, Highway, Catalog Number K1100, Argentina) and the relevant inverse pair of primers Lac-Inv-S and Lac-Inv-AS (Table 1).

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.

RNA isolation and Amplification of Lac DNAc by PCR

P. brevispora cultures were grown in ME liquid medium (Fonseca *et al.* 2010) and induced with 0,5 mM $CuSO_4$ (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 0.1 M Tris, 0.02 M EDTA

(Fonseca *et al.* 2014a). The samples were treated with DNases previous to retrotranscription step. The first strand of cDNA was synthesized using MMuLV-Transcriptase (RevertAid Reverse Transcriptase (200 U/µL), Life-Technologies, Catalog Number EP0441, 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.* (2014). 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 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 *lac I* gene from *P. brevispora* in *P. pastoris* through yeast shuttle vector

The cDNA of *lcc1* 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 (Catalog Numbers V17320 and V17520 respectively, Invitrogen, USA). Two recombinant plasmids were obtained: pPIC3.5K /*lac I*, containing the *lac I* cDNA including the native signal sequence, and pPIC9.K /*lac I*, in which the cDNA sequence encoding the native LacL signal peptide was exchanged for that encoding the *Saccharomyces cerevisiae* α-mating factor signal peptide. Plasmids DNA were digested with *Sac* I (Thermo Scientific, Catalog Number ER1131, USA) 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^{TM} Kit, Catalog Number K1730-01, Invitrogen, USA). Vectors without *lac I* 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 twenty or so

transformants were screened on minimal methanol plates supplemented with 0.2 mM ABTS for development of green color. One (pPIC3.5K /*lac I* and pPIC9.K /*lac I*) of the recombinants was selected on the basis of development of intense green color in plate assay to continue in liquid media. Inocula was prepared by transferring cells from minimal dextrose (MD) 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). Cultures were grown at 30°C in an orbital shaker (200 rpm) 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 minimal methanol (BMM). Cells harvested from the inoculum were directly resuspended in BMM to an OD_{600} of 1.0. The culture was monitored for 6 days for production of extracellular laccase with the induction of the promoter being maintained by daily addition of 0.5% (v/v) methanol.

Bioinformatic and phylogenetic analyses of *lac I* protein sequence

The obtained nucleotide sequence of laccase was translated carried out using ExPASy: Translate tool program (http://web.expasy.org/translate/). This was compared to the sequence obtained by cDNA sequencing. The obtained sequence was then *in silico* analyzed using online available bioinformatic tools. The signal peptide identification was carried out by using Signal P (http://www.cbs.dtu.dk/services/SignalP/), and the putative glycosylation sites were identified by means of NetNGlyc (http:// www.cbs.dtu.dk/services/NetNGlyc-1.0/output.php). The theoretical properties of the protein were obtained with the ProtParam software (http://web.expasy.org/protparam/).

Translated aminoacidic sequence deduced of cDNA clone of *lac I* was compared to other fungal laccase aminoacidic sequences with BLAST (http://www.ncbi.nih.gov/blast). The searched protein sequences included the most similar (Max identity 65-75%) and those with moderate identities (Max identity 45-46%) from homobasidiomycetous and the lower level homologous laccases (Max identity 42%) of *Rhizoctonia solani* (*Thanatephorus cucumeris*) in the order

Ceratobasidiales (Homobasidiomycetes). Analysis included 38 retrieved laccase amino acids sequences and also a laccase protein sequence from *Fusarium oxysporum* (Max identity 34%), an Ascomycota used as outgroup.

All sequences were analyzed with BLASTp, BioEdit and CLUSTAL W before phylogenetic tree construction. Phylogenetic analysis was carried out with the T.N.T program (Goloboff 1999). Gaps (indels) were treated as a 5th state, since they represented insertion-deletion events. Because of the reduced data set, the heuristic searches were implemented using 1000 RAS, saving one tree *per* TBR.

