DESCRIPTION OF A LACCASE GENE FROM *PLEUROTUS OSTREATUS* EXPRESSED UNDER SUBMERGED FERMENTATION CONDITIONS

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In this work, a gene (lacP83) encoding a *Pleurotus ostreatus* laccase isoenzyme expressed in submerged fermentation conditions is described. A 2,887 bp sequence was obtained from a genomic library of *P. ostreatus* by using a PCR inverse strategy. The coding sequence, 1,527 bp long, showed 17 exons and encoded a protein of 509 amino acids, with a putative signal peptide and conserved copper binding domains. The promoter region of the lacP83 gene (466 bp upstream of ATG) contains putative binding transcription factors such as MRE, XRE, a defense response element, and a stress response element. The protein and gene sequences of lacP83 showed, respectively, 90 to 96% and 78 to 92% of similarity to laccases of *Pleurotus* previously reported. However, it showed differences in its apparent molecular weight and promoter sequence.

Keywords: Laccases; Pleurotus ostreatus; *Promoter sequence; Submerged fermentation*

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INTRODUCTION

Laccases (EC 1.10.3.2) are glycoproteins classified as multi-copper oxidases, which catalyze one-electron oxidation of a wide range of inorganic and organic substances, coupled with reduction of oxygen to water (Thurston 1994). Laccases are widely distributed in fungi, higher plants, bacteria and insects (Gochev and Krastanov 2007), and recently evidence has been presented for the distribution of laccases among archaea and their probable functions (Sharma and Kuhad 2009). The most studied come from white rot fungi. There is a large diversity of laccases that have very different physicochemical properties (Gianfreda et al. 1999; Mayer and Staples 2002). Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, ortho-, and para-diphenols, and can also oxidize other substrates such as aromatic amines, syringaldazine, and nonphenolic compounds to form free radicals (Bourbonnais et al. 1997; Li et al. 1999; Robles et al. 2000). These features are suitable for several different applications in industrial effluents disposal, medical diagnostics, bioremediation to degrading pesticides and explosives in soils, delignification processes in paper industries, and in cosmetics formulation as an additive (Kunamneni et al. 2008).

On the other hand, the existence of different laccase isoenzymes and multiple genes that encode them in various fungi has been reported (Yaver and Golightly 1996; Mansur et al. 1997; Smith et al. 1998; Giardina et al. 1999). Laccases are regulated by several factors such as pH, temperature, ions, presence of inducers, etc. (Collins and Dobson 1997; Muñoz et al. 1997; Yaver et al. 1999). Copper has proven to be an excellent inducer, increasing the transcription of laccase genes (Collins and Dobson 1997; Karahanian et al. 1998; Palmieri et al. 2000; Soden and Dobson 2001; Galhaup et al. 2002).

Laccases from *Pleurotus ostreatus* have been widely studied; there are reports on the characterization of six isoenzymes (with protein sequences and/or gene coding sequences). However, there are some isoenzymes not yet characterized in *P. ostreatus*. Pezzella et al. (2009) reported a 150 kbp genomic fragment with seven genes for laccases, and Giardina et al. (2010) reported, based on the analysis of the *P. ostreatus* genome, a putative number of 12 genes for laccases inside this genome. The different physiological functions for each of the laccase isoenzymes across the fungal life cycle could explain their presence in the genome of this organism. In this research, a gene for a laccase isoenzyme (called lacP83) from *P. ostreatus* ATCC32783 was obtained by joining several partial PCR products. This laccase isoenzyme was observed only under submerged fermentation conditions (Téllez-Téllez et al. 2008).

EXPERIMENTAL

Materials

A strain of *P. ostreatus* from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used.

