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Isolation of two laccase genes from the white-rot fungus *Pleurotus eryngii* and heterologous expression of the *pel*3 encoded protein

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Abstract

In this paper we report the cloning and nucleotide sequence analysis of two new laccase genes from the white-rot fungus *Pleurotus eryngii*, named *pel*3 and *pel*4. Comparison of the protein sequences deduced from these genes with laccases previously described in *P. eryngii* indicates that these genes codify for new laccases in this fungus. We described the expression of *pel*3 gene in two different *Aspergillus niger* strains. Both the laccase signal peptide and the glucoamylase preprosequence of *A. niger* were used to target the secretion of the active enzyme. The highest levels of laccase expression were obtained by combining the last construction with an *A. niger* strain deficient in extracellular proteases secretion. The characterization of catalytic properties of the recombinant enzyme, together with the setting-up of a heterologous expression system for *pel*3, will provide the basis to study the biotechnological applications of this enzyme.

Keywords: Basidiomycete; Phenol oxidases; Copper protein; Protein sequence; Heterologous expression

1. Introduction

White-rot basidiomyctes include a group of fungi able to degrade lignin from woody and non-woody plant tissues, often with limited degradation of cellulose (Valmaseda et al., 1990). An extracellular enzymatic system, which includes different oxidoreductases, is involved in this biodegradation process together with low-molecular mass metabolites and activated oxygen species (Kirk and Farrell, 1987). These enzymes are also able to degrade aromatic pollutants causing environmental problems (Barr and Aust, 1994). Laccases and peroxidases play an important role in these processes catalyzing the one-electron oxidation of lignin aromatic units, which can subsequently progress through non-enzymatic reactions yielding bond cleavage products (Higuchi, 1990).

Laccases (EC 1.10.3.2.) catalyze the oxidation of a great variety of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor (Yaropolov et al., 1994). A relevant circumstance that increased the study of laccases during last years was the possibility to expand their activity on non-phenolic aromatic compounds in presence of low-molecular mass compounds which act as redox mediator (Bourbonnais and Paice, 1990), which include often compounds with –N=N– or –NOH groups and it is known as laccase-mediator system (LMS). The wide variety of substrates oxidized by laccases and the use of oxygen as electron acceptor make very interesting the study of these enzymes for industrial applications, including pulp bleaching in paper industry, dye decolourisation, and detoxification of environmental pollutants (Mayer and Staples, 2002). For these reasons, many efforts are being focused on molecular characterization of fungal laccases as well as the improvement of the expression levels using homologous or heterologous expression systems. On this regard, several approaches have been described in order to successfully express basidiomycetous laccases using fungal host, both yeast (Piscitelli et al., 2005; Jonsson et al., 1997; Jolivalt et al., 2005; Guo et al., 2005) and molds (Record et al., 2002; Kiiskinen et al., 2004). In this work we used *Aspergillus niger* as host for expression of *Pleurotus eryngii* laccase as a well known system to overproduce heterologous proteins of industrial interest (Conesa et al., 2000; Punt et al., 2002).

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The white-rot fungus *P. eryngii* causes a preferential degradation of wheat lignin (Valmaseda et al., 1990). This fungus secretes several laccases implicated in degradation of phenolic and non-phenolic aromatic compounds (Muñoz et al., 1997), the later in presence of mediators (Martínez et al., 1994). The role of these enzymes in transformation of 2,4-diclorophenol and benzo(*a*)pyrene has been recently reported (Rodríguez et al., 2004). In order to obtain laccases with an increased activity against these kind of compounds for their application in biotechnological processes, a heterologous source of these enzymes is mandatory.

The aim of this work is to provide the first molecular characterization of *P. eryngii* laccase genes, to create the basis for its application through heterologous expression system, and to characterize the recombinant enzyme.

2. Materials and methods

2.1. Organisms and culture media

P. eryngii CBS 613.91 was maintained on malt extract agar at 4 °C. *Escherichia coli* strains DH5α and MRA XL-Blue (P2) were purchased from Stratagene and maintained on LB with 20% glycerol (10 g/L Bacto Tryptone; 10 g/L NaCl; 5 g/L yeast extract; and 200 mL/L glycerol) at −80 °C until use.

A. niger strain N402 (Bos et al., 1988), and *A. niger* MGG029ΔΔ (*prtT* Δ*glaA*::*fleo*^{r}, Δ*aamA*::*pyrG* (Weenink et al., 2006), deficient in extracellular proteases, were maintained at 4 °C as suspension of spores in 9 g/L NaCl.

A. niger transformants were grown at 30 °C and selected on acetamide (0.6 g/L) plates containing 10 g/L glucose, 0.5 g/L $MgSO_4$, 0.052 g/L KCl, 0.15 g/L KH_2PO_4, and 1 mL/L of 1000× trace elements solution (22 g/L $ZnSO_4{\cdot}7H_2O$, 5 g/L $MnCl_2{\cdot}4H_2O$, 5 g/L $FeSO_4{\cdot}7H_2O$, 1.6 g/L $CoCl_2{\cdot}6H_2O$, 1.6 g/L $CuSO_4{\cdot}5H_2O$, 7.5 g/L $Na_2MoO_4{\cdot}2H_2O$, and 50 g/L EDTA). For the screening of laccase-producing transformants, this medium was supplemented with 1 mM 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS). For laccase production and purification, *Aspergillus* transformants were grown at 30 °C and 200 rpm on liquid medium (1 g/L casamino acids, 5 g/L yeast extract, 0.5 g/L $MgSO_4$, 0.052 g/L KCl, 0.15 g/L KH_2PO_4, and 1 mL/L of 1000× trace elements solution) using different concentrations of glucose (10–100 g/L) and polyvinylpyrrolidone (PVP, 0–20 g/L). The effect of pH in the culture medium was also evaluated.

