AEM
Journals.ASM.org

Transcriptional and Enzymatic Profiling of *Pleurotus ostreatus* Laccase Genes in Submerged and Solid-State Fermentation Cultures

Raúl Castanera,[a] Gúmer Pérez,[a] Alejandra Omarini,[a] Manuel Alfaro,[a] Antonio G. Pisabarro,[a] Vincenza Faraco,[b,c] Antonella Amore,[b] and Lucía Ramírez[a]

Genetics and Microbiology Research Group, Department of Agrarian Production, Public University of Navarre, Pamplona, Spain[a]; Department of Organic Chemistry and Biochemistry, Complesso Universitario Monte S. Angelo, University of Naples Federico II, Naples, Italy[b]; and School of Biotechnological Sciences, University of Naples Federico II, Naples, Italy[c]

The genome of the white rot basidiomycete *Pleurotus ostreatus* includes 12 phenol oxidase (laccase) genes. In this study, we examined their expression profiles in different fungal strains under different culture conditions (submerged and solid cultures) and in the presence of a wheat straw extract, which was used as an inducer of the laccase gene family. We used a reverse transcription-quantitative PCR (RT-qPCR)-based approach and focused on determining the reaction parameters (in particular, the reference gene set for the normalization and reaction efficiency determinations) used to achieve an accurate estimation of the relative gene expression values. The results suggested that (i) laccase gene transcription is upregulated in the induced submerged fermentation (iSmF) cultures but downregulated in the solid fermentation (SSF) cultures, (ii) the *Lacc2* and *Lacc10* genes are the main sources of laccase activity in the iSmF cultures upon induction with water-soluble wheat straw extracts, and (iii) an additional, as-yet-uncharacterized activity (Unk1) is specifically induced in SSF cultures that complements the activity of Lacc2 and Lacc10. Moreover, both the enzymatic laccase activities and the *Lacc* gene family transcription profiles greatly differ between closely related strains. These differences can be targeted for biotechnological breeding programs for enzyme production in submerged fermentation reactors.

Pleurotus ostreatus (Jacq.: Fr.) Kumm. (*Dikarya*, *Basidiomycota*, *Agaricomycotina*, *Agaricales*) is a white rot basidiomycete that lives as a saprophyte on dead or decaying wood. White rot basidiomycetes degrade their substrate by secreting different enzymes, of which those involved in lignin degradation are of specific interest (13, 30). In this study, we examined the transcriptome and activity profile of the phenol oxidase (laccase; EC 1.10.3.2) family of lignin-degrading enzymes under different culture conditions. Laccases catalyze the oxidation of various aromatic substrates with a subsequent reduction of molecular oxygen to water (41). The broad substrate specificity of laccases permits their use in multiple biotechnological and industrial applications as inexpensive biologically and environmentally friendly tools for the pretreatment of lignocellulose for bioethanol production, pulp bleaching, dye degradation, and xenobiotic transformation and detoxification (20).

The genome of *P. ostreatus* includes 12 laccase genes (browse the sequence at http://www.jgi.doe.gov). Six appear clustered at the subtelomere region of chromosome VI, and the others map to chromosomes IV, VI, VII, VIII, and XI (two genes) (33). However, only six *P. ostreatus* laccase isozymes, POXA1b (Lacc6), POXA1w, POXA2, POXA3a (Lacc2), POXA3b, and POXC (Lacc10), have been biochemically characterized to date (10, 11, 32). Among them, Lacc2, Lacc6, and Lacc10 are the most studied, and their genes have been expressed heterologously in yeast (8, 40). Furthermore, two new laccase isoforms in solid fermentation cultures of *P. ostreatus* have been recently described (16), although they remain uncharacterized.

Information is available on the biochemical characteristics of some *P. ostreatus* laccases, their transcriptional regulation by metals, aromatic compounds, and nitrogen and carbon (39), and the evolution of their activity in liquid cultures (44). However, only one published study has described the expression profile of this gene family by the use of real-time quantitative PCR (qPCR) (12), and that study examined only one strain.

Reverse transcription followed by qPCR (RT-qPCR) represents the most powerful technology to quantitatively amplify trace amounts of mRNA (14, 36). RT-qPCR is considered to be the gold standard for measuring gene expression (42) because of its high sensitivity and specificity, robust reproducibility, and wide dynamic range (36, 37). This technique requires the careful selection and validation of reference genes (internal standards), which are processed in parallel with the target gene (26). Moreover, it is critical to determine the amplification efficiency (35, 43), which is used in mathematical models for the accurate estimation of the expression levels.

In this paper, we present an in-depth study of the laccase gene family expression profile in five *P. ostreatus* strains cultivated under submerged fermentation (SmF) and solid-state fermentation (SSF) conditions and the effect of an aqueous wheat straw extract on this profile. For this study, we focused on the careful selection and validation of reference genes and their numbers. The results showed different laccase transcription profiles in the solid and liquid cultures and revealed that the *Lacc2* and *Lacc10* genes are more highly expressed and are the main source of laccase activity in the submerged cultures whereas, in the solid cultures, Lacc2 and

Received 15 December 2011 **Accepted** 20 March 2012
Published ahead of print 30 March 2012
Address correspondence to Lucía Ramírez, lramirez@unavarra.es.
Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.07880-11

Lacc10, together with a third laccase isozyme (Unk1), are the main sources of laccase activity.

