Revised for *Fungal Genetics and Biology*

Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: Differential regulation in lignocellulose medium and effect of temperature and pH

Elena Fernández-Fueyo[a], Raul Castanera[b], Francisco J. Ruiz-Dueñas[a], Maria F. López-Lucendo[a], Lucia Ramírez[b], Antonio G. Pisabarro[b] and Angel T. Martínez[a,*]

[a]*Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28006 Madrid, Spain*
[b]*Department of Agrarian Production, Universidad Pública de Navarra, E-31006 Pamplona, Spain*

ABSTRACT

Pleurotus ostreatus is an important edible mushroom and a model lignin degrading organism, whose genome contains nine genes of ligninolytic peroxidases, characteristic of white-rot fungi. These genes encode six manganese peroxidase (MnP) and three versatile peroxidase (VP) isoenzymes. Using liquid chromatography coupled to tandem mass spectrometry, secretion of four of these peroxidase isoenzymes (VP1, VP2, MnP2 and MnP6) was confirmed when *P. ostreatus* grows in a lignocellulose medium at 25 ºC (three more isoenzymes were identified by only one unique peptide). Then, the effect of environmental parameters on the expression of the above nine genes was studied by reverse transcription-quantitative PCR by changing the incubation temperature and medium pH of *P. ostreatus* cultures pre-grown under the above conditions (using specific primers and two reference genes for result normalization). The cultures maintained at 25 ºC (without pH adjustment) provided the highest levels of peroxidase transcripts and the highest total activity on Mn^{2+} (a substrate of both MnP and VP) and Reactive Black 5 (a VP specific substrate). The global analysis of the expression patterns observed divides peroxidase genes into three main groups according to the level of expression at optimal conditions (*vp1/mnp3* > *vp2/vp3/mnp1/mnp2/mnp6* > *mnp4/mnp5*). Decreasing or increasing the incubation temperature (to 10 ºC or 37 ºC) and adjusting the culture pH to acidic or alkaline conditions (pH 3 and 8) generally led to downregulation of most of the peroxidase genes (and decrease of the enzymatic activity), as shown when the transcription levels were referred to those found in the cultures maintained at the initial conditions. Temperature modification produced less dramatic effects than pH modification, with most genes being downregulated during the whole 10 ºC treatment, while many of them were alternatively upregulated (often 6 h after the thermal shock) and downregulated (12 h) at 37 ºC. Interestingly, *mnp4* and *mnp5* were the only peroxidase genes upregulated under alkaline pH conditions. The differences in the transcription levels of the peroxidase genes when the culture temperature and pH parameters were changed suggest an adaptive expression according to environmental conditions. Finally, the intracellular proteome was analyzed, under the same conditions used in the secretomic analysis, and the protein product of the highly-transcribed gene *mnp3* was detected. Therefore, it was concluded that the absence of MnP3 from the secretome of the *P. ostreatus* lignocellulose cultures was related to impaired secretion.

Keywords: *Pleurotus ostreatus*, manganese peroxidases, versatile peroxidases, secretome analysis, quantitative PCR, differential expression

*Corresponding author. Phone: +34 918373112; E-mail: ATMartinez@cib.csic.es

1. Introduction

Pleurotus ostreatus, the oyster mushroom, is the second in importance edible fungus worldwide (Sánchez, 2010). From an ecophysiological point of view, *Pleurotus* species belong to the group of fungi causing the so-called white rot of wood and other lignocellulosic materials, due to their ability to degrade the recalcitrant lignin polymer that protects polysaccharides in vascular plants (Ruiz-Dueñas and Martínez, 2009). Among these fungi, *Pleurotus* species are of biotechnological interest because they degrade lignin selectively (*i.e.*, with a limited attack on cellulose) when growing on cereal straw and related materials (Martínez et al., 1994). Biological delignification with lignin-degrading fungi saves energy and chemicals in the manufacture of cellulose pulp from woody (Young and Akhtar, 1998) and non-woody (Camarero et al., 1998) plant feedstocks, and can be also of interest in the production of second generation bioethanol (Salvachúa et al., 2011).

Since the sequencing of white-rot *Phanerochaete chrysosporium* genome in 2004 (Martinez et al., 2004), many other basidiomycete genomes have been sequenced, up to a total of 90 by the JGI (http://www.jgi.doe.gov), as those of *Postia placenta* (first brown-rot fungal genome) (Martinez et al., 2009), *Ceriporiopsis subvermispora* (first selective white-rot fungal genome) (Fernández-Fueyo et al., 2012) and *P. ostreatus*. According to the genomic data, ligninolytic peroxidases - including lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) - are exclusive of lignin-degrading white-rot basidiomycetes, being absent from polysaccharide-degrading brown-rot basidiomycetes (Floudas et al., 2012). The above distribution of ligninolytic peroxidase genes in basidiomycete genomes confirms its central role in lignin biodegradation. *In vitro* degradation of lignin was first reported for LiP (Tien and Kirk, 1983; Hammel et al., 1993) and recently for VP (Fernández-Fueyo et al., 2014), being ligninolytic enzymes largely investigated because of their biotechnological interest (Martínez et al., 2009).

The existence of multiple isoforms (isoenzymes) is a well-known phenomenon among degradative enzymes secreted by fungi, already described years ago for ligninolytic peroxidases (Farrell et al., 1989; Glumoff et al., 1990). However, the biochemical and operational differences between isoenzymes remain largely unknown. The availability of genomes provides evidence on the large and widespread duplication of peroxidase genes in white-rot basidiomycetes (Martinez et al., 2004; Floudas et al., 2012; Fernández-Fueyo et al., 2012; Ruiz-Dueñas et al., 2013). Our preliminary *in silico* analysis of the *P. ostreatus* genome (Ruiz-Dueñas et al., 2011) indicated the presence of nine genes encoding five MnP and four VP isoenzymes, and the absence of genes encoding LiP and generic peroxidases (a fourth non-ligninolytic peroxidase family at the class II of the superfamily of plant-fungal-prokaryotic peroxidases) (Ruiz-Dueñas and Martínez, 2010). The heterologous expression of these nine genes showed the existence of three VPs and six MnPs in *P. ostreatus*, including an unusual MnP that had been *in silico* classified as a VP, with significant differences in their pH and temperature stabilities suggesting environmental regulation (Fernández-Fueyo et al., 2014).

Media composition and growth conditions strongly affect the production of ligninolytic enzymes and the extent of lignin degradation, e.g. no ligninolytic peroxidase activity is produced by *Pleurotus* species in the synthetic medium used to produce these enzymes in *P. chrysosporium*, while activity was found in peptone and lignocellulose cultures (Martínez et al., 1996). Transcriptional regulation of *vp* and *mnp* genes has been investigated in *Pleurotus* species (Ruiz-Dueñas et al., 1999; Cohen et al., 2001). Differential expression is also characteristic among the members of a gene family, including ligninolytic peroxidase isoenzymes (Salame et al., 2010; MacDonald et al., 2011; Wymelenberg et al., 2011). Recently, the effect of natural (lignocellulosic) *vs* simple (glucose) C sources has been addressed in different transcriptomic studies on sequenced wood-rotting basidiomycetes (Martinez et al., 2009; Sato et al., 2009; Wymelenberg et al., 2011; Fernández-Fueyo et al., 2012). Reverse transcription followed by quantitative PCR (RT-qPCR)

represents the most powerful technology to quantitatively amplify trace amounts of mRNA (Heid et al., 1996; Pfaffl, 2004). Moreover, RT-qPCR is considered the gold standard for measuring gene expression (Qin et al., 2006) because of its high sensitivity and specificity, robust reproducibility, and wide dynamic range (Pfaffl and Hageleit, 2001). However, this technique requires the careful selection and validation of reference genes (internal standards), which are processed in parallel with the target gene (Ling and Salvaterra, 2011). Moreover, it is critical to determine the amplification efficiency (Pfaffl, 2001; Ramakers et al., 2003), which is used in mathematical models for the accurate estimation of the expression levels.

In the present study, we analyze the ligninolytic peroxidases secreted by *P. ostreatus* when grown in a culture medium with lignocellulose as the sole carbon (and nitrogen) source, by activity estimation and isoenzyme identification by nanoflow liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS) of a whole secretome hydrolyzate. Then, we designed specific primers for each of the corresponding transcripts, and used RT-qPCR to quantify the differential expression of the nine peroxidase genes when the cultures were transferred to extreme pH (3 and 8) and temperature (10 ºC and 37 ºC) conditions, compared with those maintained at optimal conditions (25 ºC and pH 5.5), using two reference genes that were validated for the present qPCR experiments. Finally, an intracellular proteomic study was performed to explain some differences between the results from the previous transcriptomic and secretomic analyses.

2. Material and methods

2.1. P. ostreatus *strain and genome*

Monokaryotic *P. ostreatus* PC9 (CECT-20311) was used in this study. This strain is a protoclone obtained by dedikaryotization from the commercial dikaryon N001 (CECT-20600) (Larraya et al., 1999). The fungus was maintained in 2% malt extract agar.

The genomic sequence of *P. ostreatus* PC9 is available at the JGI website (http://genome.jgi-psf.org/PleosPC9_1), together with that of the second monokaryon PC15, after sequencing in a project coordinated by A.G. Pisabarro. The manual annotation of nine ligninolytic peroxidase gene models in the PC9 genome, including intron positions and N/C termini, has been already described (Ruiz-Dueñas et al., 2011).

In addition, 1 kb promoter fragment from each of the nine *P. ostreatus* (PC9) ligninolytic peroxidase genes was manually analyzed looking for the presence of conserved regulatory and other promoter elements (Janusz et al., 2013) (see Supplemental Information section S1, for the specific sequences searched).

2.2. Fungal growth in lignocellulose medium

Lignocellulose cultures were carried out at 25 ºC on 5 g of a mixture of milled wheat straw and small poplar chips (particle size < 4 mm, ratio 1:1) soaked with 35 mL of distilled water (unadjusted pH 5.5) in glass flasks at a surface to volume ratio of 1 cm^{-1} (hereinafter referred as standard conditions). Inocula consisted of 15 mL of homogenized actively growing mycelium from liquid cultures grown at 200 rpm in the dark in M7GY, comprising (per liter) 2 g ammonium tartrate, 0.5 g $MgSO_4 \cdot 7\ H_20$, 1 g KH_2PO_4, 0.5 g KCl, 10 g glucose, and 1 ml element's trace solution (0.1 g $Na_2B_4O_7 \cdot H_2O$, 0.07 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.01 g $MnSO_4 \cdot 4\ H_2O$, 0.05 g $FeSO_4 \cdot 7\ H_2O$, 0.01 g $(NH_4)_6Mo_7O_2 \cdot 4\ H_2O$ per liter) (Castanera et al., 2012).