2.2. Preparation of DNA probes and genomic library screening

P. eryngii was grown in glucose–peptone–yeast extract medium (20 g/L glucose; 5 g/L peptone; 2 g/L yeast extract; 1 g/L KH_2PO_4; 0.5 g/L $MgSO_4{\cdot}7H_2O$; pH 5.5) at 28 °C and 160 rpm. The mycelium was harvested after 6-day incubation, freeze-dried, and genomic DNA was isolated by phenol:chloroform:isoamylic alcohol (25:24:1) extraction and isopropanol precipitation (González et al., 1992). An λEMBL3 genomic library was prepared according to Ruiz-Dueñas et al. (1999).

Two DNA digoxigenin (DIG)-labeled probes were prepared by polymerase chain reaction (PCR) using *P. eryngii* genomic DNA as template (1 μg) and the oligonucleotides 5′-TCRATMGTCCCGYCAAGCTGG-3′ and 5′-SAARGWRTGWCCGTCGATMGAG-3′ (100 pmol) with BamHI and EcoRI cleavage sites, respectively, included in their 5′ terminal regions for subsequent cloning. These degenerated primers correspond to conserved sequences from laccase genes of *Pleurotus* species (Giardina et al., 1995, 1999; Palmieri et al., 1997; Soden et al., 2002). Two fragments of 580 bp (probe 1) and 530 bp (probe 2) were amplified, purified and cloned into BamHI/EcoRI sites of pBluescript SK (±), and used as probes in DIG labeling reactions (DIG labeling and detection kit II, Roche).

Appropriated dilutions of the genomic library were plated, using *E. coli* MRA XL-Blue (P2) as host, on LB medium containing 10 mM $MgSO_4$. Plates were lifted to Ø15 cm nylon membranes (Roche) by standard procedures (Sambrook and Russell, 2001), and hybridized overnight at 42 °C in DIG Easy Hyb solution (Roche) after a prehybridization of 1 h at the same temperature. The membranes were washed and processed to detect hybridized DIG label, autoradiographed with Hyperfilm MP film (Amersham Pharmacia Biotech) and positive clones selected.

Purified DNA from positive recombinant phages was digested with EcoR1, BamH1 and Sal1 (New England Biolabs) and DNA fragments hybridizing with probes 1 and 2 were detected by Southern blot (under the same conditions described for library screening). These positive fragments were isolated, cloned into pBluescript SK (±) and maintained into *E. coli* DH5α. DNA sequencing of positive fragments was carried out in an ABI PRISM 377 automatic sequencer (PerkinElmer).

2.3. mRNA isolation and generation of cDNA

Total RNA was isolated from 4-day-old *P. eryngii* mycelium grown in glucose–peptone–yeast extract medium using Ultraspec RNA (Biotecx Laboratories, Inc.). Polyadenylated RNA was purified using mRNA purification kit (Pharmacia). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the Qiagen OneStep RT-PCR kit. Polyadenylated RNA (2 μg) was used as template. The primers pairs used (0.6 μM final concentration each) were 5′-ATGTTTCCAGGCGCACGG-3′ and 5′-CTAAGCTATGCCACCTCTG-3′ for *pel*3, and 5′-ATGGCGGTTGCATTCATTGC-3′ and 5′-CTATACACGACTCTCGTTCG-3′ for *pel*4, corresponding to the start and stop codons of the cDNA. A fragment of approximately 1.5 kbp in each reaction was amplified and cloned into pGEM-T easy cloning vector (Promega) for maintenance and sequencing.

2.4. Software for sequence analysis of P. eryngii laccase genes

SignalP was used for theoretical signal peptide determination (Dyrlov Bendtsen et al., 2004). The Genomatix

Table 1
Vectors used for laccase expression in *A. niger* host

		Expression vector	
		pPEL3	pPEL3G
Primers	Forward	521F	524F
	Reverse	524R	
Cloning vector		pAN52-1*Nco*1[a]	pAN52-4[b]
Cloning site restriction fragments		*Bsp*HI-*Bam*HI	*Bss*HII-*Bam*HI
Cloning site vectors		*Nco*I-*Bam*HI	*Bss*HII-*Bam*HI

Sequence of the primers used for cDNA amplification and cloning. Restriction sites are underlined. Stp, stop codon.

521F	ttc atc ATG ATT TTT CCA GGC GCA CGG ATT M I F P G A R I
524F	gcc aag CGC GCT GCC ATC GGG CCC ATT GCC A I G P I A D M
524R	cgc gga tcc CTA AGC TAT GCC ACC TCT Stp A I G G R

For each vector are indicated the restriction sites included in the primers, the *Aspergillus* plasmid used, and the restriction sites used for cDNA cloning.
[a]EMBL accession number Z32697.
[b]EMBL accession number Z32750.

suite was used for theoretical promoter sequence analysis (http://www.genomatix.de). BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST) were used to search similarities among different laccase sequences.

2.5. Laccase expression vectors and A. niger transformation

Two laccase expression vectors, pPEL3 (containing the complete cDNA sequence of *P. eryngii pel*3 laccase gene) and pPEL3G (where the signal peptide was replaced by the 24 amino acid glucoamylase preprosequence from *A. niger*), were prepared by PCR cloning (Table 1). In the constructions, the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*A) promoter and the *trp*C terminator from *A. nidulans* were used to drive the expression of the laccase encoding sequence.

A. niger strains were transformed following the method described by Punt and van den Hondel (1992) using the laccase expression vectors and p3SR2, containing the acetamidase gene (*amd*S) as selection marker (Kelly and Hynes, 1985). Transformants were selected on acetamide plates, and subsequently laccase-producing transformants were tested using ABTS-containing acetamide plates and the liquid medium described above.

2.6. Purification and characterization of the recombinant enzyme

Laccase activity was assayed using 5 mM ABTS in 100 mM sodium acetate buffer, pH 5 ($\varepsilon_{436} = 29{,}300\ M^{-1}\ cm^{-1}$). The enzymatic reactions were carried out at room temperature (~25 °C). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate min^{-1}. Extracellular protein was determined by the Bradford method using the Bio-Rad Protein Assay and bovine serum albumin as standard.