MATERIALS AND METHODS

Fungal strains, culture conditions, and experimental design. Five *P. ostreatus* strains were used in this study: the dikaryotic strain N001, the monokaryotic protoclones PC9 and PC15 described previously by our group (21, 23, 24), and the dikaryotic strains FF and SS, which have fast- and slow-growing phenotypes, respectively, and were produced by mating compatible spores from N001 and separating the resulting cultures by their linear growth rates on solid agar media. The FF and SS strains are almost homozygous for each of the two N001 haplotypes of chromosome VIII. This chromosome carries a strong quantitative trait locus (QTL; $R^2 = 20.27$) that controls the linear growth rate on solid culture media (22). The FF strain carries the haplotype present in the PC9 strain, and the SS strain carries that of PC15.

SmF culture growth was performed in triplicate in 135 ml of chemically defined liquid medium (M7GY) comprising (per liter) 2 g of ammonium tartrate, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of KH_2PO_4, 0.5 g of KCl, 10 g of glucose, and 1 ml of trace element solution [0.1 g of $Na_2B_4O_7 \cdot H_2O$, 0.07 g of $ZnSO_4$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ per liter]. The cultures were grown in orbital shakers at 150 rpm in the dark at 24°C and covered with synthetic cotton filters to enable air exchange.

In the induction (induced SmF [iSmF]) experiments, 50 ml of aqueous wheat straw extract warmed to 24°C was added to the SmF cultures at the beginning of the stationary-growth phase (ninth day of culture). The wheat straw extract was prepared by agitating lyophilized wheat straw in distilled water (10% [wt/vol]) for 5 h at 25°C. The extract was then filtered, sterilized for 20 min at 121°C, and kept frozen until used. The samples from the iSmF cultures were harvested 48 h after induction.

SSF culture growth was performed in triplicate using glass flasks and 10 g of lyophilized wheat straw hydrated to 70% relative humidity. Extracellular enzyme fractions were harvested every 2 days until day 11, when the mycelium was harvested for RNA extraction. By day 11, the five strains had fully colonized the substrate, and the impact of the exponential-growth phase on laccase transcription should have been minimal.

Nucleic acid extraction and reverse transcription. The mycelium from the SmF and iSmF cultures was harvested by pressure filtration and was scraped from the surface of the SSF. Mycelia from the three biological replicates were pooled and ground in a sterile mortar using liquid nitrogen and stored at −80°C until use. Total RNA was extracted from 100 mg of frozen tissue by the use of a fungal RNA E.Z.N.A kit (Omega Bio-Tek, Norcross, GA), and its integrity was determined by electrophoresis on 1% (wt/vol) agarose gels. Duplicate samples were used for nucleic acid concentration measurements, which were quantified using a Nanodrop 2000 spectrophotometer (Thermo-Scientific, Wilmington, DE). Total RNA purity was estimated using the 260-nm/280-nm ratio, and only samples with values between 1.9 and 2.1 were used. The RNA was concentrated and purified using an RNeasy MinElute Cleanup kit (Qiagen Iberia S.L., Madrid, Spain) to achieve a concentration of 1 μg liter^{-1}. One μg of total RNA was reverse transcribed to cDNA in a 20-μl volume by the use of an iScript cDNA synthesis kit (Bio-Rad Laboratories S.A., Alcobendas, Spain) with the following reaction mixture: 1× cDNA synthesis kit buffer, 1 μl of reverse transcriptase H+, and 1 μg of total RNA; the volume of the mixture was brought to 20 μl using nuclease-free water. The reaction conditions for the cDNA synthesis were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The cDNAs were stored at −20°C.

Primer design and verification. The PCR primers were designed using PrimerQuest software (Integrated DNA Technologies) with the genome sequences of protoclones PC9 version 1.0 and PC15 version 2.0, which can be obtained from the JGI database (http://www.jgi.doe.gov) (Table 1). The primer sequences were selected to be specific for each of the members of the laccase gene family, and this criterion outweighed the position in the sequence of the gene (i.e., intron/exon boundaries). PCR specificity was validated *in silico* by performing a Primer-BLAST search against all PC9 and PC15 model transcripts, and the RT-PCR products were sequenced and compared with the filtered laccase gene models to confirm the specificity of each primer pair.