To study the expression of the nine ligninolytic peroxidase genes when temperature turned to be nearly limiting for fungal growth, twenty-four 7-day-old flask cultures were transferred to 10 ºC or 37 ºC (twelve flasks per condition) and incubated for additional 24 h to study the effect of

temperature change on the transcript levels along the time (twelve additional flasks were maintained at 25 ºC and used as reference). In a similar way, other twenty-four 7-day-old cultures were adjusted to pH 3 or 8 (twelve flasks per condition) by adding 0.2 M Brighton&Robin buffer pH 2 or 9, respectively, and incubated for 24 h to study the effect of extreme pH on the transcription levels (the twelve flasks maintained at 25 ºC, without pH adjustment, were used as reference). In both cases, three replicate flasks were sampled after 1, 6, 12 and 24 h, combined and analyzed as described below.

2.3. Enzymatic activities

For enzymatic activity measurements, three technical replicates (1 mL each) were collected from each biological replicate and centrifuged at 15,000 rpm for 5 min at 4 °C to remove debris. Peroxidase activity was determined using a Biomate5 (Thermo Scientific) spectrophotometer for following the oxidation of 3 mM Mn^{2+}, 10 µM Reactive Black 5 (RB5) and 5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 0.1 M sodium tartrate, at different pH values, in the presence/absence of 0.1 mM H_2O_2. Mn^{2+} oxidation was followed at pH 5 for the formation of Mn^{3+}.tartrate complex (ε_{238} 6.5 mM^{-1} cm^{-1}). RB5 and ABTS oxidation were assayed at pH 3.5 by monitoring RB5 disappearance (ε_{598} 30 mM^{-1} cm^{-1}) and formation of ABTS cation radical (ε_{436} 29.3 mM^{-1} cm^{-1}), respectively. Reactions were at 25 °C being initiated by the addition of H_2O_2. One enzymatic activity unit was defined as the amount of enzyme that oxidizes 1 µmol of substrate in 1 min.

2.4. Analysis of P. ostreatus *extracellular and intracellular proteomes*

For secretomic analysis, total extracellular proteins in the filtrate of a *P. ostreatus* culture grown in lignocellulose medium for seven days under standard conditions were concentrated, dialyzed against 10 mM sodium acetate (pH 4.3), and centrifuged. The soluble proteins were concentrated and impurities removed by a short polyacrylamide gel electrophoresis (PAGE) run, followed by Colloidal Blue Kit (Invitrogen) staining. The total protein band was cut and destained using 50 mM ammonium bicarbonate in 50% acetonitrile (ACN), reduced with 10 mM dithiothreitol for 30 min at 56 ºC, alkylated with 55 mM iodoacetamide in obscurity for 30 min (24 ºC) and digested with 12.5 $ng.\mu l^{-1}$ trypsin in 50 mm ammonium bicarbonate, overnight at 30°C. Peptides were extracted at 37°C using 100% ACN and them 0.5% trifluoroacetic acid, dried, cleaned using ZipTip with 0.6 µl C18 resin (Millipore), and reconstituted in 5 µl of 0.1% formic acid in 2% ACN.

The tryptic peptides were analyzed in an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to a nanoEasy high-performance liquid chromatography (HPLC) equipment (Proxeon). Peptides were first trapped onto a C18-A1 ASY-Column 2 cm precolumn (Thermo Scientific), and then eluted onto a Biosphere C18 column (75 µm inner diameter, 15 cm long and 3 µm particle size) (NanoSeparations) using a 130 min gradient from 0-45% buffer-B (buffer-A: 0.1% formic acid in 2% ACN; buffer B: 0.1% formic acid in pure ACN) at a flow rate of 250 $nl.min^{-1}$. Mass spectra were acquired in the positive ion mode and data dependent manner selecting the 20 most intense ions for fragmentation using CID (collision induced dissociation). Full scan MS spectra (*m/z* 300-1600) were acquired in the Orbitrap with a target value of 1,000,000 at a resolution of 30,000 (at *m/z* 400) and MS2 spectra were acquired in the linear ion trap with a target value of 10,000 and normalized collision energy of 35%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 s.

Acquired spectra were searched against the *P. ostreatus* PC9 genomic database from JGI (PleosPC9_1_GeneModels_Filteredmodels2_aa) using Sequest search engine through Proteome Discoverer (version 1.4). As for the search parameters, precursor and fragment mass tolerance were set to 10 ppm and 0.8 Da, respectively. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was set as a dynamic modification. Two missed cleavages were allowed. Identified peptides were validated using Percolator algorithm with a q-value threshold of 0.01.

The intracellular proteome from the *P. ostreatus* lignocellulose culture under standard conditions was also analyzed. With this purpose, the mycelium was harvested, ground in a sterile mortar in the presence of liquid N_2, and resuspended in 0.1 M TRIS, pH 7.0. The suspension was homogenized for 10 min and centrifuged at 15,000 rpm for 15 min. The supernatant was processed as described above for the extracellular secretome.

2.5. Nucleic acid extraction, reverse transcription, primer design and real-time qPCR

The mycelia from 7-day-old *P. ostreatus* lignocellulose cultures, maintained at standard (25 ºC, pH 5.5) and extreme temperature (10 ºC and 37 ºC) and pH (pH 3 and pH 8) conditions for 1, 6, 12 or 24 h, were harvested, frozen and ground in a sterile mortar with liquid N_2. Total RNA was extracted from ~200 mg of deep frozen tissue using Fungal RNA E.Z.N.A Kit (Omega Bio-Tek, Norcross, GA) and its integrity estimated by denaturing electrophoresis on 1% (w/v) agarose gels. Nucleic acid concentrations were measured using a Nanodrop™ 2000 (Thermo Scientific, Wilmington, DE), and the purity of the total RNA was estimated by the 260/280 nm absorbance ratio. Samples were DNAse treated using 1 U of RQ1 DNase (Promega, Madison, WI) per µg of RNA.

One µg of total RNA was reverse-transcribed into cDNA in a 20 µl volume using the iScript cDNA Synthesis kit (Bio-Rad, Alcobendas, Spain). A set of specific primers was designed for amplification of the transcripts from the nine ligninolytic peroxidase genes identified in the genome (**Table 1**). Primers corresponding to the reference gene panel were designed using the filtered model transcripts sequences of PC9 (http://www.jgi.doe.gov) and the PrimerQuest℠ tool (Integrated DNA Technologies, Madrid, Spain). PCR products were purified and sequenced to confirm primer specificity.

RT-qPCR experiments were performed using a CFX96 (Bio-Rad) thermal cycler. SYBR green fluorescent dye was used to detect product amplification. Each reaction was set to a final volume of 20 µl and contained 1X IQ SYBR green Supermix from Bio-Rad, 300 nM forward and reverse primers, and 1 µl of 1:25 dilution of RT product in nuclease-free water. The amplification program was the following: 5 min at 95 °C, 40 cycles of 15 s at 95 °C and 30 s at 63 °C, followed by 1 min at 95 ºC, and a final melting curve with increments of 0.5 °C every 5 s in a linear gradient of 78-95 °C. High temperature fluorescence acquisition (78 ºC) was performed to eliminate the impact of PCR artifacts in cDNA quantification, whose absence was confirmed by melting-curve analysis. Baseline correction and crossing-point (Cp) acquisition were performed using the Bio-Rad CFXManager. The reactions were performed in triplicate in 96-well microtiter plates, and no-template controls were included for each master mix (one for each primer set). The amplification efficiencies were sample-estimated by linear regression from a Window-of-Linearity set in the exponential phase of the fluorescence history, which was plotted in log scale using the LinReg tool (Ramakers et al., 2003).

2.6. Selection of reference genes, quantification of RT-qPCR data, and statistical analyses

Taking advantage from previous studies (Castanera et al., 2012; 2013) ten genes of different functional classes were selected as reference candidates on the basis of their expression stability in *P. ostreatus*. The expression of the ten genes was evaluated in five samples corresponding to our experimental conditions. GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms were applied to rank the ten candidates according to their expression stability, and a reference index consisting of the geometric mean of the best-performing candidates was used for RT-qPCR data normalization.

Then, the transcription level of each peroxidase gene was expressed using two approaches: **i)** Relative quantities (RQ) in comparison to a stable reference index, and **ii)** Expression relative to a paired calibrator sample. Data pre-processing was performed using Genex software (MultiD Analyses, Göteborg, Sweden; http://www.multid.se) and included, among others, efficiency calibration, reference gene normalization, calculation of RQs, and calculation of the level of expression of a sample relative to its paired calibrator condition (e.g. gene expression after 1 h at 10 ºC *vs* 1 h at 25 ºC) using the delta-delta Ct method of Livak and Schmittgen (2001) (eqs. 1-4, respectively).

$$Cp_E = Cp_U \frac{\log(1+E)}{\log 2} \qquad \text{(eq. 1)}$$

$$\Delta Cp_E = Cp_{GOI} - \frac{1}{n}\sum_{i=1}^{n} Cp_{RGi} \qquad \text{(eq. 2)}$$

$$RQ = 2^{-\Delta Cp_E} \qquad \text{(eq. 3)}$$

$$Expression = 2^{-(\Delta\Delta Cp_E)} \qquad \text{(eq. 4)}$$

In the above equations, CP_E represents the efficiency corrected Cp. CP_U is the uncorrected Cp, and E is the amplification efficiency; ΔCp_E is the level of expression of a gene in comparison to the reference index (arbitrary units); Cp_{GOI} is the efficiency-corrected Cp of the gene of interest (peroxidases) and Cp_{RGi} is the efficiency-corrected Cp of each reference gene. RQ is the equivalent linear form of ΔCp_E.

For multivariate analysis, heatmap and hierarchical clustering was performed using the Ward´s algorithm and Euclidean distance available in Genex software. For this purpose, RQs were log2 transformed and autoscaled to classify genes according to their expression profiles without taking into account the amplitude of the changes (Stahlberg et al., 2008; Bergkvist et al., 2010). Finally, the REST random pairwise reallocation test was used to reveal statistical differences between the expression of each peroxidase gene under the different temperature and pH treatments in comparison to its paired control sample (corresponding to 1, 6, 12 or 24 h under standard conditions).