Culture liquid from laccase-positive transformants was separated from mycelia by filtration, 20-fold concentrated, and dialyzed against 10 mM sodium phosphate buffer, pH 6.5, by ultrafiltration (Filtron 5-kDa-cutoff membrane). Then the sample was applied to an ion-exchange cartridge (HiTrap Q FF, GE Healthcare), equilibrated with 10 mM sodium phosphate buffer, pH 6.5, and eluted at a flow rate of 1.5 mL min^{-1} with the following gradient of NaCl in the same buffer: 0.1–0.4 M, 100 min; 0.4–1.0 M, 10 min; and 1.0–1.0 M, 10 min. Fractions containing laccase activity were pooled, concentrated, and dialyzed by ultrafiltration against 10 mM sodium acetate buffer, pH 5.5 (Amicon Ultra 5-kDa-cutoff membrane). Then, the concentrate was applied to an anion exchange column (Mono-Q HR 5/5, Pharmacia) in 10 mM sodium acetate buffer, pH 5.5, and eluted at a flow rate of 0.8 mL min^{-1} with the following gradient of NaCl in the same buffer: 0.10–0.22 M, 60 min; 0.22–1.00 M, 10 min; and 1.00–1.00 M, 10 min. Fractions containing laccase activity were pooled, concentrated and loaded onto a size exclusion column (Superdex 75, Pharmacia) equilibrated with 10 mM sodium acetate buffer, pH 6.0, containing 150 mM NaCl, and eluted at a flow rate of 0.5 mL min^{-1}.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels was performed by the method of Laemmli, 1970, using trypsin (21.5 kDa), carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa) as molecular mass standards (Bio-Rad). Protein bands were stained using the Silver Staining Plus kit (Bio-Rad).

The dependence of laccase activity on the pH was assayed in the range of 2–7.5 using 5 mM ABTS and 10 mM 2,6-dimetoxiphenol (2,6-DMP, $\varepsilon_{469} = 27{,}500\ M^{-1}\ cm^{-1}$) as substrates, and 100 mM citrate-phosphate-borate buffer. pH stability was determined at 4 °C using 5 mM ABTS as substrate in the same buffer. Optimal temperature was investigated at 4, 30, 45, and 65 °C in 100 mM citrate-phosphate-borate buffer, pH 7.0, using 5 mM ABTS as substrate in the same buffer. Kinetic constants K_m and k_{cat} for ABTS oxidation were calculated at room temperature with 24.5 μg/mL of recombinant protein in 100 mM citrate-phosphate buffer, pH 3.0.

3. Results

3.1. Laccase cloning and description of the nucleotide and amino acid sequences

An λEMLB3 genomic library of *P. eryngii* was built and subsequently screened with two homologous DIG-labeled DNA probes (probes 1 and 2). DNA restriction fragment analyses of λEMLB3 positively hybridizing clones revealed a 6 kbp EcoRI fragment hybridizing with probe 1, and two positive EcoR1 and BamH1 fragments of 9 and 6 kbp, respectively, hybridizing with probe 2, which were cloned and sequenced. DNA sequences of approximately 3500 bp encoding two putative laccases, named *pel*3 and *pel*4, were identified in these fragments. In Figs. 1 and 2 are shown the sequences of these genes. Both sequences included a region upstream of the start codon of 770 and 880 bp long, respectively, which showed several features of eukaryotic promoters.

Comparison of the cDNA fragment obtained by RT-PCR with the genomic DNA sequence of *pel*3 confirmed the presence of 19 introns, with a size between 46 and 63 bp. After introns removal,

a 1596 bp long ORF was deduced from the *pel*3 gene. This ORF encodes a protein precursor of 532 amino acids. The translation of the first 69 bp after the initiation codon ATG resulted in a 23 amino acid putative signal peptide that showed the typical structure for the sorting of secreted proteins in eukaryotes. The theoretical molecular mass of the mature protein was 54 kDa. A potential *N*-glycosylation site was found in position 444. The expected Cu^{2+} ligands found in all blue copper oxidases, 10 histidines and 1 cysteine, are conserved in PEL3 laccase: H75, H77, H120, H122, H404, H407, H409, H460, H462, H466, and C461. Genomic DNA sequence of *pel*3 was deposited in GenBank with the accession no. AY686700.

The genomic sequence of *pel*4 was incomplete and possibly interrupted by, at least, 15 introns. Several intents were made to obtain the complete genomic sequence of *pel*4 through PCR amplification, but the results were not successful. The translation of cDNA generated from RT-PCR reaction showed a protein of 533 amino acids length. The first 20 amino acid correspond to a predicted signal sequence. The mature protein had a theoretical molecular mass of 56 kDa. Two potential *N*-glycosylation sites were identified at positions 342 and 434. As in the case of PEL3 sequence, all the expected copper ligands were identified: H64, H66, H109, H111, H394, H397, H399, H450, H452, H456, and C451. The cDNA sequence