RT-qPCR. Real-time quantitative PCRs were performed using a Bio-Rad CFX96 thermal cycler. SYBR green fluorescent dye was used to detect the product. Each reaction was set to a final volume of 20 μl and contained 1× IQ SYBR green Supermix (Bio-Rad Laboratories S.A., Alcobendas, Spain), 100 nM forward and reverse primers, and 10 ng of cDNA in nuclease-free water. The amplification program was as follows: 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 65°C and a final melt curve with increments of 0.5°C every 5 s from 65°C to 95°C. The primer specificities were validated by analyzing the melt curves. Raw fluorescence data were monitored, and the baselines were corrected using Bio-Rad CFX Manager. A multivariable, nonlinear regression model was applied to compute optimal Cp (crossing-point) values. 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). RT-negative controls for every sample (RNA template) were run using the reference gene *Lacc3* as the target. Background DNA contamination was estimated and expressed as a percentage of the relative quantities of the reference gene *Lacc3* according to equation 1:

$$\%\ \text{of genomic DNA background} = \frac{E^{-\text{Cp}_{\text{DNA}}}}{E^{-\text{Cp}_{\text{NA}}}} \times 100 \qquad (1)$$

where Cp_{DNA} and Cp_{NA} represent the Cp values obtained in the qPCRs performed using the RNA control and the retrotranscribed RNA with the endogenous DNA background, respectively. The SmF cultures were used as calibrators for the RT-qPCR relative quantifications.

Quantification strategy and qPCR data normalization. The mathematical model used for the relative quantifications of the transcripts was based on the PCR efficiencies and the mean Cp deviations between the sample and control groups. The reaction efficiencies were estimated using the LinReg tool for each sample and a Window of Linearity data set from the exponential phase of the fluorescence plotted in log scale (43). The relative quantities (RQ) were calculated using the calibrated efficiency model (equation 2) and were normalized to a robust index composed of data from three experimentally validated reference genes according to the mathematical model proposed by Pfaffl (35) (equation 3). Measures of the expression stability of the candidate genes (M) were calculated using the GeNorm tool (52). The optimal number of control genes for data normalization was determined by analyzing the pairwise variation among the sequential normalization factors (NF), which were calculated by step inclusion of additional reference genes from our panel, as proposed by Vandesompele et al. (52). Finally, the expression ratios were tested by random pairwise reallocation using REST software (38), which provided 95% confidence intervals and *P* values as follows:

$$\text{RQ} = (E\ \text{target})^{\Delta\text{Cp}\ (\text{control sample})} \qquad (2)$$

$$\text{Relative expression ratio} = \frac{(E\ \text{target})^{\Delta\text{Cp}\ (\text{control sample})}}{(E\ \text{target})^{\Delta\text{Ctp}_{\text{ref}}\ (\text{control sample})}} \qquad (3)$$

where "*E* target" represents the PCR amplification efficiency of the gene of interest and ΔCp represents the difference between the Cp values for that gene under control and experimental conditions.

Enzyme analysis. In the case of the SmF and iSmF cultures, triplicate 2-ml samples were collected from each biological replicate and centrifuged at 1,0000 rpm for 3 min at 4°C to remove debris. In the SSF cultures, three biological replicate experiments (parallel cultures) per strain per time point were used. In this case, the enzymes released to the medium were extracted from the solid culture by adding 40 ml of sodium acetate buffer (50 mM, pH 5.0) and shaking the extraction mixtures at 150 rpm for 4 h at 10°C as described by de Souza-Cruz et al. (5). Following the extraction, the liquid fraction was collected and centrifuged to remove debris, and the subsequent supernatant was used for enzyme determina-

TABLE 1 PCR primers, amplicon length, and amplification efficiency for the laccase and reference genes

Gene	Transcript identification no.[a]		Chromosome	Primer category	Primer sequence	Amplicon length (bp)	Amplification efficiency
	PC15	PC9					
Laccase genes							
Lacc1	1043420	90578	VI	Fw	GGTACATCCTAGCACCCAATG	80	1.74 ± 0.04
				Rv	GACGAGATCAGTTTCCAAGAGG		
Lacc2	1067328	116143	VIII	Fw	CCCTGGCAGATTGGTATCATG	142	1.74 ± 0.00
				Rv	ATGACAGCGTAAGGGACAAG		
Lacc3[b]	1102751	123288	VI	Fw	TCGTTTCCGTCTCGTTTCTC	134	1.69 ± 0.04
				Rv	CTGCGAAGATTTGGATGCTG		
Lacc4	1077328	65894	6	Fw	CCCCATCCTTTCCATCTTCAC	72	1.79 ± 0.04
				Rv	GTAGTTATACACCGAGCTTCCG		
Lacc5	1094975	90812	XI	Fw	CGCATTTGCCGCTTTCTT	136	1.67 ± 0.03
				Rv	GGTGACTAGGACTGAGTATCTC		
Lacc6	1113032	81104	VI	Fw	GTACAACTACGAAAACCCCG	140	1.68 ± 0.02
				Rv	CAAGGTCAAGATGCCAGT		
Lacc7	1077468	60400	VI	Fw	GTTGATAGCCTCCAGATCTTCG	142	1.72 ± 0.02
				Rv	GTAGGATGGCGGAGTTGATG		
Lacc8[c]	1106925		VII	Fw	CATTGGCTGTGACTCGAA	137	1.59 ± 0.04
				Rv	GGATCAGAGAATAGCGTTGG		
Lacc9	1089733	81107	VI	Fw	CTATCCTTCGGTATGCTGGTG	145	1.81 ± 0.05
				Rv	ATATTGATGTCTGCGCCTCC		
Lacc10	1089723	81117	VI	Fw	CCTACTTCCCCTTTGGCTATC	122	1.79 ± 0.07
				Rv	ATGACGAGCAAAGAGTGACC		
Lacc11	1043488	90573	VI	Fw	CCTGAATGGTCTGATCTCTGC	93	1.82 ± 0.05
				Rv	CCTATGACTTGGGCTCTTCG		
Lacc12[b]	1094965	90834	XI	Fw	GTACTCATTTTCGGCTCCTG	84	1.76 ± 0.06
				Rv	CCACGTAGTCCATCGCAATA		
Reference genes[c]							
cyt-c	1113744	112752	VIII	Fw	GCCTCATAAAGTCGGTCCTAAC	127	1.92 ± 0.08
				Rv	CTCAAATAGGGTGTTCTCGTCC		
actin1	1087906	114148	I	Fw	AGTCGGTGCCTTGGTTAT	129	1.72 ± 0.10
				Rv	ATACCGACCATCACACCT		
actin2	1114037	90915	VIII	Fw	GCCGTGATCTTACCGACTA	134	1.57 ± 0.04
				Rv	CTCCTGCTCAAAGTCCAA		
cyph	1058252	72928	VII	Fw	GACATTGCTATCGACTCCCAG	84	1.69 ± 0.10
				Rv	GAAATTCCTTGCAGTCTTGGG		
GAPDH	1090663	87982	IX	Fw	ACCTTGAGATTACGACCC	139	1.83 ± 0.03
				Rv	GTTGTGGCGTGGATTGTC		