2.7. VP1 purification from lignocellulose cultures and comparison with recombinant VP1

The supernatants of standard lignocellulose cultures were concentrated (Amicon 10-kDa-cut-off) and dialysed against 20 mM sodium acetate, pH 4.3, with 1 mM $CaCl_2$. Insoluble material was eliminated (15,000 rpm for 30 min) and the solution further dialysed (10 mM sodium tartrate, pH 5.5, with 1 mM $CaCl_2$). The most abundant peroxidase was purified using an ÄKTA HPLC system (GE Healthcare), in three consecutive steps. The first separation was performed on a HiTrap Q Fast Flow 5-mL cartridge at a flow rate of 1 $mL.min^{-1}$. After 12 mL, the retained proteins were eluted with a 0-35% NaCl gradient in 50 mL, followed by 50-100% gradient in 10 mL and 100% NaCl in 15 mL. Peroxidase activity was followed by Mn^{2+}, RB5 and ABTS oxidation in the presence of

H_2O_2, as described above, and the appropriate fractions were pooled, concentrated and dialyzed against 10 mM sodium tartrate (pH 5). Then, size-exclusion chromatography in a Superdex-75 HR 10/30 column with 10 mM sodium tartrate (pH 5), containing 100 mM NaCl, at 0.2 mL.min^{-1}, was performed. Appropriate fractions were processed as described above, and loaded into a Mono-Q high-resolution 5/5 column at a flow rate of 0.8 mL.min^{-1}, using a 0-30% NaCl gradient in 40 mL, followed by 100% NaCl in 10 mL. The purified peroxidase was dialyzed as above, and its homogeneity confirmed by sodium dodecyl sulfate (SDS)-PAGE in 12% gels stained with Coomassie brilliant blue R-250 (Sigma). The N-terminal sequence was analyzed by sequential Edman degradation in a Procise 494 protein sequencer (PerkinElmer).

For comparison with the enzyme purified from lignocellulosic cultures, the gene encoding VP1 from the *P. ostreatus* (PC9) genome (model 137757) was synthesized (by ATG Biosynthetic), cloned in pET23a (Novagen) and expressed in *E. coli* BL21 (DE3) pLysS. Cells were grown for 3 h in Terrific Broth, induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for 4 h. The apoenzyme accumulated in inclusion bodies and was solubilized using 8 M urea. *In vitro* refolding was performed using 0.16 M urea, 5 mM Ca^{2+}, 20 μM hemin, 0.5 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.1 mg.mL protein, at pH 9.5 (Pérez-Boada et al., 2002). Active enzyme was purified by Resource-Q chromatography using a 0-300 mM NaCl gradient (2 mL.min^{-1}, 20 min) in 10 mM sodium tartrate, pH 5.5, containing 1 mM $CaCl_2$.

Oxidation of Mn^{2+}, RB5 and ABTS by the purified VP1 from *P. ostreatus* lignocelulose cultures, and the recombinant VP1 expressed in *E. coli*, was investigated as described in section 2.3 (using ~0.01 μM enzyme). Ten mM veratryl alcohol (VA) and 2.5 mM 2,6-dimethoxyphenol (DMP) oxidation were followed under the same conditions, but using pH 3 tartrate for veratraldehyde (ε_{310} 9.3 mM^{-1} cm^{-1}) formation, and pH 3.5 tartrate for dimeric coerulignone (ε_{469} 55 mM^{-1} cm^{-1}) formation, respectively.

3. Results

3.1 Analysis of secreted ligninolytic peroxidase isoenzymes by nLC-MS/MS

The secretome of *P. ostreatus* grown in lignocellulose medium under standard conditions was analyzed by nLC-MS/MS of total tryptic peptides to identify the ligninolytic peroxidase isoenzymes produced and secreted. The VP and MnP isoenzymes identified from the presence of unique peptides (at least 2 per protein and sample), corresponding to the isoenzyme sequences in the available genome, are shown in **Table 2** (and Supplemental **Table S1**). In this way, VP1, VP2, MnP2 and MnP6 were identified, with VP1 appearing as the most abundant isoenzyme in the lignocellulose medium (highest PSM number). No MnP1 and MnP4 peptides were detected, and one MnP3, MnP5 and VP3 peptide was found, but the isoenzyme presence could not be confirmed by a second unique peptide.

3.2 Analysis of the promoter region of the nine P. ostreatus *ligninolytic peroxidase genes*

The 5´-upstream sequence of the ligninolytic peroxidase genes in the *P. ostreatus* (PC9) genome contains different responsive elements such as SP-1, AP-1, AP-2 and GATA, together with putative heat-shock elements (HSE), metal-response elements (MRE), xenobiotic-response elements (XRE) and cAMP response elements (CRE), which may be implicated in regulation of their expression by environmental conditions (a detailed description of the promoter region in the nine peroxidase genes is included in a Supplemental section, and summarized in **Table S2**). Regarding HSEs, they are the most extended elements, being present in seven of the nine promoters (HSEs absent in *vp3*

and *mnp1*). Concerning MREs, *mnp6, vp1,* and *vp3* were present only one, while *vp2* contains two elements. Finally, the presence of XREs is limited to *vp2* and *mnp3*.

3.3. Identification and validation of reference genes for qPCR analysis

The GeNorm algorithm identified *Actin1* and *Pep* as the most stable genes along all the conditions assayed, displaying an expression stability value (M-value) of 0.45. NormFinder ranked *Actin1* as the most stable gene, showing a standard deviation of 0.23 cycles. The accumulated standard deviations of increasing number of reference genes were analyzed to determine the optimal number of genes to be used (Supplemental **Figs. S1** and **S2**). As a consequence of this analysis, *Actin1* and *Pep* were selected as reference index for data normalization, displaying an accumulated standard deviation of 0.21 cycles (**Fig. S2**).

A dramatic co-downregulation in the expression of every reference gene was observed in the pH 3 samples (**Fig. S1**). This fact entailed the following implications: **i)** due to the unstable expression of the reference index at this condition, the RQ approach (eq 3) could not be quantitatively compared with the others, although the classification of genes according to their relative expression changes was unaffected by this issue; and **ii)** the expression of pH 3 samples was normalized using total RNA as internal standard, instead of reference genes, to avoid this limitation in the delta-delta Ct approach (eq 4).

3.4. Differential transcription of peroxidase genes: Exploratory analysis of the expression profiles

For analyzing the transcriptional regulation of ligninolytic peroxidases by temperature and pH, *P. ostreatus* was first grown in lignocellulose medium under standard conditions. Then, we monitored by RT-q-PCR using specific primers (**Table 1**) the time-course (1-24 h) of transcriptional changes of the nine peroxidase genes after modifying the temperature (from 25 ºC to 10 ºC and 37 ºC) and pH (from pH 5.5 to pH 3 and pH 8). Two approaches were used for this purpose: **i)** an exploratory analysis using multivariate methods to uncover similar expression trends among genes and samples; and **ii)** a classical expression analysis using the delta-delta Ct method under a paired experimental design (treatment *vs* control) where controls are the different sampling times of *P. ostreatus* growing under the standard conditions.

The transcription of peroxidase genes was firstly analyzed under standard conditions in four sampling times along 24 h. The aim was to obtain an expression map of *P. ostreatus* ligninolytic peroxidase family under lignin degradation conditions. The analysis resulted in an expression heatmap associated with a dendrogram of hierarchical clusters (**Fig. 1A**) classifying genes and samples according to their similar profiles. Three groups of genes were clearly differentiated in that sense, displaying high (group I: *mnp3* and *vp1*), medium (group II: *mnp2*, *vp2*, *mnp6*, *vp3* and *mnp1*) or low expression (group III: *mnp4* and *mnp5*) in the lignocellulose medium. The expression trends of group II genes fluctuated along the 24 h, making the alternate sampling times cluster together.

3.5. Effect of temperature and pH conditions on the expression of ligninolytic peroxidase genes

A similar analysis to that performed for the standard growth conditions, was performed in all samples after the temperature and pH modifications to uncover a putative coregulation of the ligninolytic peroxidase genes. The hierarchical clustering dendrogram obtained from the averaged expression during the 24 h period shows the gene expression trends under the modified temperature and pH conditions (**Fig. 1B**, bottom). Interestingly, the expression groups obtained under standard conditions (**Fig. 1A**) were maintained with the only exception of *vp1*, which was re-classified as

member of group II (**Fig. 1B**, bottom). The expression profiles along the 24 h sampling period (**Fig. 1B**, top) allowed us to understand the rationale of the above gene classification. *mnp3* expression (group I) was higher than the reference (mean expression of the two reference genes) after every treatment. Genes of group II displayed a relative expression greater than the reference in pH 3 treatment (and for *vp1* and *mnp2* at 25 ºC). Nevertheless, the expression in pH 8 and temperature treatments remained lower than the reference, and displayed similar trends among the members of the two sub-groups. Finally, *mnp4* and *mnp5* (group III) displayed lower expression than the reference after every treatment, although it was higher after pH treatment in comparison to temperature treatment.

Then, the individual effect of the different environmental conditions and times on the ligninolytic peroxidase expression were analyzed by referring the transcriptional levels observed at different times (1-24 h) after changing the culture temperature (from 25 ºC to 10 ºC and 37 ºC) and pH (from pH 5.5 to pH 3 and pH 8) to those obtained under the standard conditions (**Fig. 2**). Changing the temperature and pH led to a transcriptional downregulation of most ligninolytic peroxidase genes genes, with a few significant exceptions discussed below.

Concerning temperature effects, in those cultures placed at 10 ºC (**Fig. 2A**) the expression decreased gradually along the time, becoming statistically significant in every peroxidase gene after 24 h. The expression change was more patent for the three *vp* genes, which attained -5.3 to -5.9 fold decreases (with respect to the 25 ºC control). By contrast, the expression of *mnp3* was the less altered, fluctuating between a slight upregulation during the first 12 h and a final downregulation of -1.7 fold after 24 h. Cultures exposed to 37 ºC (**Fig. 2B**) showed an interesting transcriptional profile, as they displayed wide and alternate up/down regulations along the four sampling times in genes of groups II and III. Again, *mnp3* showed a different profile, its expression being similar (at 1, 12 and 24 h) or greater than the control (2.0 fold increase after 6 h).

The pH 3 treatment (**Fig. 2C**) led to a general downregulation of the ligninolytic peroxidase genes. This fact could be observed just 1 h after the pH modification (reaching -11.4 fold decrease) and was maintained over the 24 h period (up to -10.9 fold decreases). Regarding pH 8 (**Fig. 2D**), most genes showed a similar downregulation, which increased with time. However, *mnp4* and *mnp5* expression (group III) was significantly upregulated by pH 8. Group II genes displayed a strong repression from the 6 h period, with the highest alkaline downregulation corresponding to genes *mnp2* (-6.6 fold) and *vp1* (-7.0 fold). Finally, *mnp3* (group I) showed a gradual repression along the time, reaching a -3.5 fold decrease after 24 h at pH 8.