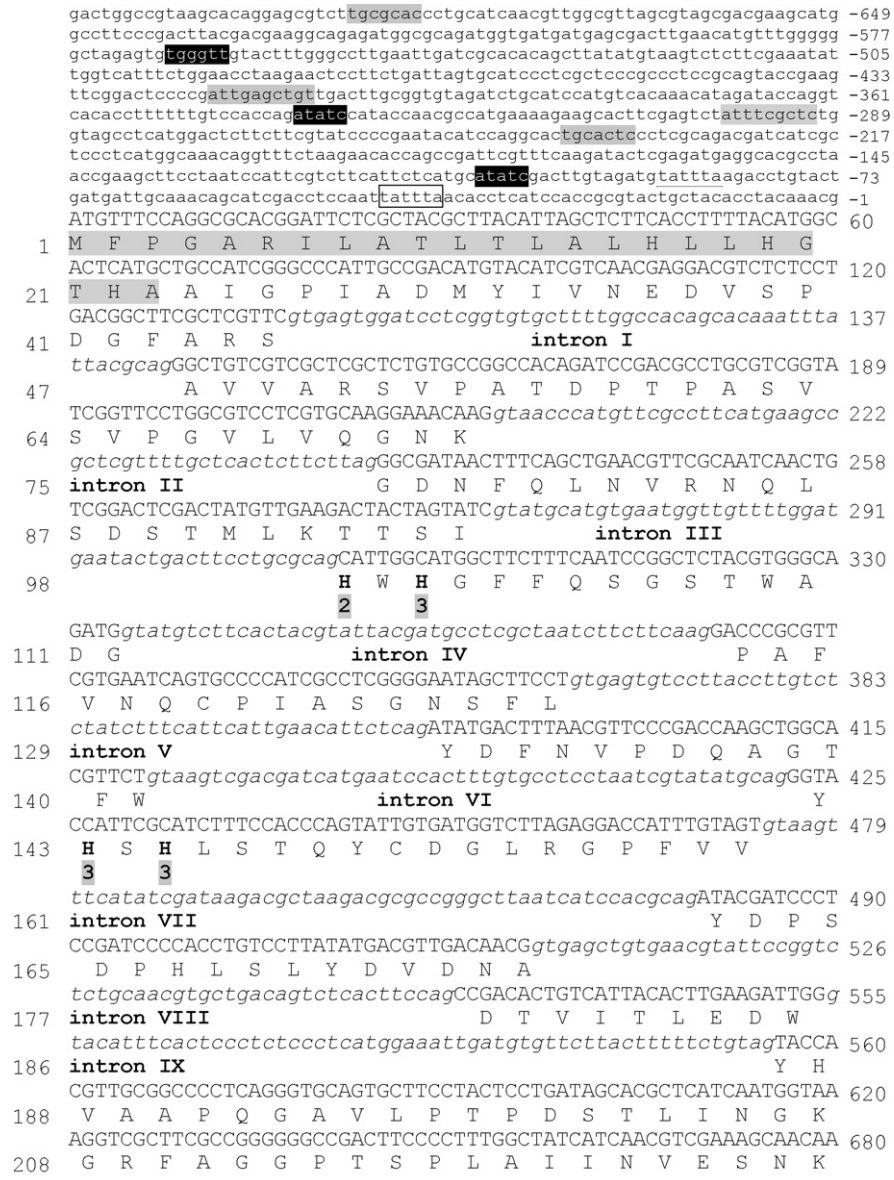

Fig. 1. Genomic sequence of *pel*3 gene, including the deduced amino acid sequence of the encoded protein PEL3. Introns are showed in italics. Residues implicated in copper coordination are showed in bold and numbered depending on the type of copper that coordinate. The *pel*3 promoter region analysis showed different consensus sequences: (i) the TATA box, at position −43 to −36, (ii) one copper-sensing sequence VTVBVGCTGW (Uldschmid et al., 2002), at position −419 to −410, (iii) one yeast copper-response element HTHXXGCTC (Labbé and Thiele, 1999), at position −299 to −291 and (iv) two putative metal response elements (MREs), in agreement with previously released consensus sequence TGCRCXC (Thiele, 1992), at positions −242 to −236 and −694 to −688 and three elements analogous to the consensus sequences TGGGT and ATATC described in other laccases promoters (Soden and Dobson, 2003) (black squares). A polyadenilation signal at 3′ end is closed on straight-line square.

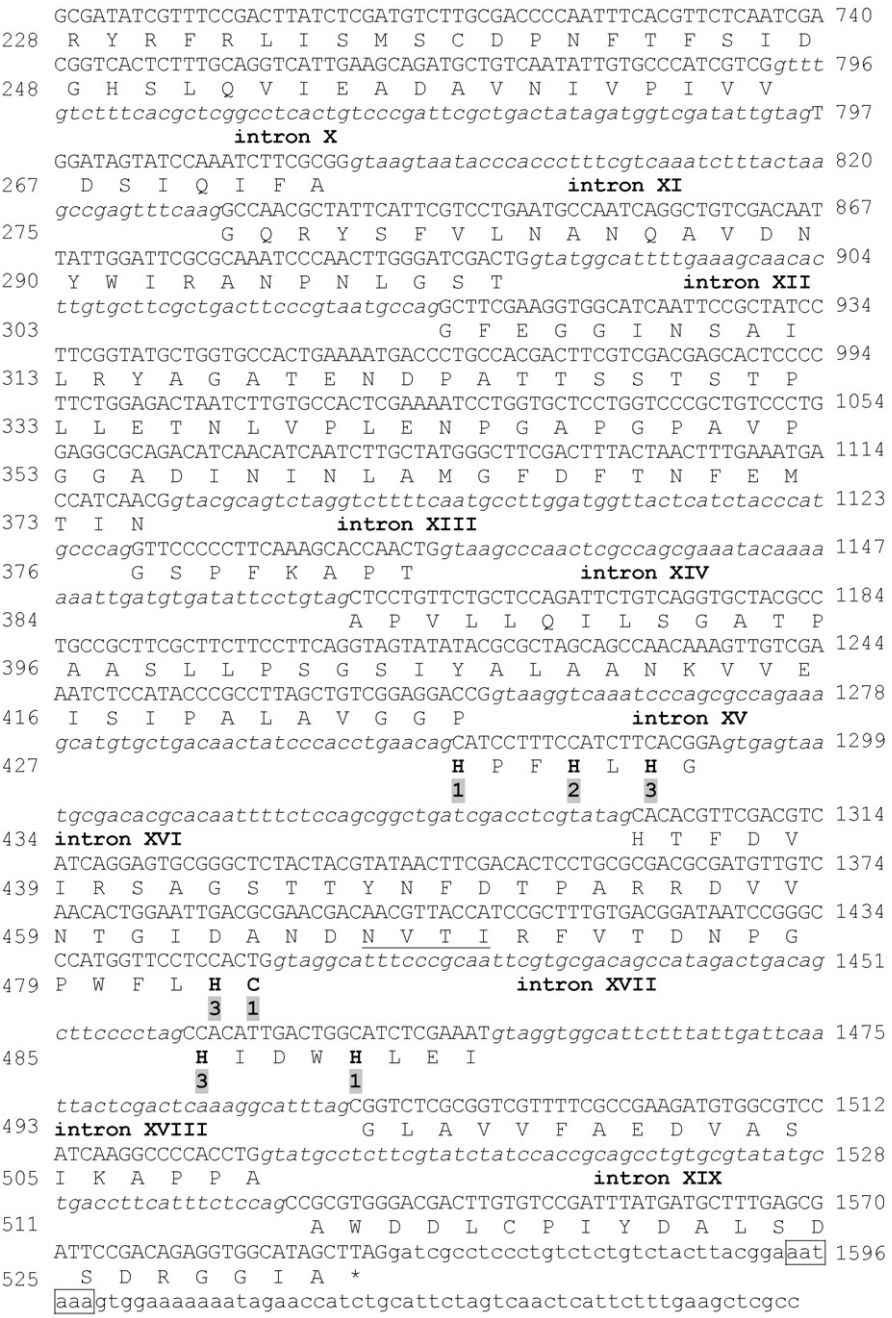

Fig. 1. (*Continued*).

of *pel*4 was deposited in GenBank with the accession no. ABB30169.