Methods

Culture conditions

Mycelial plugs (4 mm diameter) taken from the periphery of colonies of *P. ostreatus* grown for 7 d at 25 °C in Petri dishes containing potato dextrose agar were used as inoculum. A liquid medium, previously optimized for the production of laccases by this fungus in submerged fermentation, was prepared containing (in g/L): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$-$5H_2O$, 0.25; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$, 0.05; and $ZnSO_4$-$7H_2O$, 0.001 (Téllez-Téllez et al. 2008). The pH was adjusted to 6.5 using 0.1 M NaOH. Triplicate 250 mL flasks containing 50 mL of culture medium were inoculated with three mycelial plugs each. The cultures were incubated at 25°C for 23 days on a rotary shaker at 120 rpm (Téllez-Téllez et al. 2008). Samples were taken every 24 h after the third day of growth. The enzymatic crude extract (ECE) was obtained by filtration of the cultures using filter paper (Whatman No. 4).

Enzyme assays

Laccase activity was determined by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol as substrate (DMP). The assay mixture contained 950 μL substrate (2 mM DMP in 0.1 M phosphate buffer at pH 6.0) and 50 μL ECE, which were incubated

at 40 ºC for 1 min (Téllez-Téllez et al. 2005). One enzymatic unit (U) of laccase activity is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The activity was expressed in U/L of ECE.

Semi-purification of laccase isoenzyme

The ECE obtained after 19 days of fungal growth was used for the semi-purification process. The laccase isoenzyme that showed the lowest molecular weight (approx. 56 kDa) determined by SDS-PAGE (Laemmli 1970) in the ECE, was semi-purified in a three-step method. The first step was isoelectro-focusing using a chamber of 60 mL and ampholytes in the pH range of 3 to 10 (Rotofor, Bio-Rad Laboratories). After that, proteins were eluted from an ionic exchange column (Econo-Pac High Q anion exchange cartridge, Bio-Rad) with a NaCl gradient (0-0.5 M) for 10 min at a flow rate of 1.5 ml min^{-1} in Tris-HCl (25 mM, pH 8.1) buffer. Finally, the fractions were loaded onto an anion exchange column (Bio-Scale Q5, anion exchange cartridge, Bio-Rad, Laboratories) and eluted with a NaCl gradient (0-1 M) for 30 min at a flow rate of 1.5 mL min^{-1} in Tris-HCl buffer (50 mM, pH 8.1). The samples were dialyzed after each purification step (12,400 Da, Sigma D9527).

Zymogram analysis

The laccase activity was also detected through zymograms, using the modified technique SDS-PAGE (Laemmli 1970). The running gel contained 100 g acrylamide/L and 27 g bis-acrylamide/L. The stacking gel contained 40 g acrylamide/L and 27 g bis-acrylamide/L. Each EE (30 µL approx.) was mixed with sample buffer without a reducing agent for the disulfide bonds. Without heating, the samples were placed in gels (thickness 1.5 mm) of Mini-Protean III electrophoresis system (BioRad) and then 150 V was applied for 1 to 1.25 h. After the electrophoresis, gels were washed with deionized water on an orbital shaker (20 to 30 rpm) for 2 to 2.5 h, and the water was changed every 30 min to remove SDS. Finally, the gels were incubated at room temperature in substrate solutions (2 mM DMP). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 2 h.

N-terminal protein sequencing and probe amplification

Semi-purified laccase was electroblotted on PVDF (polyvinyl difluoride) membranes and sequenced by automated Edman degradation on a Protein Sequencer Procise 491 (Applied Biosystems, Inc, Foster City, CA) done at the Institute of Neurobiology, UNAM-UAQ (National Autonomous University of Mexico-Autonomous University of Queretaro, Mexico). The N-terminal sequence (AIGPTGDMYIVNEDV) was used to design gene-specific primers (Table 1) to be used for probe amplification. For this purpose, the amplification conditions included a first denaturation step at 94 °C for 3 min; 30 cycles at 94 °C for 25 s; 60 °C for 1 min, and 72 °C for 1 min, and an extra polymerization step at 72 °C for 10 min. Adaptor-specific primers are primers included in Genome Walker kit. Gene-specific GW primers are designed from the first PCR amplification of lacP83 gene, for use in PCR inverse strategy. Finally, cDNA-specific primers were used to obtain cDNA from the mRNA.