[a] Transcript identification numbers correspond to the PC15 version 2.0 and PC9 version 1.0 genomes available at www.jgi.doe.gov.
[b] The *Lacc3* and *Lacc12* genes were also used as reference genes.
[c] Only present in the PC15 genome.

tion. In all cases, three technical replicates of each sample were used to determine each datum.

The laccase activity was determined spectrophotometrically by measuring the oxidation of 2,6-dimethoxyphenol (DMP; $\varepsilon_{480} = 49{,}600\ M^{-1}\ cm^{-1}$) following a 60-s incubation at room temperature. For these incubations, 90 to 450 μl of the sample was added to 500 μl of a 10 mM DMP solution in 0.1 M sodium acetate buffer (pH 5.0) and made up to achieve a 1-ml final volume with the acetate buffer. The enzyme activities were expressed in international units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

Laccase zymograms in native PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under nondenaturing conditions. The resolving and stacking gels contained 9% acrylamide–50 mM Tris-HCl (pH 9.5) and 4% acrylamide–18 mM Tris-HCl (pH 7.5), respectively. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Laccase activity was revealed by soaking the gels in a 20 mM ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) solution made using 0.1 M sodium citrate buffer (pH 3.0). Laccase isozymes purified from the dikaryotic strain ATCC MYA-2306 were used as standards (10, 11, 32).

Biomass determination. Three biological replicates of the SmF and iSmF cultures were taken on every second day of culture. The mycelium was harvested by filtering these samples with 10-μm-pore-size nylon filters followed by dehydration for 48 h at 50°C, and the samples were subsequently weighed.

Phenol and reducing sugar determinations. The phenol content of the wheat straw extract was estimated using Folin-Denis reagent (9) as proposed by Zhang et al. (55), and the reducing sugar content of the extract was determined using the DNS method (31).

Statistical analysis. All laccase activity and biomass measurements were subjected to Student's *t* tests to determine the statistical significance of differences.

RESULTS

Parameters used for accurate laccase RT-qPCR gene expression quantification. (i) Reference gene selection. Seven functionally

different genes (*Cyt-c*, *Actin1*, *Actin2*, *Cyph*, *GAPDH*, *Lacc3*, and *Lacc12*) were tested for use as internal reference genes under our experimental conditions (see Fig. S1 in the supplemental material). Their expression stability (*M*) was calculated for three culture conditions (SmF, iSmF, and SSF) using the FF and PC15 strains because of their different phenotypes. The seven genes had *M* values ranging from 0.8 (*Cyt-c*) to 0.15 (*Lacc3* and *Lacc12*; see Fig. S2 in the supplemental material). Lower *M* values indicate a more stable gene expression profile (52), and our results revealed that *Lacc3*, *Lacc12*, and *Cyph* had the lowest expression variability among the seven genes tested.

(ii) Optimal number of reference genes. To determine the optimal number of reference genes, we studied the pairwise variation of NF (52) determined for *n* relative to $n + 1$ reference genes ($V_{n/n+1}$; see Fig. S3 in the supplemental material). This approach enabled us to set the ideal number of reference genes to three, because the inclusion of a fourth gene ($V_{3/4}$) led to a small (0.006) variation in *V*. Moreover, we noted that the NF_n-NF_{n+1} pairwise variation correlated positively with the number of reference genes included (data not shown). Consequently, *Lacc3*, *Lacc12*, and *Cyph* were chosen as the reference gene set for data normalization in this study.