To assess the support for the identified groups, Bootstrap and Parsimony Jackknifing tests were performed (Moncalvo *et al.* 2000). Both analyses included 1000 resampled matrices. For each resampled matrix, 100 RAS + TBR cycles were performed.

Purification and characterization of laccase

The supernatant (300 mL) was harvested from a BMM culture of the recombinant *P. pastoris* GS115-lac (6 days old). The supernatant was obtained from the culture by centrifugation at 6000 g for 10 min; afterwards, the centrifuged sampled was purified by the ultrafitration using a filter Pierce Concentrator 20 ml/20 K (Pierce® Concentrator 20K MWCO 20Ml, Catalog Number 89887A, Thermo Scientific, USA). The resulting filtrate thus obtained was subjected to the total protein precipitation with ammonium sulphate at 100 % saturation. The protein was dissolved in 0.1 M sodium acetate buffer (pH 3.6) and dialyzed 12 h against the same buffer. The purity of the enzyme was 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, Catalog Number 161-0374, Bio-Rad, 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 was performed as described by Fonseca *et al.* (2010). Staining was

carried out after native PAGE by incubating the gel in 0.1 M sodium acetate buffer containing 5 mM of DMP or ABTS before detecting laccase activity. After incubating the gel for 5min, 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 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} =7.5 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.

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 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 time for 6 h. Residual activity was calculated considering as 100% the maximal enzymatic activity at optimal pH and temperature, and was expressed as percent of the remaining 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}). 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). *Km* 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. 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 mM) as some potential laccase inhibitors were 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.

Dyes decolorization

Nine different dyes were used for this study. The reaction mixture (2 ml) contained 100 mM acetate buffer pH 3.6, individual dye (each 50 mg/l 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*(Ao - At)/Ao. Where Ao 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 hybridize 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 in a 1563-pb 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 (AATAAA) (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-HTHNNGCTGD-3 (Zhu and Thiele 1996), an inverted metal response element (MRE) 5-TGCRCNC-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 Figure 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. AH28-2 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); additional four Cys and His were also found to be conserved, and these would be likely involved in binding to 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, being Ala the most replaceable aa at this position, in contrast with low potential redox laccases which harbor VSG replacing LEA tripeptide. The finding of the LNA tripeptide in Lac I (see Figure S2, green box), in association to the presence of Leu at the T1 copper binding position, led us to suggest that *P. brevispora* BAFC 633 Lac I would be a high-redox potential Lac.

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* (Figure 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 transformant 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 construcs pPIC9.K/lac I, pPIC3.5K/lac I, were then fermented in BMM liquid medium at 30ºC and induced by addition of 0.5% methanol daily and different copper concentration. After 6 days growth, the laccase activities reached 30U/L and 500 U/L for pPIC9.K/lac I and pPIC3.5K/lac I respectively (Figure 3).

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 (Figure 4), a higher

value higher than the predicted masses of 54.14 kda. The zymogram shows the active laccase with DMP and ABTS. (Figure 4)

The optimal pH values of Lac I was 3.6 and the optimal temperature values was 50°C for DMP (Figure 5 A,B respectively). The recombinant laccases were stable at temperature of 40 and 50ºC for 4 h maintaining above the 50% of activity while at 30°C it was for 6 h (Figure 5, C). 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 case of pH 5.8 after 5 h (Figure 5, D). It was very sensitive to SDS and EDTA (Table 2). The metal ions such as Ca^{2+}, K^{+}, NH^{4+}, Mn^{2+} Increased the Lac I activity while $Zn^{2+}$and Cu^{2+} 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 (kcat Km^{-1}) of purified Lac I was higher for ABTS than DMP.

The effect of various water miscible organic solvents (acetone, ethanol, dimethylsulfoxide or DMSO), was investigated on laccase activity and the results are shown in Figure 6 A. Lac I was stable in ethanol up to 3h at 70% (v/v) concentration retaining 90% activity. Lac I in the presence of acetone and DMSO retained until 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 mM of concentration Figure 6 B.