The alignment of the two *P. eryngii* laccase sequences showed a 56.7% of identity. The residues that bind copper and the sequences surrounding these residues are conserved, as in the rest of laccase sequences, except for a methionine residue that is present in PEL4 sequence (M396) but in PEL3 sequence is substituted by a phenylalanine (F406).

The comparison of the deduced amino terminal sequence from these proteins showed important differences between them and those previously described in *P. eryngii* (Muñoz et al., 1997), indicating that *pel3* and *pel4* codify for new laccases in this fungus.

3.2. Expression and characterization of the recombinant *P. eryngii pel3*

The *A. niger* N402 and MGG029ΔΔ strains were transformed with pPEL3 and pPEL3G expression vectors (Table 1). Positive transformants (30 with pPEL3 construct in N402 strain, 9 with pPEL3G in the same strain and 10 with pPEL3G in MGG029ΔΔ strain) were tested on ABTS-acetamide agar plates for laccase activity. The positive recombinant *A. niger* strains which produce highest ABTS-oxidation halo on plate were analyzed in liquid cultures for laccase production. Preliminary results showed very low levels of laccase activity in liquid media (10 g/L glucose, 0% PVP, and initial pH 6.2) with

all the recombinant *A. niger* strains assayed. Different culture conditions, glucose concentration and addition of PVP to the medium were analyzed. The best conditions for laccase production were obtained when the initial pH in the medium (around 6.2) decreased to 2.5 and 100 g/L of glucose and 20 g/L PVP were added.

The low pH in the culture and the presence of PVP promoted a transition in the form of growth from aggregated mycelia (pellets) to dispersed mycelia, which is in agreement with an increase in protein secretion (Archer et al., 1995; Carlsen and Anders, 1996). In these conditions, the highest laccase production (2.4 U/mg protein) was achieved after 115 h incubation with *A. niger* MGG029ΔΔ strain transformed with pPEL3G compared to values obtained with the same construction on N402 strain (1.3 U/mg) or pPEL3 construction on the N402 strain (0.4 U/mg). Experiments to develop the expression of *pel*4 cDNA were carried out using the same strategy, but not successful results were obtained, may be due to the instability of the recombinant enzyme.

To isolate and characterize the recombinant PEL3 laccase the culture supernatant of *A. niger* MGG029ΔΔ-pPEL3G was used. Three chromatographic steps, two on anion exchange columns and one on exclusion size column were necessary to achieve an apparent homogeneity on SDS-PAGE (Fig. 3A). The molecular mass of the recombinant enzyme was estimated on 58 kDa by SDS-PAGE.

In relation with the pH dependence of the enzyme activity, the oxidation of ABTS decreased with the pH, showing the higher rates at low pH values; nevertheless, the oxidation of 2,6-DMP showed a bell-shaped curve, with an optimum about pH 6 (Fig. 3B). The pH stability study (Fig. 3C) showed that 80–100% of laccase activity remained at pH range 5–7 after 3 h of incubation and more than 80% remained at pH 6–7 after 24 h. The activity decreased with the pH showing less than 5% of