Construction and screening of a P. ostreatus genomic DNA library

Mycelium from *P. ostreatus*, grown in 250 mL of PMY medium (in g L^{-1}: glucose, 40; $NaNO_3$, 3; yeast extract, 2; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; and $FeSO_4.7H_2O$, 0.01) at pH 6.0 and incubated at 30 °C for 4 days, was harvested, washed, and frozen in liquid N_2. Chromosomal high-molecular weight DNA from the mycelium was prepared as described by Specht et al. (1982). DNA was partially digested with *Sau*3AI (37 °C for 1 h, 0.03 U of enzyme per mg of DNA), and fragments in the molecular size ranging between 9 to 18 kbp were isolated through a sucrose gradient. Purified fragments were ligated into vector λDASH II, previously made compatible to the DNA fragments by linearization with *Bam*HI. Ligations were packaged by using the Gigapack II Plus packaging system (Stratagene, La Jolla, CA). Propagation and amplification of the genomic library were performed by infecting *Escherichia coli* XL-Blue MRA P2 cells, and the library was stored at 4 and -70 °C, according to manufacturer's instructions.

About 40,000 PFU from the amplified genomic library was hybridized with the probe obtained in the above section, and one recombinant positive bacteriophage was selected after three duplicate hybridization steps.

Gene sequence

An inverse PCR alternative approach was used for serial gene amplification in order to avoid those problems that arise upon ligation of the DNA fragment obtained from digested bacteriophage in plasmids (Genome Walker-GW, Clontech, USA).

Bacteriophage DNA was used as the DNA template for amplification, and *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I were used as the restriction enzymes, according to the manufacturer's protocol. After digestion and ligation with the adaptors, 1 µL of the diluted ligation reaction was used for a PCR reaction (Eppendorf Master Cycler Gradient, Brinkmann Instruments, Inc., NY, USA), by using the high-fidelity Elongase enzyme (Invitrogen). Conditions were 94 °C, 25 s; 72 °C, 3 min for 7 cycles; 94 °C, 25 s; 67 °C, 3 min for 32 cycles, and an additional cycle at 67 °C for 10 min. Gene-specific GW primer (1R) and an adaptor-specific primer (AP1) were used at this time (Table 1). The PCR products were diluted 50-fold, and 1 mL of diluted PCR products was used as the template for a second, nested, PCR. The PCR conditions were: 94 °C, 25 s; 72 °C, 3 min for 5 cycles; 94 °C, 25 s; 67 °C, 3 min for 20 cycles, and an additional cycle at 67 °C for 10 min; Gene-specific GW primer (1R) and a nested adaptor specific primer (AP2) were used (Table 1). First and second PCR cycles were repeated with the different endonuclease-restricted DNA reactions, changing the Gene-specific GW primer (Table 1), until the completion of the laccase gene sequence.

Isolation of total RNA and cDNA synthesis

Mycelium of cultures after 19 days of growth was harvested, rinsed with 0.9% NaCl and frozen. RNA was isolated from frozen mycelium by using the TRIZOL (Invitrogen) extraction. DNA was degraded with RQ1 DNase kit (Promega) to avoid interference in the next steps of cDNA synthesis. cDNA synthesis was performed through three PCR steps using cDNA-specific primers (Table 1) by rapid amplification of cDNA ends, according to specifications of the OneStep RT-PCR (QIAGEN) kit.