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 discolored whit different efficiency, the Thymol Blue the most discolored with 90% (Table 4). 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 2010) and given that nucleotide sequences of the copper binding regions are highly

conserved, they have been frequently used for PCR primers design in order to clone laccase gene fragments in several basidiomycetes (D'Souza *et al.* 1996). Based on this knowledge, a laccase gene harboring 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 antioxidant response element (ARE), a nitrogen-responsive element (NIT) and an ACE-like element. Overall response elements are differentially distributed throughout the promoter sequence and some of them are characteristic of laccase sub-families, 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 are exposed to toxic concentrations of Cd, Cu and Zn (Whitelaw *et al.* 1997). Some authors (Karahanian *et al.* 1998; Mansur *et al.* 1998; Klonowska *et al.* 2001; Galhaup *et al.* 2001) found other promoters containing multiple putative MRE sites with consensus sequences 5-TGCRCNC-3 (Thiele *et al.* 1992). Although MREs are included in the promoters of *poxc* and *poxa 1b* in *P. 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; 1991 Hill and Li).

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 *Saccharomyces cerevisiae* as a recognition site for the ACE1 transcription factor which

responds to Cu(I) and Ag(I), but not to Zn(II) (Gralla *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 *Pleurotus 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) were found three times in the gene promoter of *P. radiata* (Saloheimo *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 (TGACNNNGC) 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), $CytP_{450}$ genes, glutathione-S-transferase and NAD(P)H: quinone oxido-reductase 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 promoter regions of *P. sajor-caju* (Soden 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 *et al.*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 introns 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.* 2013). 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 (Canters 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 (Figure 2). Both *P. brevispora* BAFC 633 and *P. radiata* laccase-encoding genes are apparently ortologus as a speciation event. As depicted in Figure 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 (P_{AOX1}) 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* (Colao *et al.* 2006, Brown *et al.* 2002, Jönsson *et al.* 1997; Soden *et al.* 2002).

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 than 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 others recombinant laccase (Boa et al. 2012, Gu et al. 2014).

The Lac I was stable at temperature of 40 and 50ºC 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 (SDS) 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 Ca^{2+}, K^{+}, NH^{4+}, Mn^{2+} increased the Lac I activity while Zn^{2+} and Cu^{2+} decreased the activity. In this sense recombinant laccase of *Coprinus comatus* was strongly inhibited by Fe^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} and Co^{2+} and activated in 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 Lac1 at the same extent. The differences in dyes 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 triarylmethane type dye, which is used in dyes 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

We declared that all authors of this manuscript have no conflicts of interest to this work.

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TABLE 1 Oligonucleotide primers used in this study

Primer name	**Oligonucleotide sequence 5´- 3´**	**Purpose**	**Reference**
LacII-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
LacI-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
LacInv-S	TGRAARAANCCRTGCCARTG	Used for LD-IPCR	This work
LacInv-AS	CAYTGYCAYATHGAYTTYCA	Used for LD-IPCR	This work
LacEs-S	TATACAGTTGGTGGTTCACCT	Used for cloning and confirmation of *lac I* sequence	This work
LacEs-AS	TATGGGACAGAGYTGSTCCCAAGC	Used for cloning and confirmation of *lac I* sequence	This work
NlacIs/ps-S	CTCGAGGAATTCATCACAGGACCTATTGGG	Used for cloning cDNA *lac I* sequence without the signal peptide	This work
NlacI	CTCGAGGAATTCGAGAGATGCTCTC	Used for cloning	This work

	CCTAGTTCTTGTCGCA	cDNA *lac I* sequence with the signal peptide	
NlacKL-	GGTCTAGCGGCCGCTTAGTTTTGTC	Used for cloning	This work
AS1	CCAGAACCTTCTG	cDNA *lac I* sequence	

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

Table 2: Effects of ions and inhibitors on Lac I activity.