3.6. Contribution of each isoenzyme to the global peroxidase expression

Changes in the expression of the nine *P. ostreatus* ligninolytic peroxidase genes under the different temperature and pH conditions were further analyzed by considering the percentual contribution of each of them to the total peroxidase gene expression (as averaged values for a 24-h period) under each condition (**Fig. 3**). The predominant expression of gene *mnp3* over the eight other peroxidase genes, was already noticed when analyzing the peroxidase transcriptional levels under standard conditions. However, its contribution was even higher in the cultures grown under more extreme conditions: pH 3 (25 ºC) > pH 8 (25 ºC) > 10 ºC (pH 5.5) > 37 ºC (pH 5.5) > 25 ºC (pH 5.5) (**Fig. 3A**). Under the three last conditions, which correspond to the lowest *mnp3* relative transcription levels, gene *vp1* was significantly transcribed with around 20% contribution to the total peroxidase expression, while the transcription levels of the other seven peroxidase genes were low (<5% of the total).

To better analyze the contribution of the latter genes, their transcription levels were also shown as percentages of total expression without gene *mnp3* (**Fig. 3B**). In this way, two different expression profiles could be identified under the modified temperature and pH conditions. On the

one hand, at the standard and temperature modified conditions, *vp* genes represented the majority of the total ligninolytic peroxidase expression (*mnp3* excluded): 72% at 25 ºC (with *vp1* representing 52% of the total transcription), 65% at 37 ºC and 63% at 10 ºC. On the other hand, changes in the pH led to a predominant expression of the *mnp* genes, representing 71% and 75% of the total ligninolytic peroxidase transcripts at pH 3 and pH 8, respectively (*mnp3* excluded).

For easier identification of the relative expression patterns, the above comparisons were performed on the averaged data for the 24-h sampling period after changing the temperature and pH conditions. However, additional information on the time-course of each gene expression was obtained when the four sampling times were analyzed separately. Gene *mnp3* remained as the most expressed gene at the four sampling times under each condition (Supplemental **Fig. S3**), although the relative transcriptional levels at 10 ºC progressively increased from 1 h to 24 h, and those at 37 ºC attained the highest value at 1 h and 12 h. Concerning the other eight ligninolytic peroxidase genes (Supplemental **Fig. S4**), their relative transcriptional patterns showed significant differences with time. For example, the initial transcription (1 h) of gene *mnp2* was high at both pH 8 and pH 3 but it decreased later, being accompanied by the increased expression of genes *mnp1* (pH 3) and *mnp4/mnp5* (pH 8) that finally (12/24 h) represented over 50% of the total expression of the eight genes analyzed here.

3.7. Effect of temperature and pH on the peroxidase activity in the lignocellulose cultures

In lignocellulose cultures of *P. ostreatus,* the highest extracellular ligninolytic peroxidase activities, measured with Mn^{2+}, ABTS or RB5 in the presence of H_2O_2 (by subtracting the ABTS oxidation observed in the absence of H_2O_2) were generally observed under the standard conditions (**Fig. 4** bars 1-4) in agreement with the transcriptomic results. RB5 oxidation was the most affected by the temperature and pH modification. In the 10 ºC treatments, a sharp decrease of Mn^{2+} and RB5 oxidation activity was observed after 1 h incubation (and after 6 h incubation in the case of ABTS). Mn-oxidation was the most affected activity, with a 2.6 fold reduction. There was a slightly recovery in RB5 and ABTS oxidation after 6 and 24 h, respectively (although the activities were still 2.3 and 1.5 fold lower, respectively). In the 37 ºC treatments, no strong changes of the ABTS and Mn^{2+} oxidation activities were initially produced, being similar or even higher (ABTS, 1 h) than found at 25 ºC. However, a decrease of ABTS and RB5 oxidation was produced after 12 h, while Mn^{2+} oxidation was less affected.

pH 3 turned out to be a too severe condition resulting in a complete lack of peroxidase activity. Finally, at pH 8 the ligninolytic peroxidase activities were less affected than at pH 3 but strong decreases were detected, especially immediately after the pH adjustment (8-fold reduction for Mn^{2+}, 4-fold decrease for ABTS and 11-fold reduction for RB5 oxidation, in the 1 h sample). Then, some recovery of Mn^{2+} and ABTS oxidation activities were produced (attaining 4 and 2 fold lower levels, respectively, than found at 25 ºC) but the RB5 oxidation activity maintained similar low levels during the whole 24-h period.

3.8. Analysis of intracellular ligninolytic peroxidases isoenzymes by nLC-MS/MS

As already described, MnP3 could not be confirmed in the *P. ostreatus* secretome due to detection of a single unique peptide (**Table 2**). However, *mnp3* was the most transcribed gene in the *P. ostreatus* cultures (**Fig. 1B**). To explain this apparent contradiction, mycelium of *P. ostreatus* grown on the lignocellulose medium for seven days at 25 ºC was harvested, homogenized, and the supernatant analyzed by nLC-MS/MS to identify intracellular ligninolytic peroxidase isoenzymes.

As in the secretomic analysis, the identification of intracellular proteins was based on the detection of at least two unique peptides in each sample (**Tables 2** and **S1**). VP1, VP2, MnP2, VP3,

MnP6 and MnP3 were identified in decreasing order of importance (according to PSM values). VP1 was found to be the most abundant intracellular ligninolytic peroxidase isoenzyme, as in the case of the extracellular analysis, while MnP1, MnP4 and MnP5 were not detected. More importantly, translation of the *mnp3* transcripts and intracellular presence of the MnP3 isoenzyme was confirmed.

3.9. VP1 purification

The transcriptomic, enzymatic activity, and extracellular/intracellular proteomic studies were completed by purification of the main peroxidase isoenzyme present in the *P. ostreatus* lignocellulose cultures (after 7-day growth under standard conditions). The purification process included three chromatographic steps, and concluded with Mono Q chromatography (**Fig. 5**) yielding an electrophoretically homogeneous protein, as shown by SDS-PAGE chromatography (**Fig. 5** inset). The visible spectrum of the purified enzyme showed the main Soret band (at 406 nm) and the small heme bands 502 and 640 nm, confirming that it had properly incorporated the cofactor.

The peroxidase purified from lignocellulose cultures had activity on ABTS, DMP, Mn^{2+}, VA and RB5 (**Table 3**) revealing that it corresponds to one of the three *P. ostreatus* VPs. With the aim of identifying the isoenzyme purified, the following N-terminal sequence was determined: ATXADGRTTANAXXVLFPILDDIQ (X correspond to three conserved cysteine residues). A comparison with the predicted mature N-terminal sequences of the nine ligninolytic peroxidases from the *P. ostreatus* genome (**Table S3**) showed 100% sequence identity with both VP1 and MnP3. However, its ability to oxidize VA and RB5 excluded the MnP isoenzyme, and revealed that the main ligninolytic peroxidase produced in lignocellulose cultures is isoenzyme VP1.

Finally, gene *vp1* was expressed in *E. coli*, and the catalytic activities of the recombinant enzyme compared with those of the wild enzyme from the fungal culture (**Table 3**). Differences in oxidation of substrates other than Mn^{2+}, being 2-3 fold lower for the recombinant VP1, suggest some involvement of the glycosidic moiety in VP activity. However, the similar substrate specificity range, including Mn^{2+} and high and low redox-potential aromatic compounds as substrates, confirmed the identification of the main peroxidase present in lignocellulose cultures as *P. ostreatus* isoenzyme VP1.

4. Discussion

4.1. Ligninolytic peroxidases in P. ostreatus

P. ostreatus has nine ligninolytic peroxidase genes as shown by its sequenced genome (Ruiz-Dueñas et al., 2011). According to their catalytic properties after heterologous expression, they have been classified as six MnPs and three VPs, and a definitive isoenzyme numbering has been assigned (Fernández-Fueyo et al., 2014) in agreement with that shown at the JGI portal after manual annotation (http://genome.jgi-psf.org/PleosPC9_1).

These peroxidases play an active role in lignin degradation by *Pleurotus* species as shown in different studies (Camarero et al., 1998; Salame et al., 2013). Recently, Fernández-Fueyo et al.et al.et al.et al. (Fernández-Fueyo et al., 2014) demonstrated that *P. ostreatus* VP degrades non-phenolic lignin model dimers and depolymerizes synthetic lignin as previously reported for *P. chrysosporium* LiP (Hammel et al., 1993), and suggested that VP in some Agaricales plays the same role of LiP in many Polyporales. The same authors (Fernández-Fueyo et al., 2014) have shown that *P. ostreatus* MnPs represent a subfamily of short MnPs different from those found in *P. chrysosporium* (long MnP subfamily) characterized by broader substrate specificity including Mn-

independent oxidation of phenols and other compounds, which would enable direct oxidation of lignin-related compounds.

The high number of genes encoding VP and MnP isoenzymes in the *P. ostreatus* genome points to the potential redundancy and/or diversity in their properties, and suggest differential regulation as discussed below.

4.2. Differential expression of ligninolytic peroxidase isoenzymes in lignocellulose medium

Differential expression of some peroxidase genes depending of environmental and nutritional conditions had already been described in *Pleurotus* cultures grown on different media (Ruiz-Dueñas et al., 1999; Cohen et al., 2002a; Salame et al., 2010). To study the differential expression (and environmental regulation) of the whole array of ligninolytic peroxidase genes identified in the *P. ostreatus* genome we decided to use a lignocellulose medium. Sawdust and cereal straw are the usual substrates for the commercial production of *P. ostreatus* (Sánchez, 2010), a fungus that naturally grows on deciduous trees (Phillips, 2006). Therefore, in the present study we used a combination of milled wheat straw and poplar wood chips as the only growth substrate in stationary cultures (containing a small water volume). These conditions are more similar to the fungal natural growth conditions than shaken synthetic liquid media.

To study the expression of the nine peroxidase genes in the lignocellulose medium, we determined the relative abundance of the corresponding transcripts by RT-qPCR using selected reference genes. In terms of studying the effect of different factors on transcript level, RT-qPCR has been shown to be 10^3-10^4 more sensitive than Northern blotting (Sooknanan et al., 1993). According to the pattern of expression (abundance of transcripts) at the standard growth conditions, the ligninolytic peroxidase genes of *P. ostreatus* were divided into three different groups, being *mnp3* and *vp1* the most expressed genes. This agrees with previous studies using other culture media (such as glucose-peptone medium amended with Mn^{2+}). Among them, Irie et al.et al.et al.et al. (2000) described MnP3 as the major MnP isoenzyme in *P. ostreatus*, and Salame et al.et al.et al.et al. (2012; 2013) described *vp1* (*mnp4* for these authors) and *mnp3*, together with *mnp2* (*mnp9* for these authors), as the most transcribed *P. ostreatus* PC9 genes. On the other hand, *mnp4* and *mnp5* appeared as the least expressed genes at the standard conditions used in the present study.