Table 1. Primers Used in this Work *

Gene-specific primers	
LMF:	5′–GGCAACATGTACATCGTCAACGAGGAC–3′
LMR:	5′–GAAAGATGCGARTGRTACCARAACGTKCC–3′
Adaptor-specific primers	
AP1:	5′–GTAATACGACTCACTATAGGGC–3′
AP2:	5′–ACTATAGGGCACGCGTGGT–3′
Gene-specific GW primers	
1R:	5′–AACGAAGGATAAAGACAAGGTAAT–3′
1F:	5′–TCCACTTACGAACGATTGAA–3′
2R:	5′–TTCATGGTAATCAACTTACAGAACG–3′
2F:	5′–CTAGTACACTCATCAACGGTAAAGG–3′
3R	:5′–GATGATGATGTTTTACGTCGTCCG–3′
3R	5′–TAATGTAAGCGTAGCGAGAATCCGT–3′
cDNA-specific primers	
RLacF1	5′–CGTATGTTTCCAGGCGCA–3′
RLacR1	5′–CATTCTGAGGGGCCACAA–3′
RLacF2	5′–CGACACCATCATTACACTTGAA–3′
RLacR2	5′–GAAGGCCATAGCGAGATTG–3′
RLacF3	5′–CTGGAGGCGCAGACATCA–3′
RLacR3	5′–TTGTTTGGAATGCAGATGGTTC–3′

* Gene-specific primers were designed from the protein N-terminal sequence deduced in this work.

Sequencing of PCR products

Nucleotide sequences were determined using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) on ABI automated sequencers (ABI 3100 Avant), with specific oligonucleotide primers. Similarity searches for nucleotide and amino acid sequences were done using BLAST (Altschul et al. 1990) from the National Center for Biotechnology Information's web page. Sequences obtained were aligned using the program Seqman (included in the Lasergene suite).

Laccase dendrogram

An amino acid sequence deduced for the lacP83 gene was aligned with laccase sequences by using the ClustalW Multiple alignment software of BioEdit Sequence Alignment Editor, version 7.1.3.0 (Hall 1999). Distance matrix analyses were done with the Jukes and Cantor correction (Jukes and Cantor 1969). The tree analysis was performed by using the neighbor-joining method (Saitou and Nei 1987), and the tree topology was determined by bootstrap analysis using 100 replicates, with the software Phylip 3.69 (Felsenstein 1989). Software Treeview 1.6.6 (Page 1996) was used to visualize the tree.

RESULTS AND DISCUSSION

Up to four bands with laccase activity were observed during the stationary phase of fungal growth. For the semi-purification process, the ECE was obtained at 19 days of fermentation, because this time showed the maximal laccase activity (Fig. 1) and the

presence of two or three bands with laccase activity including lacP83 (Fig. 2a). Figure 2b shows the presence of three proteins after the purification process, including the band with laccase activity of interest. Figure 2c shows only one band with laccase activity after the purification process.

lacP83 had a total size of 2,887 bp, including the promoter, introns, exons, and the signal peptide. This sequence was deposited in GenBank with accession number JF719064. The coding sequence (2,421 bp long) showed 17 exons interrupted by 16 introns, ranging in size from 47 to 65 bp. The mRNA sequence was 1,527 bp long and encoded a protein of 509 amino acids. The predicted molecular mass of lacP83 was 54.1 kDa. A putative, 22 amino acid long, signal peptide matched with the typical sequence of eukaryotic extracellular proteins (Nielsen et al. 1997).