Inhibitor	**Concentration (mM)**	**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
Ca^{2+}	1	145
Cu^{2+}	1	38
K^{+}	1	137
Mn^{2+}	1	118
NH^{4+}	1	110

Zn^{2+}	1	39

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

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

	% of enzymatic activity [a]		K_m (µM)		$V_{máx}$ (µM min^{-1})		k_{cat} (s^1)		k_{cat}/K_m (s^{-1} µmol l^{-1})	
	ABTS	**DMP**	**ABTS**	**DMP**	**ABTS**	**DMP**	**ABTS**	**DMP**	**ABTS**	**DMP**
Lac I	100 ± 1	60 ± 1	727	425	169.2	48.45	1.1×10^6	3.2×10^4	158	77

Table 4: Decolorization of synthetic dyes with recombinant Lac I after incubation for 12 h.

Dyes	**Wavelength (nm)**	**Type of dyes**	**Decolorization (%)**
Blue Dextran	624	Triazine	50±0.2
Bromophenol Blue	590	Triphenylmethane	60±0.2
Comassie R-250	595	Triphenylmethane	50±0.5
Congo Red	488	Azo	50±0.9
Gentian Violet	590	Triarylmethane	15±0.5
Methylene blue	668	Thiazine	15±0.5
Orange G	476	Azo	25±0.5
Phenol Red	430	Triarylmethane	28±0.8
Safranin	495	Phenazines	35±0.6
Thymol Blue	590	Triarylmethane	91±0.4

Victoria Blue	592	Triarylmethane	10±0.1

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

Supporting Information

Figure S1: Nucleotide and deduced amino acid sequences of *lac I* and the corresponding Lac product, respectively, from *P. brevispora* BAFC 633. TATA boxes, CAAT boxes, and two putative CAAT inverted boxes are denoted in bold. A putative ACE1 transcription factor binding site is shown in the box. Metal inverted element response is shown in dark gray (MER) (■), a heat responsive element is shown in light gray (HSE) (■), an antioxidant response element is shown in dashed lines (ARE) (---), a nitrogen responsive element is shown in doubled underlined lines (NIT) (═). Introns are denoted in lowercase. All 8 putative glycosylation sites are indicated in dotted lines and the stop codon with (*). The 20 underlined amino acids correspond to the signal peptide.

Figure S2: In-silico deduced amino acid sequences alignment of laccase of Phlebia brevispora BAFC633 with other fungal laccases. Black boxes represent the Cys residues present in disulphide bridges. Red boxes include the 10 His residues at the conserved copper binding domains.

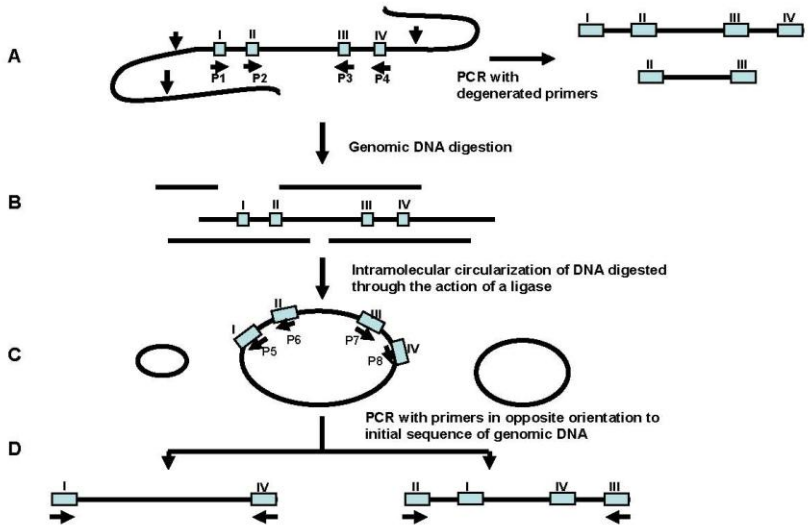

Figure 1: Molecular strategy used to obtain *P. brevispora* BAFC 633 lac gene. Primers designed by D'Souza et al. (1996) and specially designed for the present work, were used to amplify the copper-binding region I (P1) and IV (P4), respectively. To amplify the gene region between copper-binding region II (P2) and III (P3), primers design was based on the conserved sequences at the copper-binding regions for fungal laccase (A).On the other hand, gDNA was thoroughly digested with BamHI restriction enzyme (B) and was self-ligated (C); the self-ligation products were used as templates for LD-IPCR using the relevant inverse primer pairs Lac-Inv-S (P6) and Lac-Inv-AS (P5) (D).