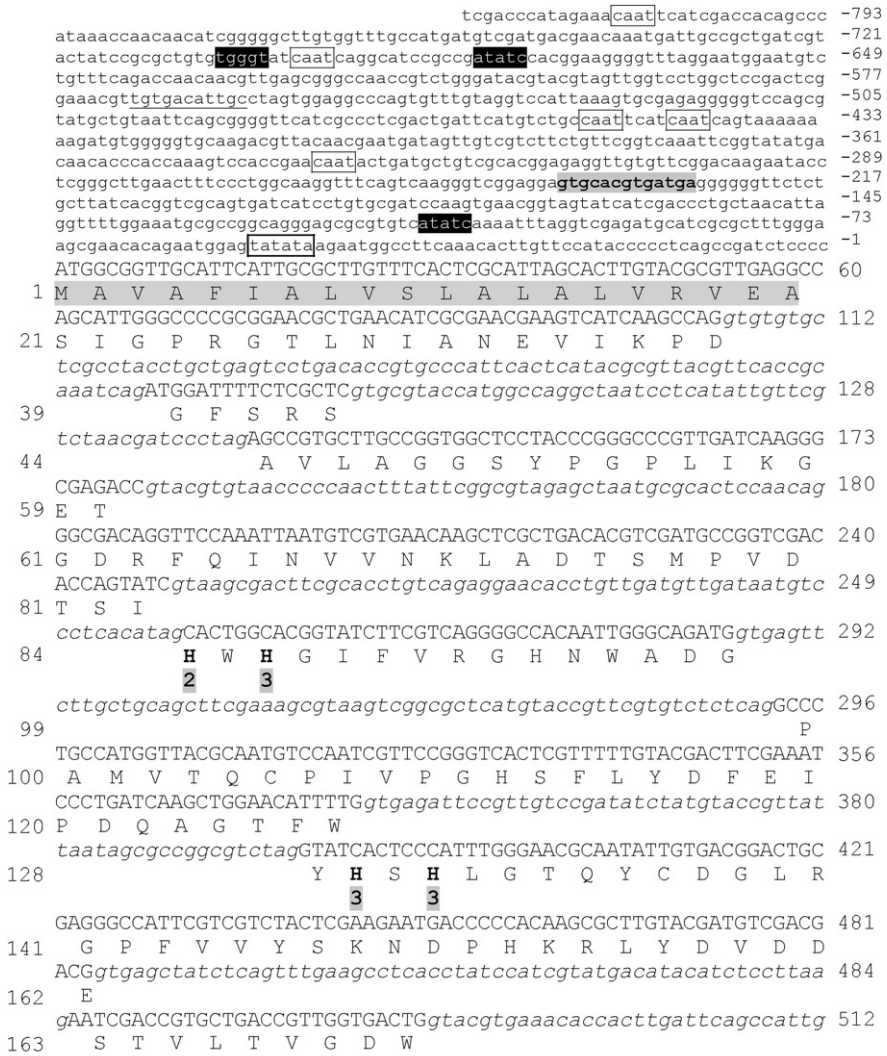

Fig. 2. Genomic and deduced amino acid sequence of *pel*4 gene. Introns are showed in italics. Residues implicated in copper coordination are showed in bold and numbered depending on the type of copper that coordinate. The deduced signal peptide of encoded protein is surrounded by a grey square. Hypothetical *N*-glycosilation sites are underlined. Different consensus regulatory sequences are highlighted upstream the ATG codon: five CAAT boxes and one TATA box (straight-line squares), one putative regulatory element GTGCACGTGATGA in response to phosphate starvation (Lemire et al., 1985) (in bold on grey square), one antioxidant response element RGTGACNNNGC analogous to previously described in mammalian cells (Rushmore et al., 1991) (underlined), and three elements analogous to the consensus sequences TGGGT and ATATC described in other laccase promoters (Soden and Dobson, 2003) (black squares).

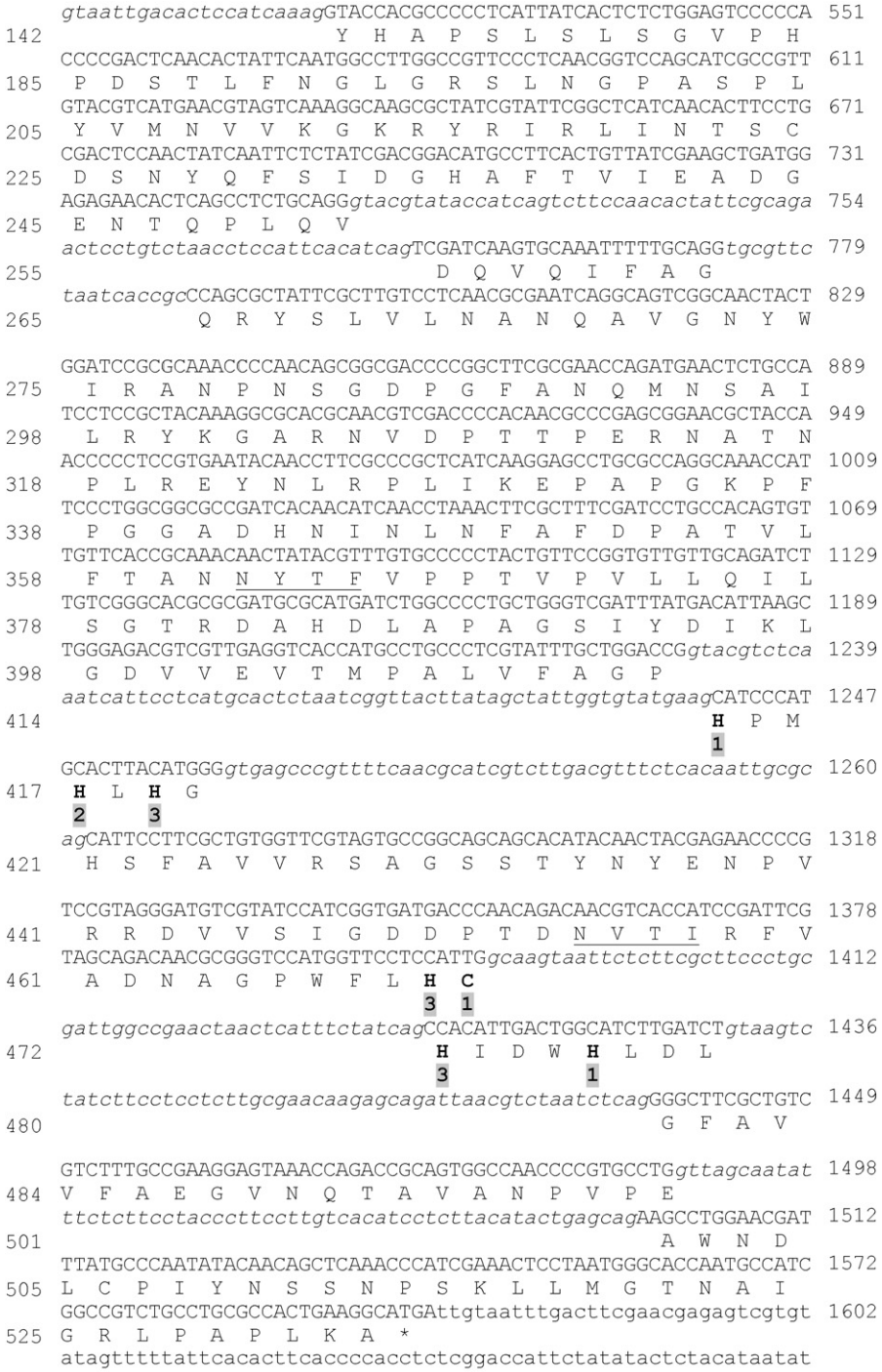

Fig. 2. (*Continued*).

remaining activity after 24 h at pH 2–3. Thermal stability study, carried out at pH 7.0 to avoid the damaging effect of acidic pH, showed that the enzyme retained all the activity at 4 and 30 °C after 90 min incubation (Fig. 3D), but it decreased to less than 20% after 24 h of incubation at 30 °C (data not shown). The enzyme had a half-life of 30 min at 45 °C, showing around 30% activity after 90 min. No activity was found after 30 min at 65 °C. The kinetic constants K_m and k_{cat} using ABTS, a substrate often used in laccase specificity studies, were 0.42 mM and 1.8 s^{-1}, respectively.