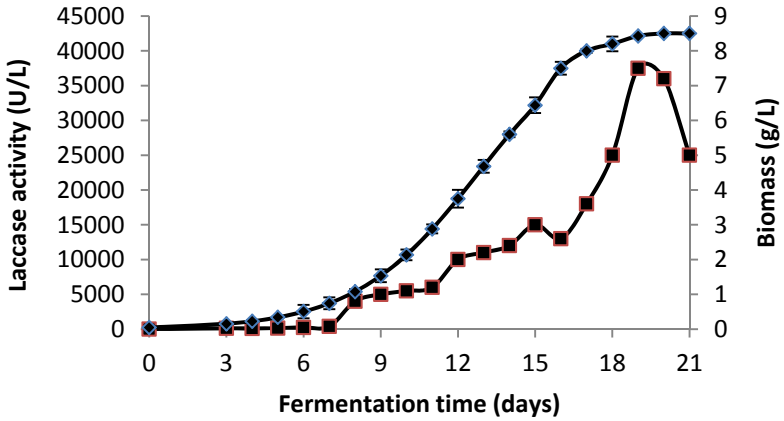

Fig. 1. Lacasse activity (■) and growth (♦) of *Pleurotus ostreatus*

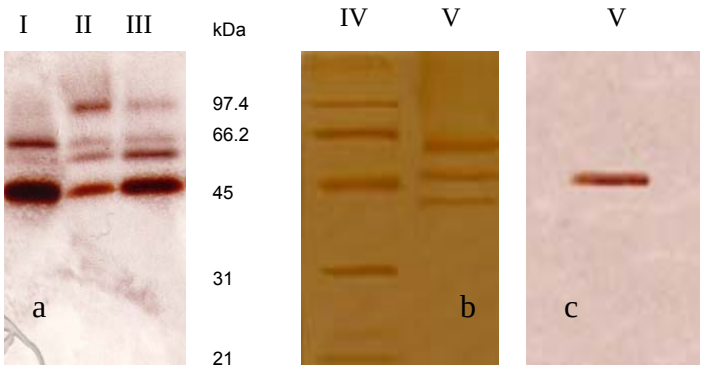

Fig. 2. a. Zymogram with ECE; b. SDS-PAGE of the semi-purified enzyme extract; c. Zymogram with semi-purified *P. ostreatus* isoenzyme lacP83. Line I, II, III: ECE obtained at 19, 20 and 21 days respectively; Line IV: Molecular marker; Lines V: sample obtained from ECE produced at 19 days, after the purification process.

A region spanning 469 bp before the start codon was analyzed to study the promoter sequence. Three potential sites of MREs (metal response elements) regulation

were found at positions -22 to -29, -181 to -189 and -356 to -365. Three possible XRE (xenobiotic response element) sites were located at positions -113 to -119, -283 to -289, and -381 to -375. One potential defense response site was marked at position -406 to -393 and one stress response element at position -462 to -468. The TATA box was located at -92 to -98 (Fig. 3).

It has been reported that laccase production in many fungi is controlled by different types of regulation, mainly by various physiological factors, so that different transcriptional activation mechanisms are involved. These depend upon transcription factors that bind to specific sites in the promoters of the genes. The lacP83 promoter has four possible sites of MREs, which are similar to the consensus sequence (5'-TGCRCNC-3') identified in the promoter of the metallothionein gene (mt) (Thiele 1992).

-469 GCCCCCTCGCAGTACCGAGTTCGGACTCCCCGTTTGAGCTGTTGACTTCCGGTGTAGATC
-409 TGCATCCATGACAACATAGATACCAGGGTCACGCCTTTTTGTCGTCCCGCGCGGATATCC
-349 ATACCAACGCCAGGAATAGAAGCGCTTTGAGTCTATTTCGCTCTAGTAGTCTTCCATTCC
-289 TCACGGGCTCTTCTTCGCATCTCCGAATACACCCAGGTTATGCACTGACTCGCGGACGAT
-229 CATCGCTCCCTTACGACAAACGGATCTCTAACAACACCGCCCAATCCGGTTCAAGATCCT
-169 CGAGATGAGGTACGCCTAACCGAAGCTTCCCAATCCGTTCATCTTTGTTCTCATGCATAT
-109 CGTTCTATAGGTATATTTAAGACGTGCACGGACGACTGGAAAACATCATCATCGACCTCC
-49 AATTATTTAACATCTCATCCAGCGCGTACTGTTACACCTACAAACG**ATG**

Fig. 3. Nucleotide sequence of the promoter region of the gene lacP83 from *P. ostreatus*, extending 466 bp upstream from the start codon (bold letters), TATA box is underlined. The putative response elements in the promoter region are indicated. Metal response elements; Xenobiotic response element; Defense response; Stress response element.