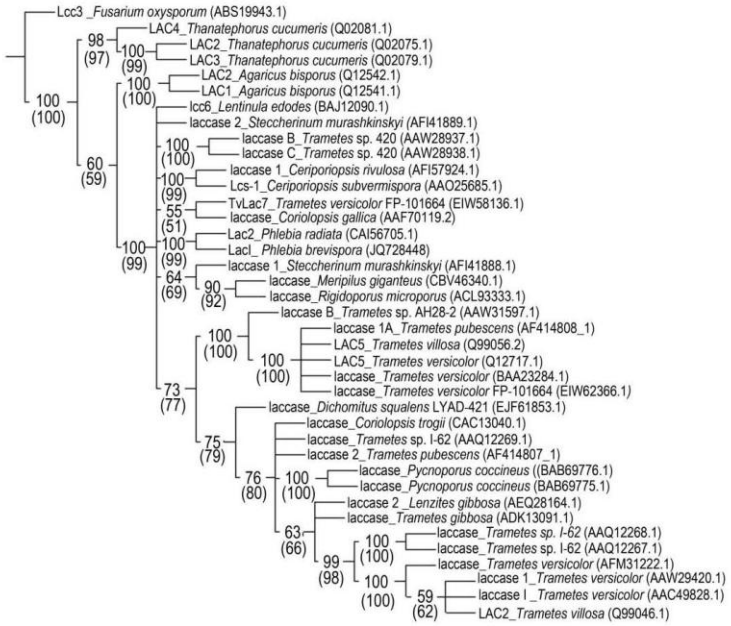

Figure 2: Phylogenetic tree of the evolutionary relationships based on the sequence alignment of the Lac I of the *Phlebia brevispora* BAFC 633 with homologous amino acids sequences obtained from the NCBI GenBank. Group support, assessed with 1000 Bootstrapping and Parsimony Jackknifing replicates. Numbers above branches correspond to Jackknife support. Bootstrap supports are given in parentheses. Phylogenetic tree performed using maximum likelihood methods

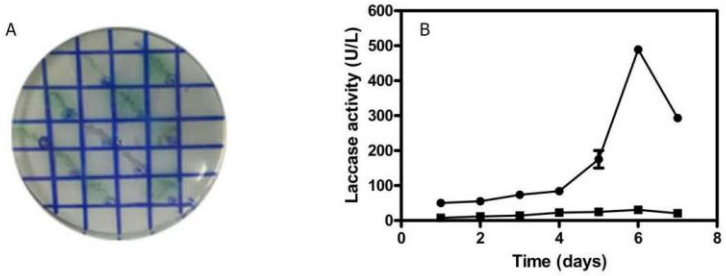

Figure 3: A) Example of detection of laccase activity on BMM agar plate containing $CuSO_4$ and ABTS in some transformants obtained. The green color indicates the presence of laccase. B) Laccase activity in BMM liquied medium whit native signal peptide (■) and whit using the α-factor signal peptide (●)