(iii) Amplification efficiency. The PCR amplification efficiency was calculated for the reference genes and for the members of the laccase gene family by the use of the LinReg PCR tool (43). The efficiency values ranged from 1.59 to 1.82 for the *Lacc* gene set (standard deviation [SD] = 0.23) and from 1.57 to 1.92 (SD = 0.44) for the reference gene set (Table 1). The standard deviations of amplicon efficiencies in comparisons of different samples ranged from 0.01 to 0.06 for the *Lacc* genes and from 0.03 to 0.10 for the reference genes.

(iv) Genome DNA contamination. All samples were tested for DNA background contamination. The mean DNA content in the samples was 1.94%. All of the samples had contamination values lower than 5%, with the exception of strain N001 in SSF (7.51%) and strain SS in SmF (5.36%; see Table S1 in the supplemental material).

Transcriptional profile of the laccase gene family. (i) Submerged fermentation cultures. We determined growth curves (biomass accumulation) under the conditions used for the SmF and iSmF experiments to analyze the behavior of the five strains in liquid cultures. The five strains showed a linear increase in biomass during the first 7 days of culture; by the 9th day of culture, all of the strains had entered the stationary phase of growth (see Fig. S4 in the supplemental material), and they had reached similar levels of biomass content by day 11 in the SmF cultures (see Fig. 2). The addition of the wheat straw extract on day 9 of culture produced significant differences in the accumulated fungal mass by day 11 in the iSmF cultures (see Fig. 2). The increases in biomass differed between strains and ranged between 1.57 and 22.57 g $liter^{-1}$ in the SS and PC9 strains, respectively. We analyzed the composition of the wheat straw extract, and we found that it contained 4.01 ± 0.22 g $liter^{-1}$ reducing sugar and 0.24 ± 0.03 g $liter^{-1}$ gallic acid equivalents (an estimation of the amount of polyphenolic compounds).

The laccase gene family expression profiles were quantified on day 11 using 10 of the 12 genes, because the *Lacc3* and *Lacc12* genes had been used in the reference set. In Fig. 1, we show the effect of the addition of wheat straw extract to the iSmF cultures on the transcriptional profiles of the members of this gene family (see Table S2 in the supplemental material). We observed that 65% of the analyzed *Lacc* genes were differentially expressed compared with the control ($P < 0.05$) after the addition of wheat straw extract, with 46% of the genes being upregulated and 19% downregulated (Table 2). The proportion of differentially regulated genes was higher in the monokaryotic strains than in the dikaryotic strains (84% versus 52%, respectively). Furthermore, this proportion was also higher when the fast-growing strains were compared as a group with the slow-growing strains (75% versus 50%, respectively). The genes showing the greatest upregulation in all the strains were *Lacc2* and *Lacc10* (see Fig. S5 in the supplemental material). The highest upregulation was observed in *Lacc2* in the PC9 strain (7.7-fold upregulation) and in *Lacc10* in the N001 strain (5.2-fold upregulation; Fig. 1).

(ii) Solid-state fermentation cultures. To profile the laccase gene expression in SSF cultures, the cultures were incubated for 11 days as described above. By that time point, all of the strains had completely colonized the substrate. The transcriptional profile of the 10 *Lacc* genes in SSF differed greatly from that observed in the SmF and iSmF cultures (Fig. 1; see also Table S3 in the supplemental material). Our results revealed that 42% of the genes were differentially expressed compared with the control conditions (SmF), with only 4% of the genes being upregulated and 38% downregulated (Table 2). Similar to the results seen with the iSmF cultures, the proportion of differentially regulated genes was higher in monokaryons than in dikaryons (53% versus 34%) and in fast-growing versus slow-growing strains (50% versus 30%).

Enzymatic laccase activity profile in SmF, iSmF, and SSF cultures. (i) Laccase activity in SmF, iSmF, and SSF cultures. To complement the results observed when analyzing the laccase family transcription profile in iSmF, we monitored the differences in laccase enzymatic activity associated with the addition of wheat straw extract. The laccase activity released into the culture medium was assayed on day 11 in the SmF and iSmF cultures. The activity measured in the control cultures (SmF) ranged between 0 and 0.73 U $liter^{-1}$ for the SS and FF strains, respectively (Fig. 2). However, significant ($P < 0.05$) increases in activity were observed in four of the five fungal strains following the addition of wheat straw extract, and enzymatic activities of more than 40 U $liter^{-1}$ were recovered for three of the strains. Notably, no laccase activity was detected in the PC15 iSmF cultures, despite a large increase in biomass following the addition of the inducer to this strain.

The activity extractable from the SSF cultures was measured at different time points (Fig. 3). This activity was found to be highly variable between the biological replicates. Laccase activity was detected in four of the strains studied, and it was not detected in cultures of the monokaryotic PC15 strain. The N001 and PC9 strains exhibited similar evolutions of laccase activity over time, whereas the dikaryotic FF and SS strains displayed a more erratic evolution of activity.