Transcription of many laccase genes is regulated by copper ions (Collins and Dobson 1997; Fernández-Larrea and Stahl 1996; Palmieri et al. 2000). Díaz et al. (2011) reported the effect of Cu in the liquid culture medium on the laccase activity of several strains of *Pleurotus ostreatus.* The maximal laccases activity was very high in presence of Cu. In particular, the strain Po83 showed 37490 and 1086 U/L with and without Cu respectively. Giardina et al. (1999), obtained a laccase production of 30000 U/L, growing *Pleurotus ostreatus* (ATCC MYA-2306) in nutrient-rich medium, with addition of Cu, whereas laccase production resulted between 0.5 and 4 U/L in the presence of Cu traces. The addition of 25.0 mM $CuSO_4$ in the solid culture of *Pleurotus pulmonarius (Fr) Quélet*, increased the level of laccase from 270 to 1420 U/L (Kirst et al. 2006). In this study, the maximal laccase activity obtained was around 30-fold higher than those observed in fermentation carried out under the same conditions but without addition of Cu (data not shown).

XRE transcription factors are involved in the induction of laccases of fungi by aromatic compounds (Soden and Dobson 2001). The promoter of *Trametes pubescens* does not show XRE transcription factors, and compounds such as 2,5-xylidine or catechol (laccase inducers) do not induce laccase activity (Galhaup et al. 2002). Apparently, the promoters of the laccase family of *Pleurotus ostreatus* are poorly preserved (Pezzella et al. 2009). The promoter analyzed in this work shows three MRE regulatory sites, whereas LACC1 promoter has only one, LACC10 has four, and LACC4 has five. XRE transcription factors present only in LACC10 and LACC2 promoters. The binding site for the stress response element was found only in LACC2.

Sequences for copper binding domains were also conserved in this gene, as in other laccase genes. Histidines and the only cysteine residue were conserved, and the alignment of this region with the seven sequences reported for *P. ostreatus* laccase genes (Fig. 4) shows a complete identity of the lacP83 copper binding domain with LACC9 (Giardina et al. 1995), and minimal differences from the rest of them.

Twelve putative laccase genes have been identified in the recently sequenced *P. ostreatus* genome (http://www.jgi.doe.gov/sequencing/why/50009.html (Pezzella et al. 2009). The size found for the laccase gene in this study (lacP83) is in the range reported in NCBI (GeneBank) for the laccase genes of *P. ostreatus*. For example, Giardina et al. (1995) and Moussa (2011) reported the LACC9 of 2619 bp (AB514560.1), and Giardina et al. (1996) reported the LACC10 gene, with a size of 2608 bp (Z49075.2). Giardina et al. (1999) reported the LACC6 of 3371 bp in length (AJ005017.2). The divergence of the laccase genes could be due to the role of isoenzymes in fungi (Lundell et al. 2010). In some cases the corresponding cDNA or protein has not been found (Pezzella et al. 2009). LACC12 (55 kDa by SDS-PAGE) has been found only in fruiting bodies of *P. ostreatus* (Lettera et al. 2010).

Protein	T1	T2	T3	T4
LACC9	HWHGFFQSGSTWADGPAFVNQCPIGTFWYHSHLSTQYCDGLRGPFHPFHLHGHGPWFLHCHIDWHLEIGLAVVF
LACC10	HWHGFFQAGSSWADGPAFVTQCPIGTFWYHSHLSTQYCDGLRGPFHPFHLHGHGPWFLHCHIDWHLEIGLAVVF
LACC4	HWHGLYQEKTTWADGPAFVTQCPIGTFWYHAHLGTQYCDGLRGPFHPFHLHGHGPWFLHCHIDWHLEIGLAVVF
LACC6	HWHGLFVKGHNWADGPAMVTQCPIGTFWYHSHLGTQYCDGLRGPFHPIHLHGHGPWFLHCHVDWHLEIGLAVVF
LACC2	HWHGLFQHKTSGMDGPSFVNQCPIGNYWYHSHLSTQYCDGLRGSFHPFHLHGHGAWFLHCHIDWHLEAGLAVVF
LACC12	HWHGFYQKGSNWADGPAFVTQCPIGTFWYHSHLSTQYCDGLRGVFHPFHLHGHGPWFLHCHIDWHLEIGLAIVF
lacP83	HWHGFFQSGSTWADGPAFVNQCPIGTFWYHSHLSTQYCDGLRGPFHPFHLHGHGPWFLHCHIDWHLEIGLAVVF
Consensus	HWHG********DGP**V*QCP_G**WYH*HL*TQYCDGLRG*FHP*HLHGHG*WFLHCH*DWHLE*GLA*VF