4.3. Environmental regulation of ligninolytic peroxidase isoenzymes by temperature and pH

Two extreme temperature and pH conditions were selected to study the environmental regulation of the nine ligninolytic peroxidase genes in *P. ostreatus*, in connection with previous studies (Fernández-Fueyo et al., 2014). Previous observations had shown that 10 ºC and 37 ºC are the most extreme temperatures, in which this fungus continues growing. On the other hand, although the highest fungal ligninolysis and activity of ligninolytic enzymes (LiP and VP) is often produced at acidic conditions (Ruiz-Dueñas and Martínez, 2009), pH 3 always results in progressive peroxidase inactivation, as shown for most of the *P. ostreatus* isoenzymes (Fernández-Fueyo et al., 2014). The same study also showed that pH 8 inactivates these peroxidases, except the most stable isoenzymes, due to loss of structural Ca^{2+} ions and formation of bis(histidyl) heme iron complexes. Therefore, to study environmental regulation, *P. ostreatus* cultures pregrown under standard conditions were transferred to 10 ºC and 37 ºC or the medium pH adjusted to pH 3 and pH 8, and the peroxidase transcriptional levels (during the subsequent 24 h) referred to those of cultures maintained under unchanged conditions.

Modifications of temperature and pH led to a downregulation of most ligninolytic peroxidase genes, although the individual genes were differentially affected. In general, the pH modifications produced more severe effects than the temperature modifications, and *vp* genes were more affected

than *mnp* genes. Among the latter, *mnp3* appears as the most transcribed ligninolytic peroxidase gene in all the conditions, and the least downregulated by temperature modification. pH 3 seems to be a too stressful condition for the fungus, with a downregulation of more than 10 folds in all genes after only 1 h incubation. On the other hand, while all *vp* genes were dramatically downregulated at pH 8, *mnp4* and *mnp5* were upregulated under these conditions. Interestingly, MnP4 has been reported as the most stable *P. ostreatus* peroxidase isoenzyme under alkaline conditions (Fernández-Fueyo et al., 2014).

Concerning temperature, all genes except *mnp3* were downregulated at 10 ºC. Conversely, at 37 ºC most of the genes were upregulated after 24 h, with the exception of *mnp2*, *mnp6* and *mnp1*. This general upregulation at 37 ºC could be related to HSEs, which are present in all the *P. ostreatus* peroxidase promoters except in those of *mnp1* and *vp3*. The existence of HSEs has been reported in the promoters of other ligninolytic peroxidase genes, such as *P. chrysosporium mnp* (Godfrey et al., 1994; Mayfield et al., 1994). No correlation between the thermal stability of the different isoenzymes (Fernández-Fueyo et al., 2014) and the expression levels of the nine ligninolytic peroxidase genes was observed under the present experimental conditions.

4.4. Activity levels, proteomes, and enzyme purification from lignocellulose cultures

In the *P. ostreatus* lignocellulose cultures under standard conditions, the maximal peroxidase activity coincided with the highest expression of ligninolytic peroxidase genes. In most of the cases, there was also a correlation between the expression and activity levels in the modified temperature and pH conditions. Regarding pH, no activity was detected for any substrate at pH 3, where the fungus could not survive and the peroxidase expression sharply decreases. On the other hand, RB5 oxidation (a specific VP reaction) was the most affected activity at pH 8, in agreement with the higher decrease of *vp* than *mnp* transcripts. In the case of temperature, the lack of activity in the cultures transferred at 10 ºC, a temperature at which these peroxidases are fully stable (Fernández-Fueyo et al., 2014) correlates with the strong decrease of all transcript levels. Thus, it seems that a constant production of the enzyme is necessary to maintain the activity. At 37 ºC, while no strong changes in Mn^{2+} oxidation were produced, there was a decrease in ABTS and RB5 oxidation activities (after 12 h) which coincided with the stronger reduction in *vp* expression.

In previous studies, only VP1 (Sarkar et al., 1997; Kamitsuji et al., 2004), VP2 (Kamitsuji et al., 2004) and MnP3 (Cohen et al., 2001; Cohen et al., 2002b) had been purified from *P. ostreatus* cultures, suggesting that they are the most abundant ligninolytic peroxidases in the different culture conditions assayed. In our case, only VP1 was purified from lignocellulose fungal cultures, highlighting the role of VPs in lignin degradation by this fungus (Fernández-Fueyo et al., 2014). By nLC-MS/MS, a highly sensitive technique, VP2, MnP2 and MnP6 were also detected, and a single unique peptide was found for MnP3, MnP5 and VP3 (while MnP4 and MnP1 were not detected). In a similar study on *P. ostreatus* secretome (from Mn^{2+}-amended glucose-peptone medium) only MnP3, VP1 and MnP2 were detected by Salame et al.et al.et al.et al. (2013) (the two latter isoenzymes respectively called VP4 and MnP9 by these authors).

The secretome results mostly agree with the transcript levels at 25 ºC except for gene *mnp3* that showed the highest transcription level, but whose protein product could not be confirmed by a second unique peptide. However, the intracellular presence of MnP3 was shown by the nLC-MS/MS analysis of the corresponding proteome, revealing a limited secretion of this isoenzyme. A similar case was reported for a *P. ostreatus* laccase *(*POXA1b) that shows the highest transcription levels in copper-supplemented cultures, but it is not a major extracellular protein due to inefficient secretion (it appears in the cellular extract) and the action of specific proteases (Palmieri et al., 2000).

Although all the ligninolytic peroxidase genes are transcribed in the *P. ostreatus* lignocellulose cultures, those peroxidases being absent from both the intracellular/extracellular secretomes (MnP4 and MnP1) correspond to the less transcribed genes at the standard growth conditions (together with gene *mnp5*). Another possible reason for the absence of these two peroxidases from the proteomes is that MnP4 has the highest number of lysine residues (20) followed by MnP1, a fact that would difficult nLC-MS/MS identification due to the high number and small size of tryptic peptides. Moreover, Salame et al.et al.et al.et al. (2013) proposed that MnP4 (called MnP7 in this study) could be anchored to the membrane, according to the prediction of a transmembrane domain that would limit its secretion.

5. Conclusions

The main ligninolytic peroxidase genes expressed in *P. ostreatus* cultures with lignocellulose as the sole carbon (and nitrogen) source were investigated by transcriptomic, secretomic and enzyme purification studies. Genes *mnp3* and *vp1* showed the highest transcription levels, but the presence of isoenzyme MnP3 in the cultures seems affected by a limited secretion under the present conditions (as shown by comparison of the intracellular and extracellular proteomes). However, the product of gene *vp1* was the most abundant protein in the secretome and the only peroxidase purified from the *P. ostreatus* lignocellulose cultures. For the rest of genes there was also a general correlation between the level of transcripts and the secretome abundance. Expression of the *P. ostreatus* six *mnp* and the three *vp* genes was differentially affected by changes in the culture temperature and pH. Although an increase of temperature (to 37 ºC) caused a transient increase of gene expression with respect to 25 ºC (6 h sample), a general downregulation was observed after 24 h at 37 ºC, as well as in nearly all the samples from the 10 ºC treatments. Concerning the effect of pH, all the ligninolytic peroxidase genes were downregulated at pH 3 with respect to the standard conditions (pH 5.5). Finally, pH 8 downregulated all the genes but *mnp4* and *mnp5*, whose upregulation by alkaline pH constitutes an intriguing finding since most ligninolytic peroxidases are inactivated over pH 7. However, it agrees with previous studies that described the unusual alkaline stability of the MnP4 isoenzyme from the *P. ostreatus* genome.

Acknowledgements

This work was supported by the PEROXICATS (KBBE-2010-4-265397; www.peroxicats.org) and INDOX (KBBE-2013-7-613549; www.indoxproject.eu) EU projects, and by the BIO2011-26694 and AGL2011-30495 projects of the Spanish Ministry of Economy and Competitiveness (MINECO). EF-F acknowledges a Junta de Ampliación de Estudios fellowship of the CSIC, co-funded by the European Social Fund, and FJR-D acknowledges a MINECO Ramón y Cajal contract.

Supporting information

Supplemental Information includes a section on the promoter region of the ligninolytic peroxidase genes, the peroxidase peptide sequences identified in the secretome of *P. ostreatus* (**Table S1**), a list of putative regulatory elements in the different promoters (**Table S2**), the N-terminal sequence identity of the peroxidase purified from lignocellulose cultures (**Table S3**), the crossing point distribution of ten candidate reference genes at different temperature and pH conditions (**Fig. S1**), the expression stability of these ten genes using GeNorm and Normfinder (**Fig. S2**), the relative expression of the nine ligninolytic peroxidase genes at different times after changing the incubation conditions (**Fig. S3**) and the same information excluding *mnp3* (**Fig. S4**).

References

Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64**,** 5245-5250.

Bergkvist, A., Rusnakova, V., Sindelka, R., Garda, J.M.A., Sjogreen, B., Lindh, D., Forootan, A., Kubista, M., 2010. Gene expression profiling - Clusters of possibilities. Methods 50**,** 323-335.

Camarero, S., Barrasa, J.M., Pelayo, M., Martínez, A.T., 1998. Evaluation of *Pleurotus* species for wheat-straw biopulping. J. Pulp Paper Sci. 24**,** 197-203.

Castanera, R., Omarini, A., Santoyo, F., Pérez, G., Pisabarro, A.G., Ramírez, L., 2013. Non-additive transcriptional profiles underlie dikaryotic superiority in *Pleurotus ostreatus* laccase activity. PLoS One 8, 9.

Castanera, R., Pérez, G., Omarini, A., Alfaro, M., Pisabarro, A.G., Faraco, V., Amore, A., Ramírez, L., 2012. Transcriptional and enzymatic profiling of *Pleurotus ostreatus* laccase genes in submerged and solid-state fermentation cultures. Appl. Environ. Microbiol. 78**,** 4037-4045.

Cohen, R., Hadar, Y., Yarden, O., 2001. Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by $Mn2^{+}$. Environ. Microbiol. 3**,** 312-322.

Cohen, R., Persky, L., Hadar, Y., 2002a. Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. Appl. Microbiol. Biotechnol. 58**,** 582-594.

Cohen, R., Persky, L., Hazan-Eitan, Z., Yarden, C., Hadar, Y., 2002b. Mn^{2+} alters peroxidase profiles and lignin degradation by the white-rot fungus *Pleurotus ostreatus* under different nutritional and growth conditions. Appl. Biochem. Biotechnol. 102**,** 415-429.

Farrell, R.L., Murtagh, K.E., Tien, M., Mozuch, M.D., Kirk, K.T., 1989. Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. 11**,** 322-328.