(ii) Laccase isozymes in SmF, iSmF, and SSF. The released (SmF and iSmF cultures) and extractable (SSF cultures) laccase isozymes were examined using PAGE followed by *in situ* detection of enzymatic activity. The zymograms revealed the presence of three isozymes, which corresponded to Lacc2, Lacc10, and an unknown isoform (Unk1) in the SSF (Fig. 4). The Lacc2 and Lacc10 isozymes were also detected in the fast-growing strains (N001, PC9, and FF) in the iSmF and SSF cultures. No laccase isozymes

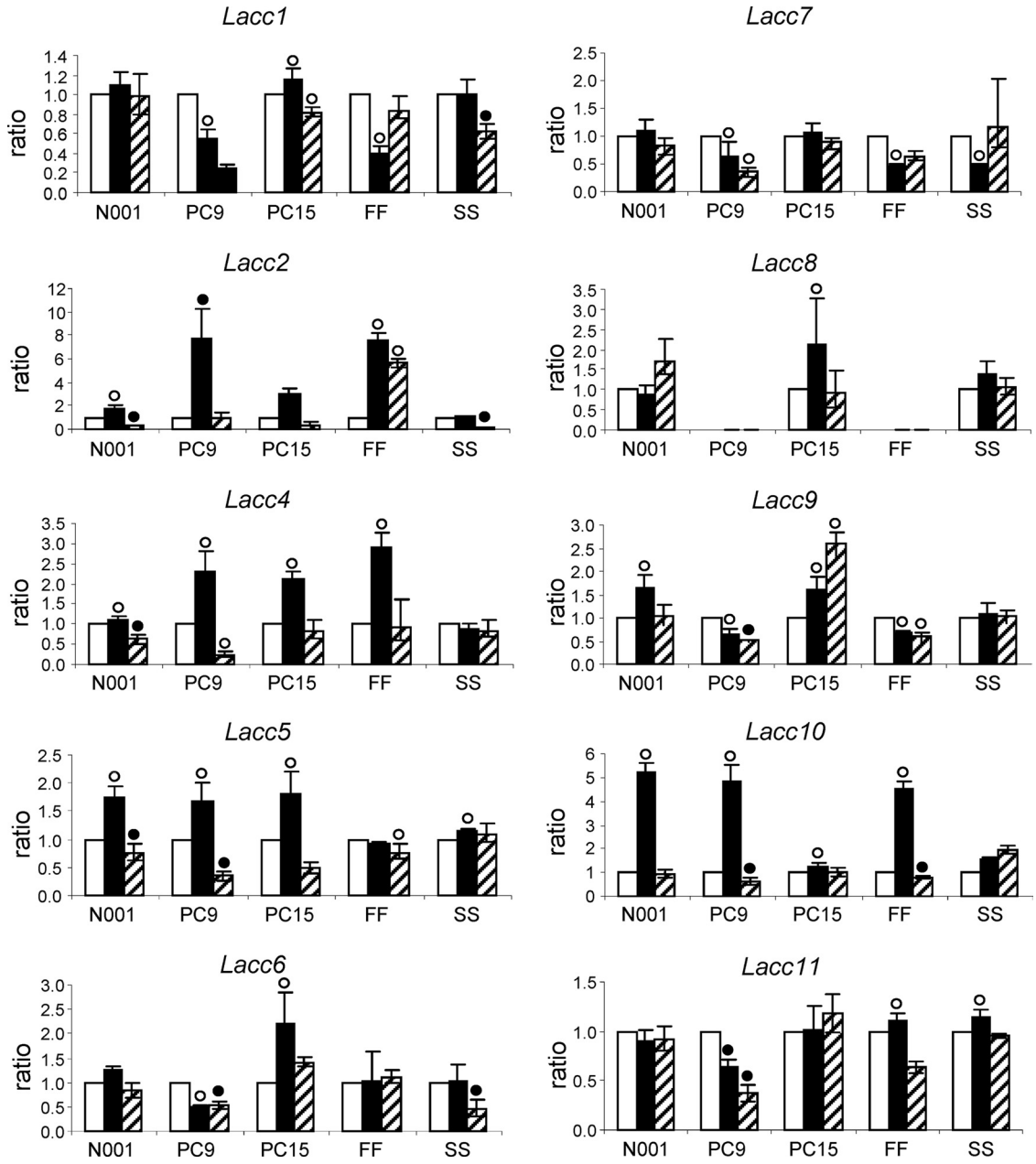

FIG 1 Relative expression ratios of 10 *Lacc* genes of *P. ostreatus* cultured in iSmF (black bars) and on SSF (striped bars) media relative to the expression in the SmF control culture (white bars), according to equation 3 in the text. The bars represent the standard errors of the means of three independent measurements of the same pooled samples (see the text). The statistical significance of the differences between sample means is indicated with open circles ($P < 0.05$) or solid circles ($P < 0.01$).

were detected in any of the SmF cultures, in the PC15 samples, or in the iSmF sample of the SS strain.

DISCUSSION

The existence of gene families comprising apparently redundant members raises questions concerning the actual function of these members. Phenol oxidases (laccases; EC 1.10.3.2) form a family of 12 genes in the white rot basidiomycete *P. ostreatus*, and they play an active role in lignin degradation (33, 34). Studying their differential expression enables the examination of the functions of the gene family members. This examination has been performed in certain cases for certain members of the laccase gene family (12, 29, 48); however, to date, not all of the gene family members have been examined. For the present study, using RT-qPCR, we report the differential expression characteristics of laccase genes in different strains and under different growth conditions.