Fig 4. Sequences for the four copper binding domains (T1, T2. T3. T4) from the *P. ostreatus* laccases LACC9 (Giardina et al., 1995), LACC10 (Palmieri et al., 1993; Giardina et al., 1996), LACC4 (Pezzella et al., 2009), LACC2 (Palmieri et al., 2003), LACC6 (Giardina et al., 1999) and LACC12 (Lettera et al. 2010).

Most of the laccase genes showed a common pattern encoding a polypeptide of 520 to 550 amino acid residues. The size for the lacP83 gene is similar to other laccase genes previously described: for the gene LACC9, a length of 1,663 bp and 529 amino acids (Giardina et al. 1995); LACC10 showed a size of 1,713 bp encoding 533 amino acids (Palmieri et al. 1993); LACC6 had an mRNA of 1,772 bp and 533 amino acids (Giardina et al. 1999); LACC2 showed a size of 1,566 bp encoding 521 amino acids (Palmieri et al. 2003); LACC1 had 1,599 bp encoding 509 amino acids (Pezzella et al. 2009). In most of the laccase genes from *P. ostreatus*, 19 introns have been reported, but in the gene described in this work only 17 introns were found. However, the sequence is relatively preserved as compared with those laccase genes in basidiomycetes.

Table 2 shows the comparison of the gene, coding sequence and amino acid sequence between lacP83 and some other laccases reported in GeneBank. Figure 5 shows the similarity of the amino acids sequences obtained for lacP83 gene and some other laccases reported in GeneBank. This analysis reported the similarity percentage of the lacP83 with other laccases (from *P. ostreatus:* LACC9, 89.97; phenol-oxidase, 90.09; LACC10, 98.22; lcck, 98.21; LACC4, 92.5; LACC1, 62.04; LACC6, 93.54; from *P. eryngii:* 98.24 and 98.23; and from *Trametes villosa:* 94.40 and *Trametes versicolor:*

92.80). After comparison in the genome sequences PC15 and PC9 of *P. ostreatus*, it was observed that the lacP83 gene is a 95% homolog of LACC9.

Table 2. Comparison between Laccase (lacP83) and Others from *Pleurotus ostreatus* Reported in GeneBank

	Homology percentage (Accession No.)		
	Gene	Coding sequence	Amino acid
LACC9	92 (AB514560.1)	95 (Z34847.1)	96 (Q12729.1)
LACC10	78 (Z49075.2)	85 (Z34848.1)	90 (Q12739.1)
lcck	78 (AB089612.1)	92 (AB08912.1)	90 (AB089612.1)