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., Ferreira, P., Floudas, D., Hibbett, D.S., Canessa, P., Larrondo, L., James, T.Y., Seelenfreund, D., Lobos, S., Polanco, R., Tello, M., Honda, Y., Watanabe, T., Watanabe, T., Ryu, J.S., Kubicek, C.P., Schmoll, M., Gaskell, J., Hammel, K.E., St.John, F.J., Vanden Wymelenberg, A., Sabat, G., Bondurant, S.S., Syed, K., Yadav, J., Doddapaneni, H., Subramanian, V., Lavín, J.L., Oguiza, J.A., Perez, G., Pisabarro, A.G., Ramírez, L., Santoyo, F., Master, E., Coutinho, P.M., Henrissat, B., Lombard, V., Magnuson, J.K., Kües, U., Hori, C., Igarashi, K., Samejima, M., Held, B.W., Barry, K., LaButti, K., Lapidus, A., Lindquist, E., Lucas, S., Riley, R., Salamov, A., Hoffmeister, D., Schwenk, D., Hadar, Y., Yarden, O., de Vries, R.P., Wiebenga, A., Stenlid, J., Eastwood, D.C., Grigoriev, I.V., Berka, R., Blanchette, R.A., Kersten, P., Martínez, A.T., Vicuña, R., Cullen, D., 2012. Comparative genomics of *Ceriporiopisis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc. Natl. Acad. Sci. USA 109**,** 5458-5463.

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., Martínez, M.J., Romero, A., Hammel, K.E., Medrano, F.J., Martínez, A.T., 2014. Ligninolytic peroxidase genes in the oyster mushroom genome: Heterologous expression, molecular structure, catalytic and stability properties and lignin-degrading ability. Biotechnol. Biofuels 7:2.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martínez, A.T., Otillar, R., Spatafora, J.W., Yadav, J.S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P.M., de Vries, R.P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Górecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J.A., Kallen, N., Kersten, P., Kohler, A., Kües, U., Kumar, T.K.A., Kuo, A., LaButti, K., Larrondo, L.F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D.J., Morgenstern, I., Morin, E., Murat, C., Nolan, M., Ohm, R.A., Patyshakuliyeva, A., Rokas, A., Ruiz-Dueñas, F.J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J.C., St.John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D.C., Martin, F., Cullen, D., Grigoriev, I.V., Hibbett, D.S., 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336**,** 1715-1719.

Glumoff, T., Harvey, P.J., Molinari, S., Goble, M., Frank, G., Palmer, J.M., Smit, J.D.G., Leisola, M.S.A., 1990. Lignin peroxidase from *Phanerochaete chrysosporium* - Molecular and kinetic characterization of isozymes. Eur. J. Biochem. 187**,** 515-520.

Godfrey, B.J., Akileswaran, L., Gold, M.H., 1994. A reporter gene construct for studying the regulation of manganese peroxidase gene expression. Appl. Environ. Microbiol. 60**,** 1353-1358.
Hammel, K.E., Jensen, K.A., Mozuch, M.D., Landucci, L.L., Tien, M., Pease, E.A., 1993. Ligninolysis by a purified lignin peroxidase. J. Biol. Chem. 268**,** 12274-12281.
Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Research 6**,** 986-994.
Irie, T., Honda, Y., Ha, H.-C., Watanabe, T., Kuwahara, M., 2000. Isolation of cDNA and genomic fragments encoding the major manganese peroxidase isoenzyme from the white rot basidiomycete *Pleurotus ostreatus*. J. Wood Sci. 46**,** 230-233.
Janusz, G., Kucharzyk, K.H., Pawlik, A., Staszczak, M., Paszczynski, A.J., 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. Enzyme Microb. Technol. 52**,** 1-12.
Kamitsuji, H., Honda, Y., Watanabe, T., Kuwahara, M., 2004. Production and induction of manganese peroxidase isozymes in a white-rot fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. 65**,** 287-294.
Larraya, L.M., Pérez, G., Peñas, M.M., Baars, J.J.P., Mikosch, T.S.P., Pisabarro, A.G., Ramírez, L., 1999. Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 65**,** 3413-3417.
Ling, D.J., Salvaterra, P.M., 2011. Robust RT-qPCR data normalization: Validation and selection of internal reference genes during post-experimental data analysis. PLoS One 6, 3.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25**,** 402-408.
MacDonald, J., Doering, M., Canam, T., Gong, Y.C., Guttman, D.S., Campbell, M.M., Master, E.R., 2011. Transcriptomic responses of the softwood-degrading white-rot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Appl. Environ. Microbiol. 77**,** 3211-3218.
Martínez, A.T., Camarero, S., Guillén, F., Gutiérrez, A., Muñoz, C., Varela, E., Martínez, M.J., Barrasa, J.M., Ruel, K., Pelayo, M., 1994. Progress in biopulping of non-woody materials: Chemical, enzymatic and ultrastructural aspects of wheat-straw delignification with ligninolytic fungi from the genus *Pleurotus*. FEMS Microbiol. Rev. 13**,** 265-274.
Martínez, A.T., Ruiz-Dueñas, F.J., Martínez, M.J., del Río, J.C., Gutiérrez, A., 2009. Enzymatic delignification of plant cell wall: from nature to mill. Curr. Opin. Biotechnol. 20**,** 348-357.
Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D.S., Schmoll, M., Kubicek, C.P., Ferreira, P., Ruiz-Dueñas, F.J., Martínez, A.T., Kersten, P., Hammel, K.E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., Bondurant, S.S., Larrondo, L.F., Canessa, P., Vicuña, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavín, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kues, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R., Cullen, D., 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc. Natl. Acad. Sci. USA 106**,** 1954-1959.
Martinez, D., Larrondo, L.F., Putnam, N., Gelpke, M.D., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D., Rokhsar, D., 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat. Biotechnol. 22**,** 695-700.
Martínez, M.J., Böckle, B., Camarero, S., Guillén, F., Martínez, A.T., 1996. MnP isoenzymes produced by two *Pleurotus* species in liquid culture and during wheat straw solid-state fermentation. In: Jeffries, T.W., Viikari, L. (Eds.), Enzymes for pulp and paper processing ACS, Washington, pp. 183-196.
Mayfield, M.B., Godfrey, B.J., Gold, M.H., 1994. Characterization of the *mnp2* gene encoding manganese peroxidase isozyme 2 from the basidiomycete *Phanerochaete chrysosporium*. Gene 142**,** 231-235.
Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., Sannia, G., 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 66**,** 920-924.
Pérez-Boada, M., Doyle, W.A., Ruiz-Dueñas, F.J., Martínez, M.J., Martínez, A.T., Smith, A.T., 2002. Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimisation of *in vitro* folding. Enzyme Microb. Technol. 30**,** 518-524.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 9.

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. In: Bustin, S.A. (Ed.), A-Z of quantitative PCR International University Line, La Jolla, CA, pp. 87-120.
Pfaffl, M.W., Hageleit, M., 2001. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol. Lett. 23**,** 275-282.
Phillips, R., 2006. Mushrooms. McMilan.
Qin, L.X., Beyer, R.P., Hudson, F.N., Linford, N.J., Morris, D.E., Kerr, K.F., 2006. Evaluation of methods for oligonucleotide array data via quantitative real-time PCR. BMC Bioinformatics 7.
Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters 339**,** 62-66.
Ruiz-Dueñas, F.J., Fernández, E., Martínez, M.J., Martínez, A.T., 2011. *Pleurotus ostreatus* heme peroxidases: An *in silico* analysis from the genome sequence to the enzyme molecular structure. C. R. Biol. 334**,** 795-805.
Ruiz-Dueñas, F.J., Guillén, F., Camarero, S., Pérez-Boada, M., Martínez, M.J., Martínez, A.T., 1999. Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus *Pleurotus eryngii*. Appl. Environ. Microbiol. 65**,** 4458-4463.
Ruiz-Dueñas, F.J., Lundell, T., Floudas, D., Nagy, L.G., Barrasa, J.M., Hibbett, D.S., Martínez, A.T., 2013. Lignin-degrading peroxidases in Polyporales: An evolutionary survey based on ten sequenced genomes. Mycologia 105**,** 1428-1444.
Ruiz-Dueñas, F.J., Martínez, A.T., 2009. Microbial degradation of lignin: How a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microbial Biotechnol. 2**,** 164-177.
Ruiz-Dueñas, F.J., Martínez, A.T., 2010. Structural and functional features of peroxidases with a potential as industrial biocatalysts. In: Torres, E., Ayala, M. (Eds.), Biocatalysts based on heme peroxidases Springer-Verlag, Berlin, pp. 37-59.
Salame, T.M., Knop, D., Levinson, D., Mabjeesh, S.J., Yarden, O., Hadar, Y., 2012. Release of *Pleurotus ostreatus* versatile-peroxidase from Mn2+ repression enhances anthropogenic and natural substrate degradation. PLoS One 7, 12.
Salame, T.M., Knop, D., Levinson, D., Yarden, O., Hadar, Y., 2013. Redundancy among manganese peroxidases in *Pleurotus ostreatus*. Appl. Environ. Microbiol. 79**,** 2405-2415.
Salame, T.M., Yarden, O., Hadar, Y., 2010. *Pleurotus ostreatus* manganese-dependent peroxidase silencing impairs decolourization of Orange II. Microbial Biotechnol. 3**,** 93-106.
Salvachúa, D., Prieto, A., Lopez-Abelairas, M., Lú-Chau, T., Martínez, A.T., Martínez, M.J., 2011. Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. Bioresource Technol. 102**,** 7500-7506.
Sánchez, C., 2010. Cultivation of *Pleurotus ostreatus* and other edible mushrooms. Appl. Microbiol. Biotechnol. 85**,** 1321-1337.
Sarkar, S., Martínez, A.T., Martínez, M.J., 1997. Biochemical and molecular characterization of a manganese peroxidase isoenzyme from *Pleurotus ostreatus*. Biochim. Biophys. Acta 1339**,** 23-30.
Sato, S., Feltus, F.A., Iyer, P., Tien, M., 2009. The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr. Genetics 55**,** 273-286.
Sooknanan, R., Malek, L., Wang, X.H., Siebert, T., Keating, A., 1993. Detection and direct-sequence identification of BCR-ABL messenger-RNA in Ph(+) chronic myeloid-leukemia. Experimental Hematology 21**,** 1719-1724.
Stahlberg, A., Elbing, K., Andrade-Garda, J.M., Sjogreen, B., Forootan, A., Kubista, M., 2008. Multiway real-time PCR gene expression profiling in yeast *Saccharomyces cerevisiae* reveals altered transcriptional response of ADH-genes to glucose stimuli. BMC Genomics 9, 170.
Tien, M., Kirk, T.K., 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science 221**,** 661-663.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 7.
Wymelenberg, A.V., Gaskell, J., Mozuch, M., Bondurant, S.S., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Grigoriev, I.V., Kersten, P.J., Cullen, D., 2011. Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* by plant species. Appl. Environ. Microbiol. 77**,** 4499-4507.