4. Discussion

Two genes and the corresponding cDNAs encoding new laccase isoenzymes in *P. eryngii* were cloned, sequenced and the respective amino acid sequences deduced. The analysis of *pel*3 promoter sequence showed a metal response element analogous to those previously described for the white-rot fungus *T. versicolor* (Uldschmid et al., 2002) and other copper sensing elements analogous to those found in yeast that could be implicated in the copper induction of the gene (Thiele, 1992; Labbé and Thiele,

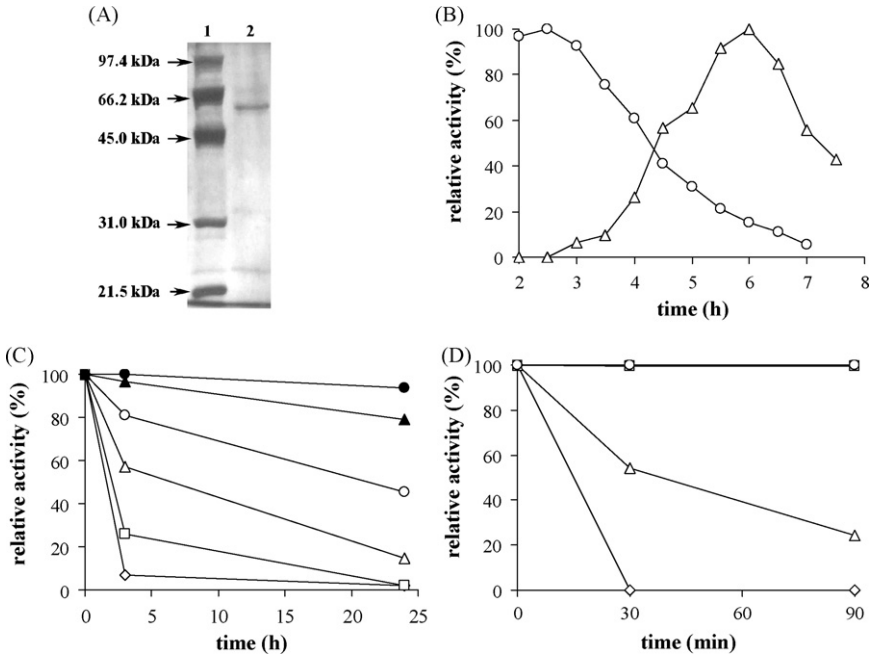

Fig. 3. (A) Silver-stained SDS-PAGE of the fraction obtained in the last step of purification of the recombinant enzyme. Lane 1 corresponds to low-molecular weight marker (Bio-Rad). (B) Optimum pH of recombinant PEL3 laccase using 5 mM ABTS (◯) or 10 mM DMP (△) as substrates. (C) Stability of the recombinant enzyme at pH 2 (◊), 3 (□), 4 (△), 5 (◯), 6 (▲), and 7 (●) using 5 mM ABTS as substrate. (D) Stability of the enzyme after incubation at different temperatures: 4 °C (□), 30 °C (◯), 45 °C (△), and 65 °C (◊) using 5 mM ABTS as substrate.

1999). Nevertheless, the promoter sequence of *pel*4 did not display any metal response element. The lack of data about the expression pattern and substrate specificity of these new laccases *in vitro* avoided us to obtain experimental evidences of a different function of these enzymes, but the differences found in regulatory elements in the promoter sequences could point out to different regulation mechanisms.

The laccases codified by *pel*3 and *pel*4 showed a high identity with other fungal laccases, especially with those reported in *Pleurotus* species. The maximum identity of PEL3 was 92.1% with *P. ostreatus* POX1 laccase (Giardina et al., 1995) and 90.6% with *P. sajor-caju* laccase 1 (Soden et al., 2002). In the case of PEL4, the maximum identity (95.3%) was found with *P. ostreatus* POXA1b laccase (Giardina et al., 1999).

The differences found between PEL3 and PEL4 sequences (57% identity), and the dendrogram obtained from the alignment of several phenol oxidases (Fig. 4), suggest that PEL3 and PEL4 belong to two different phylogenetic groups. However, both sequences belong to the cluster that included laccases from different basidiomycetes and are distant from other clusters in which are included laccases from ascomycetes, bacteria and plants. Recently, a more extensive phylogenetic analysis of different multicopper oxidases has provided the basis to reserve the name of laccases only from these enzymes produced by fungi (Hoegger et al., 2006). Although biochemical characterization of native enzymes have not been possible, according to Hoegger et al. (2006) and the results shown in Fig. 4, the new genes studied from *P. eryngii* can be considered as laccase codifying genes.

Many laccases are being studied by their possible applications in textile processing and treatment of industrial effluents, detoxification of environmental pollutants, and paper pulp bleaching (Sigoillot et al., 2003; Wesenberg et al., 2003; Baldrian, 2006). The availability of enough protein is a limited step for its use at industrial scale. Some approximations have been made successfully in yeasts (Guo et al., 2005; Piscitelli et al., 2005; Jolivalt et al., 2005). In this work we have expressed the PEL3 laccase in *A. niger*, a host for heterologous expression successfully used for other white-rot fungal laccase (Record et al., 2002). Recombinant expression of PEL3 was performed using both *P. eryngii* laccase and *A. niger* glucoamylase signal peptide sequences. The extracellular detection of recombinant laccase by using its own signal peptide indicated its functionality in vivo, but its substitution by the glucoamylase signal peptide affected positively to the secretion and production of recombinant enzyme in *A. niger*. The highest laccase levels were achieved using a strain deficient in the secretion of proteases due to a regulatory mutation (Mattern et al., 1992). The protease activity can produce problems in the heterologous expression, due to the possibility of enzyme proteolysis (van den Hombergh et al., 1997).