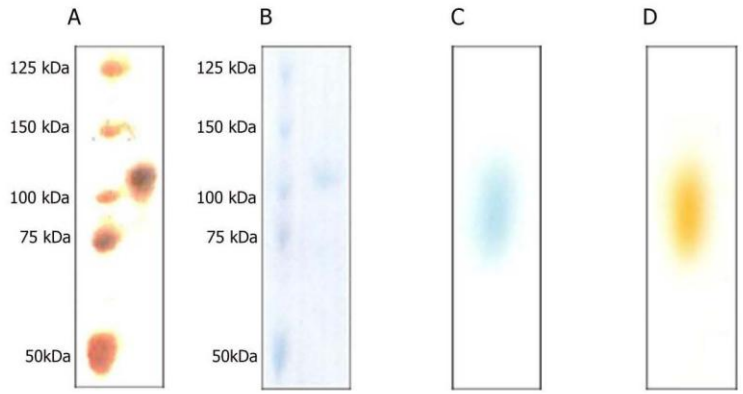

Figure 4: Molecular weight estimation of LacI purified from BMM culture supernatant after 6 days of incubation. A) SDS-PAGE (12% wt/v) after silver nitrate staining. (MW) molecular weight marker, (1) LacI purified. B) SDS-PAGE (12% wt/v) after Coomassie Brilliant Blue staining. (MW) molecular weight marker, (1) LacI purified. Zimogram (7.5% wt/v) revealed with ABTS (C) or (DMP) substrate.

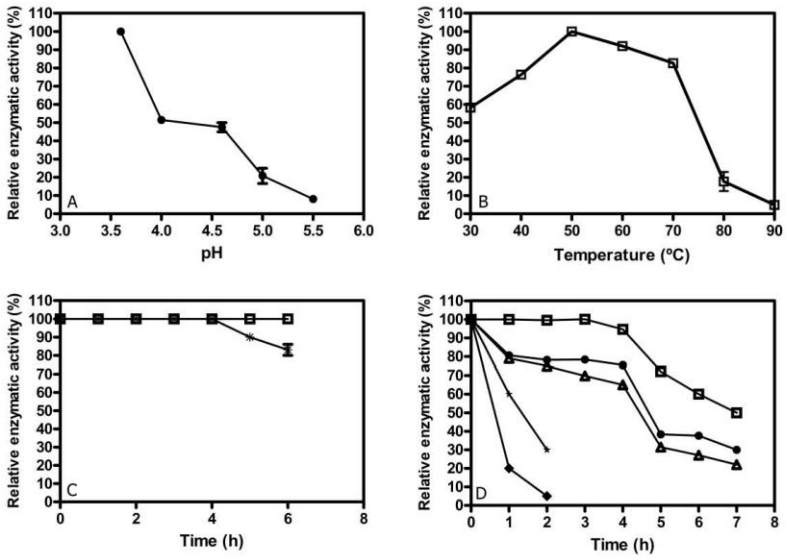

Figure 5: Effects of pH (A) and temperature (B) on the activity of LacI. And Effect of pH (C) and temperature (D) on stability of LacI. The symbols represent: pH 3.6(□) , pH 4.8 (■), pH 5.8(*), and 30 °C (□), 40 °C (●) 50 °C, (Δ), 60 °C (*), 70 °C (♦).

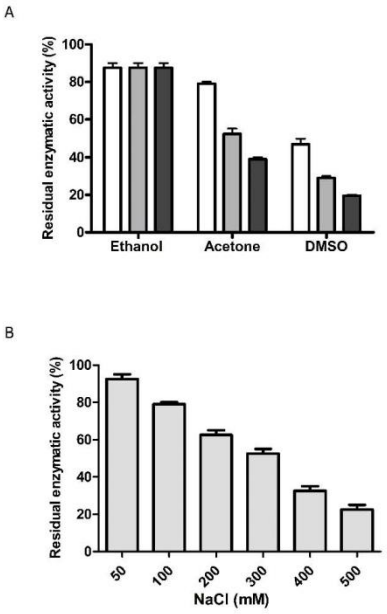

Figure 6: Residual activity of laccase after incubation of purified enzyme for 3 h A) Residual activity of laccase in the presence of different concentrations of organic solvents 40% (white bar), 50% (grey bar), 70% (black bar). B) Residual activity of laccase in the presence of NaCl concentration. The values are the mean of triplicate experiment