Young, R.A., Akhtar, M., 1998. Environmentally-friendly technologies for the pulp and paper industry. John Wiley and Sons, New York.

LEGENDS FOR FIGURES

Fig 1. Exploratory analysis of gene expression of the nine *P. ostreatus* ligninolytic peroxidases. **A)** Heatmap combined with dendrograms representing gene expression and sample (1, 6, 12 and 24 h) clustering under standard conditions (25 ºC and unadjusted pH 5.5) after seven days of culture. **B)** Averaged expression of RQs along 24 h, combined with hierarchical clustering dendrogram summarizing the gene expression trends under modified temperature and pH conditions. The pH 3 expression values were normalized with the total RNA, while the reference genes were used for normalization in the other cases.

Fig 2. Effect of changes in temperature and pH conditions on the expression levels of the nine *P. ostreatus* ligninolytic peroxidase genes. **A)** 10 ºC. **B)** 37 ºC. **C)** pH 3. **D)** pH 8. The increased or decreased expression values at 1 h (black), 6 h (magenta), 12 h (yellow) and 24 h (blue) after changing the temperature and pH conditions were referred to those obtained under the standard conditions (25 ºC and unadjusted pH 5.5). Error bars represent the standard deviations of the means of three independent amplifications of pooled samples, and asterisks mean that the changes referred to the control (standard conditions) are statistically significant at $p<0.05$.

Fig. 3. Relative expression of the ligninolytic peroxidase genes when temperature and pH conditions were changed. **A**) Relative expression of each peroxidase gene, as percentage of the total expression of the nine genes (in RQs), under the extreme temperature (10 ºC and 37 ºC) and pH (3 and 8) conditions assayed, compared with the 25 ºC (and unadjusted pH 5.5) standard conditions. **B)** Relative expressions after excluding the predominantly-expressed gene *mnp3*. Averaged values for the 24-h sampling period are shown (as means from the 1, 6, 12 and 24 h samples; see **Table S4** and **S5** for the whole set of relative transcriptional values for each gene and treatment condition with and without gene *mnp3*, respectively).

Fig. 4. Change of extracellular peroxidase activity determined using Mn^{2+} **(A)**, ABTS **(B)** and RB5 **(C)** as substrates. Effect of change in temperature and pH conditions and incubation times (after conditions change). Error bars represent the standard deviations of the means of three independent measurements of the same pooled samples.

Fig. 5. Purification of the major ligninolytic peroxidase present in the *P. ostreatus* cultures after 7-d growth in lignocellulose medium: Mono Q chromatography showing 280 nm (continuous black line) and 410 nm (continuous red line) absorbance and NaCl (dashed line) profiles, and SDS-PAGE (inset) of the purified enzyme (lane B) compared with different molecular mass standards (lane A).

Table 1
Transcript identification (gene, monokaryon and chromosome), PCR primers used (type - forward or reverse - and sequences) and amplification length and efficiency for the *P. ostreatus* ligninolytic peroxidase genes and the selected references genes

Transcript identification				Primer used		Amplification	
Gene	PC15	PC9	Chr[1]	Type[2]	Sequence	Lenght (bp)	Efficiency
Peroxidase genes							
mnp1	1096331	137760	IV	Fw	CTACAGAAGAACTTGTTCGACGAC	165	1.76
				Rv	GGCAGGGAAGTTAACCTCAGTAT		
mnp2	199510	137764	II	Fw	TGCTTCTCAAAGGAACGCTTGTGC	174	1.89
				Rv	CGAATCGCGAAACCATGAGCCTTT		
mnp3	1089546	137740	V	Fw	ATGGACAAGTTGGCTACACTCGGT	139	1.70
				Rv	CACACGCTTGCTCGATGTTGTTCA		
mnp4	1099081	121638	I	Fw	TCGGACAGAACCCAAAGAAGCTGA	127	1.85
				Rv	GCTTTGCGGCAGGATGCTTCAATA		
mnp5	199511	137765	V	Fw	GCCGCGATGTTGAAACTGTCTTTGG	171	1.85
				Rv	AGTCAAAGCGGGGAAAGGAGTAGC		
mnp6	1041740	51713	V	Fw	GCTATTGGATTCTCCCCTAAGCTCTT	81	1.86
				Rv	AGAGTGGGCCATGATGGAACCATC		
vp1	1089895	137757	VI	Fw	CTCCTGACAACAAGGGAGAAGTCC	198	1.89
				Rv	CAATCAGTTTGCTCTTGTCCTGG		
vp2	1113241	137766	VI	Fw	TATCGCTCGTCACAACATCAGT	146	1.86
				Rv	CTGGGACAAGACCATCAGGTGGA		
vp3	156336	123383	IV	Fw	TGGATTCTCTCCCACCAAAG	195	1.84
				Rv	GGGCAGTTGGAAACACCTAA		
Reference genes							
Actin1	1087906	114148	I	Fw	AGTCGGTGCCTTGGTTAT	129	1.83
				Rv	ATACCGACCATCACACCT		
Pep	1092697	115017	IV	Fw	TGATTCCAGAGGACAAGGACGCAA	148	1.70
				Rv	AAATCTTCCGCGATACGGGTCACT		

[1]Ch, chromosomes I-VI; [2]Fw and Rv, forward and reverse primer types.

Table 2
Ligninolytic peroxidases in the extracellular and intracellular proteomes of *P. ostreatus* (monokaryon PC9) grown in lignocellulose medium (25 ºC)

	nLC-MS/MS					Protein properties		
Enzyme	Score	Coverage (%)	Unique Peptides	Total Peptides	PSMs	AAs	MW [kDa]	Calculated pI
Extracellular								
VP1	715.87	33.84	6	7	165	331	34.6	4.63
VP2	242.93	16.81	3	4	96	339	35.8	4.56
MnP2	228.61	15.09	4	5	94	338	35.7	4.97
MnP6	77.11	22.78	7	7	30	338	35.7	4.88
VP3	35.97	9.67	1	2	12	331	34.6	4.63
MnP3	8.84	2.72	1	1	4	331	34.5	4.60
MnP5	8.33	5.67	1	1	2	335	35.0	4.59
Intracellular								
VP1	46.06	20.78	3	4	13	331	34.6	4.63
VP2	18.58	16.48	3	4	6	339	35.8	4.56
MnP2	16.47	12.85	2	4	6	338	35.7	4.97
VP3	14.52	14.72	2	3	5	331	34.6	4.63
MnP3	11.20	15.13	3	3	3	331	34.6	4.63
MnP6	10.63	8.61	2	3	4	335	35.0	4.59

Score, statistic reliability of protein identification; Coverage, percentage of tryptic peptides identified; PSM, number of scans where the isoenzyme was identified; AAs, amino-acid number

Table 3
Activity ($U.mg^{-1}$) of main ligninolytic peroxidase found in lignocellulose cultures (wild VP1), and the same isoenzyme after *E. coli* expression (recombinant VP1).

	Wild VP1	Recombinant VP1
Mn^{2+}	289 ± 12	259 ± 9
ABTS	574 ± 24	198 ± 13
DMP	300 ± 15	83 ± 2
VA	54 ± 4	18 ± 1
RB5	35 ± 3	12 ±1