In addition, the expression of laccase was strongly conditioned by the culture conditions. The presence of PVP, which affect the viscosity of the medium, and the initial low pH produced changes in the fungal growth, transforming the pellets to disperse mycelium and increasing the extracellular activity, according to the results previously reported (Archer et al., 1995; Carlsen and Anders, 1996).

The apparent molecular mass of recombinant protein was 58 kDa. It have been reported that fungal laccases are glycoproteins with a molecular mass among 50 and 80 kDa (Thurston, 1994). Laccases I and II, previously characterized in *P. eryngii*, are glycosylated proteins (Muñoz et al., 1997). Since the PEL3 sequence shows a *N*-glycosylation site, the difference in

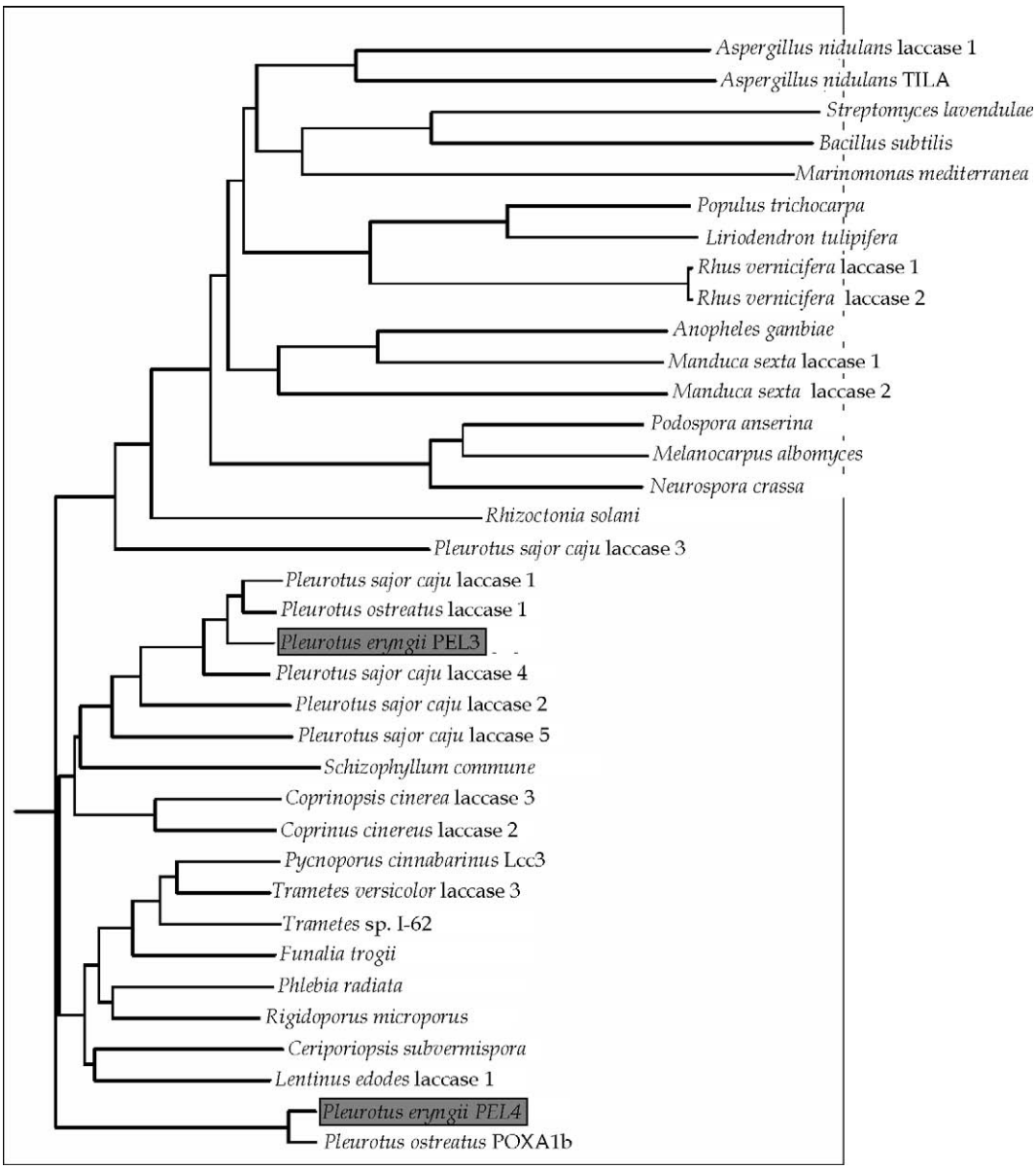

Fig. 4. Dendrogram of several phenol oxidases from different sources generated by neighbour-joined method using the Drawgram program. The original amino acid sequence alignment was created using Clustal W program. The *P. eryngii* laccases are highlighted in grey.

the M_r based in amino acid sequence and the M_r determined by SDS-PAGE could correspond to the glycosylation process.

The recombinant enzyme exhibits catalytic properties similar to those reported for other fungal laccases in relation with ABTS and 2,6-DMP oxidation (Baldrian, 2006), but differed from those previously reported in *P. eryngii* laccases (Muñoz et al., 1997). Laccases I and II isoenzymes from *P. eryngii* showed the maximum oxidation rate of ABTS at pH 4 and 3.5, respectively, differing from the optimum (pH 2.5) found with recombinant PEL3 laccase. Nevertheless, the pH stability of PEL3 was similar to *P. eryngii* laccase I, and different from laccase II, which showed more dependence on pH, being more stable at alkaline pH. The thermal stability of the recombinant *P. eryngii* laccase, as well as the K_m constant for ABTS oxidation, was similar to those previously reported in *P. eryngii* laccases I and II (Muñoz et al., 1997).

In conclusion, we have cloned two genes coding for laccases in the white-rot fungus *P. eryngii*. The analysis of the experimental data suggests that these genes code for two different laccases that have not previously described in this fungus. Moreover, one of these genes, *pel*3, has been expressed in *A. niger.* The activity of the recombinant enzyme was low, probably due to incomplete or incorrect folding. To get higher expression levels we will check other signal peptides or use alternative expression system (i.e. *Pichia pastoris*) in order to know more about this protein and study its use for biotechnological experiments.

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