RT-qPCR conditions for profiling the expression of gene families. Gene expression varies as a result of biological and experimental variation (53). The principal sources of this experimental variation include RNA extraction, the amount of starting material, random experimental errors, and the reverse transcrip-

TABLE 2 Numbers and proportions of laccase genes up- and downregulated in monokaryotic, dikaryotic, fast-growing, and slow-growing strains in induced submerged and solid fermentation cultures[a]

Laccase gene category	Strain group[b]									
	Total		mk		dk		FG		SG	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Differentially expressed	51	53	26	68	25	43	29	52	16	40
Total[c]	96		38		58		56		40	
iSmf (differential expression)[d]	31	65	16	84	15	52	21	75	10	50
Upregulated	22	46	11	58	11	38	13	46	9	45
Downregulated	9	19	5	26	4	14	8	29	1	5
SSF (differential expression)[d]	20	42	10	53	10	34	14	50	6	30
Upregulated	2	4	1	5	1	3	1	4	1	5
Downregulated	18	38	9	47	9	31	13	46	5	25

[a] *n*, numbers of laccase genes; %, proportions of laccase genes; mk, monokaryotic strains; dk, dikaryotic strains; FG, fast-growing strains; SG, slow-growing strains; iSmF, induced submerged fermentation; SSF, solid-state fermentation.
[b] The members of the strain groups were as follows: mk, strains PC15 and PC9; dk, strains N001, FF, and SS; FG, strains N001, FF, and PC9; SG, strains PC15 and SS.
[c] Total numbers of laccase genes studied in each strain group.
[d] Numbers and proportions of the laccase genes differentially ($P < 0.05$) expressed in iSmF and SSF cultures. For the proportion values (%), note that half of the total number of genes were studied in the iSmF and the other half in the SSF experiments.

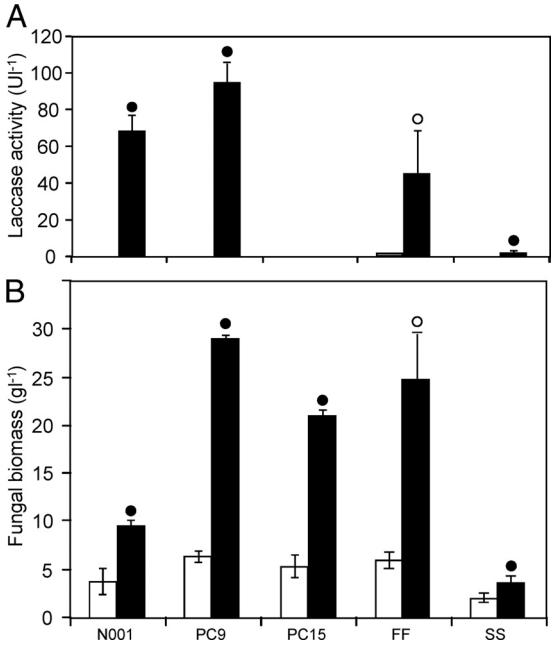

FIG 2 Extracellular laccase activity determined using DMP (A) or biomass (B) as the substrate in SmF (white bars) and iSmF (black bars) cultures. Bars represent the standard deviations of the means of the results of three biological replicate experiments. The statistical significance of the differences between sample means is indicated with open circles ($P < 0.05$) or solid circles ($P < 0.01$).

tion process (49). To measure gene expression accurately using RT-qPCR, normalization relative to an endogenous control that is processed in parallel with the target genes is required (26). Ideal endogenous reference genes should have equivalent transcript levels in all cells at every developmental stage and should be unaffected by the experimental conditions (52). Housekeeping genes, such as those encoding β-actin, β-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA, are frequently used as reference genes without any experimental validation, although fluctuations in their expression stability could cause unacceptable changes in gene expression estimations (46). In the search for appropriate internal standards for our experiment, we tested five housekeeping genes (*Cyt-c*, *GAPDH*, *Actin1*, *Actin2*, and *Cyph*) that are frequently used as standards in RT-qPCR assays and two *Lacc* genes (*Lacc3* and *Lacc12*), expression of which was stable under our experimental conditions. Our results revealed that expression of *Cyt-c*, *GAPDH*, *Actin1*, and *Actin2* was dependent on the media and culture conditions, with *Cyt-c* showing the most unstable expression. *Cyph*, *Lacc3*, and *Lacc12* expression levels were more stable under our experimental conditions (Fig. 1; see also Fig. S2 in the supplemental material), and *Lacc3* and *Lacc12* were expressed at approximately the same level as the target genes, as recommended for optimal endogenous controls (1). *Lacc12* encodes a fruit-body-specific laccase (25), and its stability under our experimental conditions was due to its basal expression level during vegetative growth. In the case of *Lacc3*, our results suggest that it is expressed constitutively. Other constitutively expressed laccases have been described in studies of *Pleurotus sajor-caju* growing in media containing different levels of carbon, nitrogen, and aromatic compounds (48).