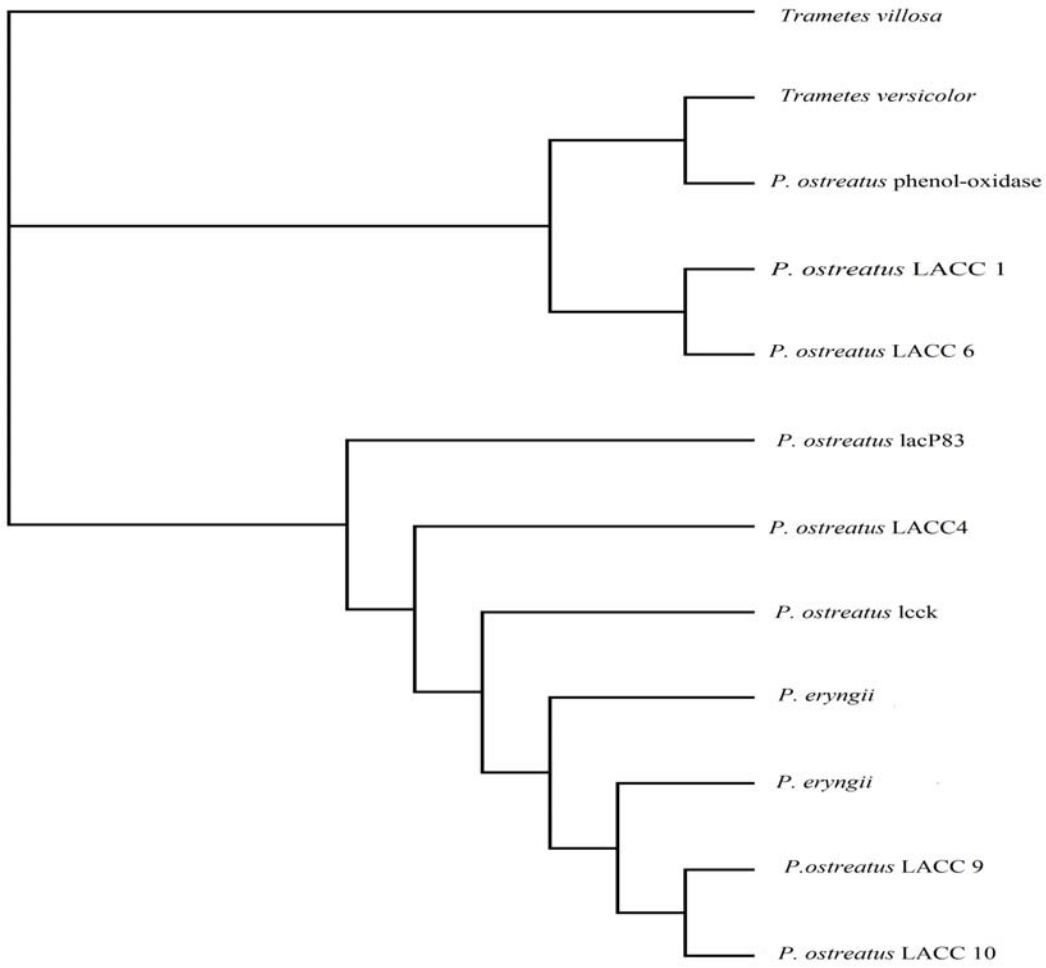

Fig. 5. Neighbor-joining tree representing the amino acid sequence similarity between lacP83 and 11 sequences retrieved from GenBank. [*P. ostreatus:* LACC9 (Q12729), phenol-oxidase (BAH90721.1), LACC10 (Q12739), lcck (BAC65099.1), LACC4 (B5MAF4), LACC1 (B5MAF5), LACC6 (O60199), lacP83 (AEO22162.1); *P. eryngii:* laccase (ADG01835.1), laccase (AAV85769.1); *Trametes villosa:* laccase (AAB47735.2), and *Trametes versicolor:* laccase (BAA23284.1)]

CONCLUSIONS

1. The lacP83 gene is a 95% homolog of LACC9; however it has similarity at the amino acid sequences level of around 98.20% with LACC10, lcck, and those used in this study from *P. eryngii*.
2. Heat shock elements have been reported in the sequence of the promoter of other laccases of *P. ostreatus*, but have not been found in the promoter of lacP83.
3. There was a consensus sequence for stress response elements, which has not been reported previously in these promoters.
4. Furthermore, none of the estimated weights for the protein (54.1 kDa calculated from the nucleotide sequence or 56 kDa observed by SDS-PAGE) has been previously assigned to a laccase from *P. ostreatus*.
5. The laccase activity obtained under submerged fermentation was higher in culture added with Cu than those without Cu.

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