Figure 1

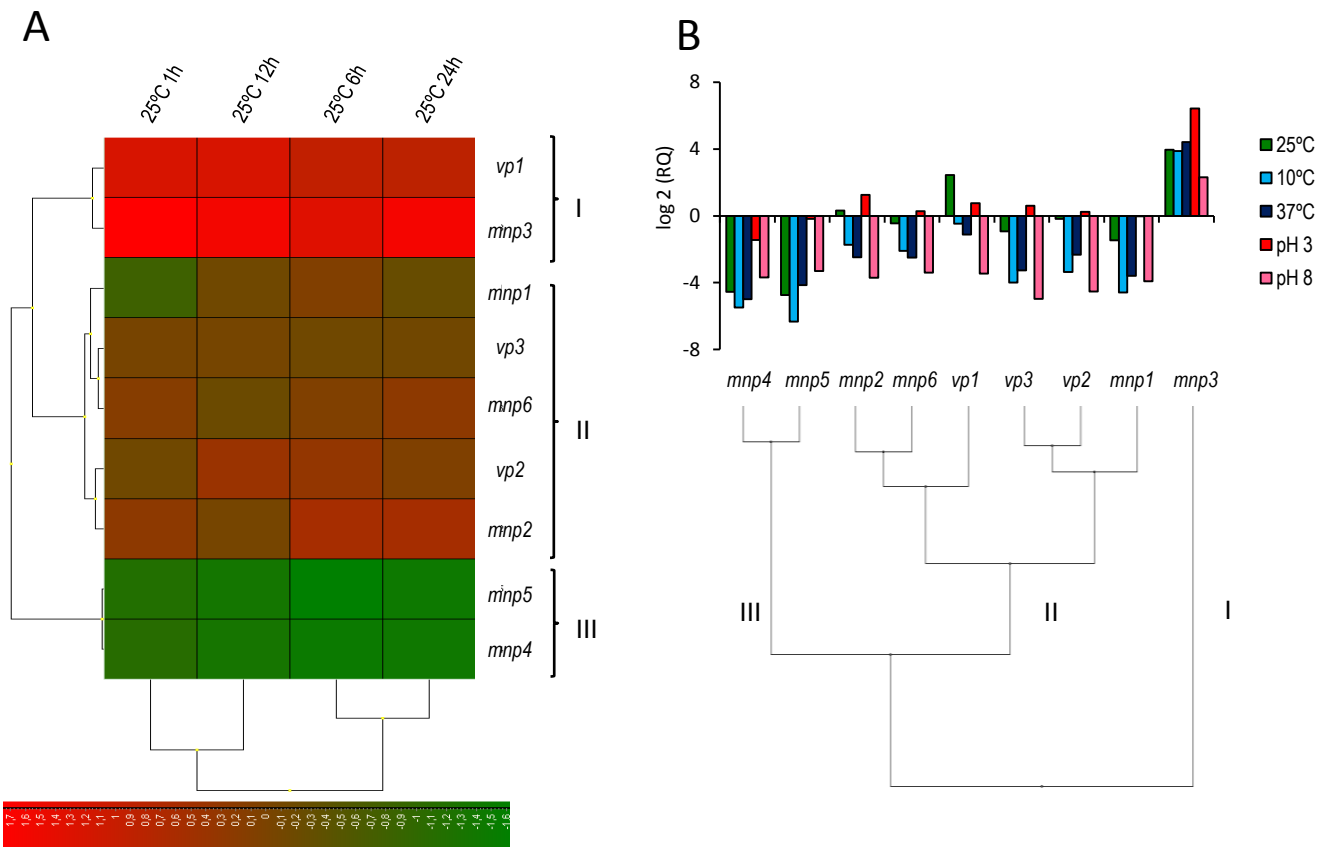

Fig 1

Figure 2

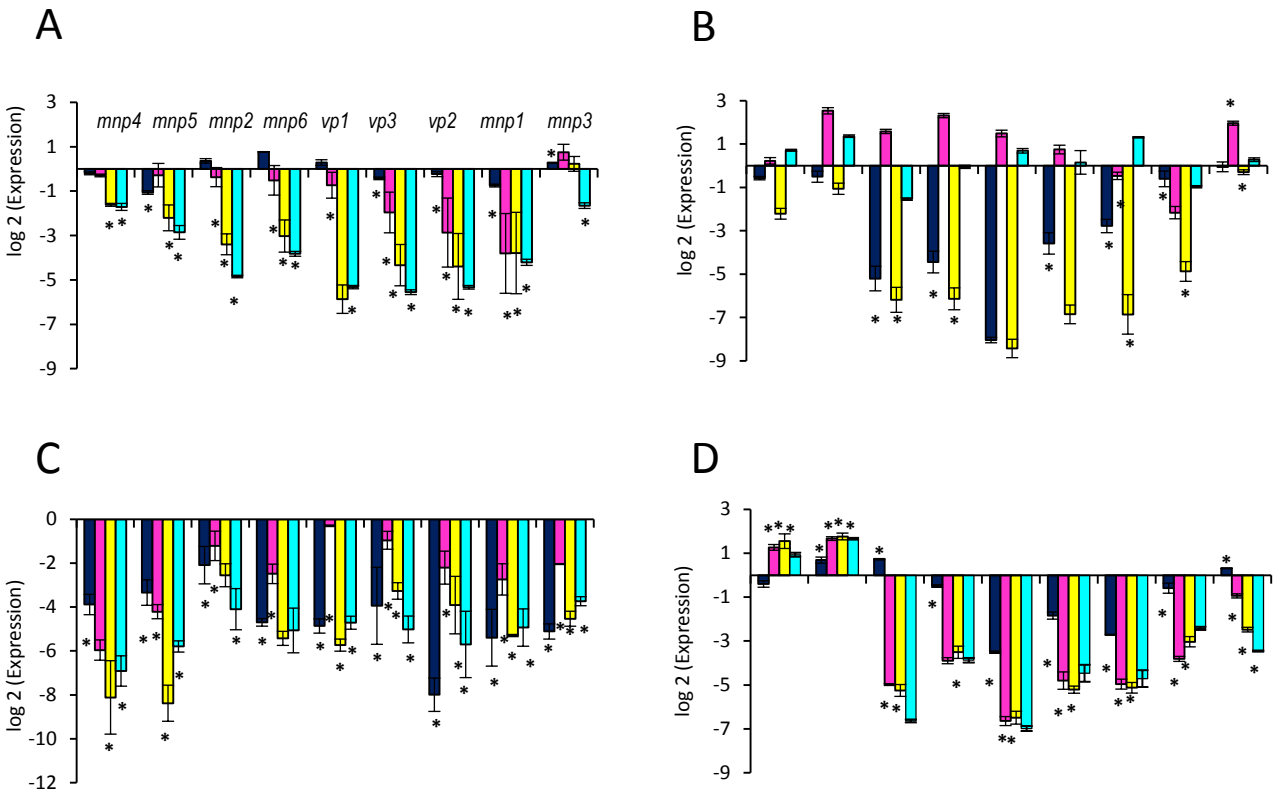

Fig 2

Figure 3

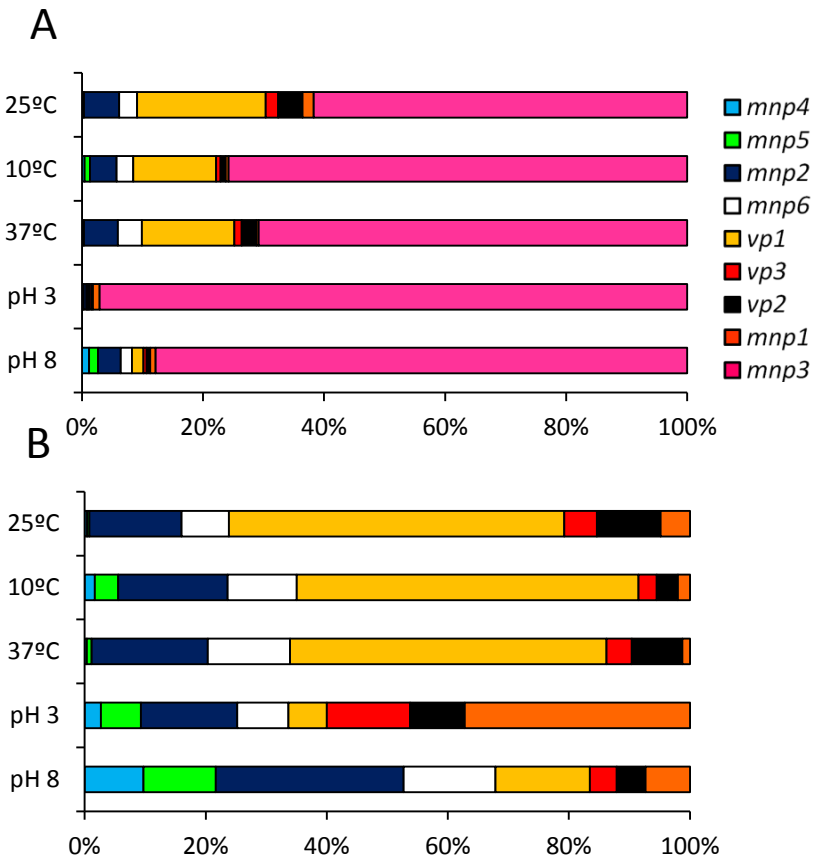

Fig 3

Figure 4

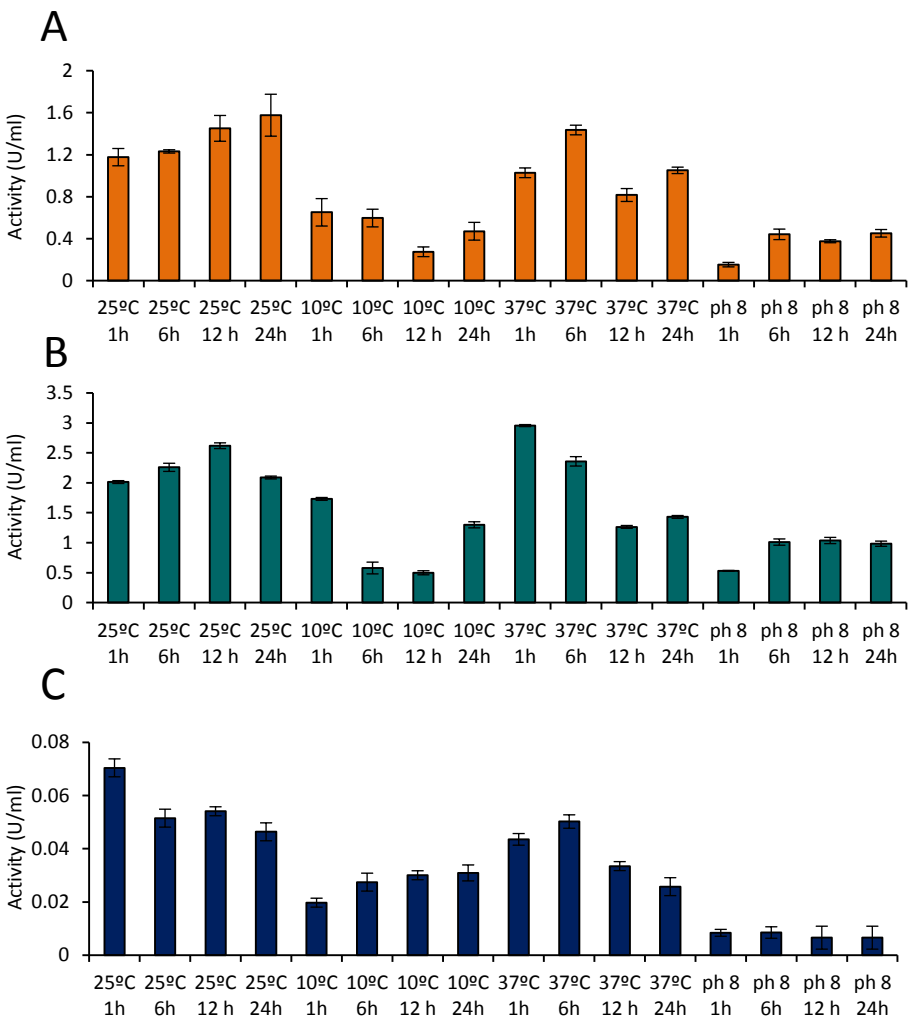

Fig 4

Figure 5

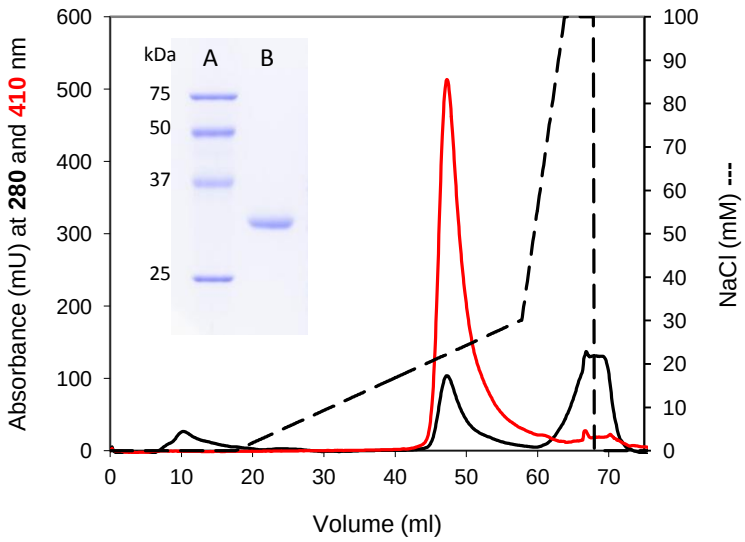

Fig 5

Supplementary Material

Click here to download Supplementary Material: FF-SI-revised.pdf