We determined the optimal number of reference genes, as proposed by Vandesompele et al. (52), and we concluded that three stable reference genes were sufficient for qPCR data normalization, as reported by other authors (6). We observed a positive linear correlation between the increase in NF_n-NF_{n+1} pairwise variation and the number of reference genes included that differed from the U-shaped correlation reported in other studies (52); this finding suggests that the sequential increase in $V_{n/n+1}$ is due to the inclusion of genes that have relatively unstable expression (27).

In summary, we suggest using the *Lacc3*, *Lacc12*, and *Cyph* genes for reference gene searches in further *P. ostreatus* RT-qPCR studies performed under similar conditions, and we stress the importance of validating the expression stability of the reference genes for each specific set of experiments (2, 52).

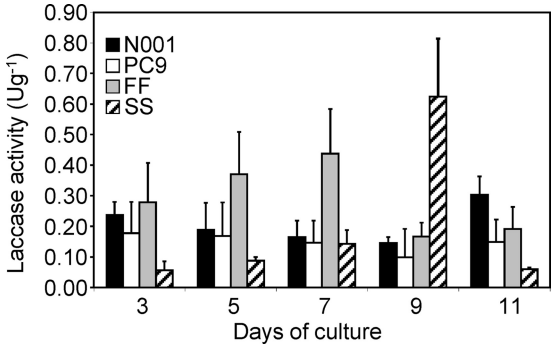

FIG 3 Laccase activity extractable from SSF cultures harvested at different times. The monokaryotic PC15 strain did not show activity during any of the experiments and is not included here. The enzymatic activity was determined using DMP as the substrate. The bars represent the standard deviations of the means of the results of three biological replicate experiments.

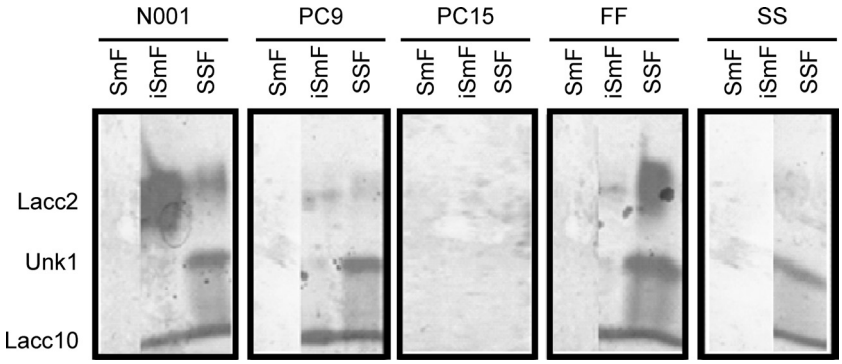

FIG 4 Zymograms of the *P. ostreatus* laccases detected in SmF, iSmF, and SSF cultures. A 37.5-μl volume of protein extract was loaded in each lane.

PCR amplification efficiency is among the major factors that affect amplification kinetics. This efficiency is influenced significantly by the primers used (2, 18), the PCR components, and sample processing (50). Additionally, cDNA integrity and the RT reaction are the main sources of variability between samples for a given amplicon. The RT itself can interfere with cDNA first-strand PCR amplification via direct interaction with specific primer-template combinations (4), thereby inhibiting polymerase activity and altering PCR efficiency. Cp values are strongly influenced by the amplification efficiency, and a moderate (i.e., 0.1) intersample efficiency difference has been shown to lead to large differences in gene expression (36). Therefore, it is important to use a PCR model that considers amplification efficiency values for each sample when studying the expression stability of the reference gene set.

PCR efficiencies are typically calculated from standard curves based on serial dilutions of a reference cDNA sample (15). This method is highly reproducible for the same transcript and tissue in an experiment, but it has two primary disadvantages: (i) the efficiency must be calculated for each amplicon (disregarding intersample variation), and (ii) it tends to overestimate the real amplification efficiency and produces values greater than 100% in some cases (36). This overestimation is common when the cDNA samples are contaminated with salt, phenol, and chloroform and/or ethanol (43). Moreover, this method is expensive and time-consuming, particularly if many genes and tissues are used. We have used an alternative approach for estimating PCR efficiency that is based on the increase in fluorescence that occurs during the exponential phase of the PCR (43). In theory, this method underestimates efficiencies compared with the dilution method because the data are calculated using the reaction linear phase, which can be restrictive (17). Nevertheless, we found this method to be sensitive and reliable because it produced amplicon efficiency values lower than 100%, with standard deviations between different samples ranging from 0.01 to 0.10.

Transcriptional profile of the laccase gene family. The expression levels of members of the laccase gene family were profiled on day 11 of culturing. In the SmF and iSmF cultures, the biomass had reached a stationary accumulation phase at least 48 h before that time point (see Fig. S1 in the supplemental material), and in the SSF cultures, the mycelium had already colonized the entire substrate by that time. For the experiments in which laccase expression was induced, a wheat straw extract was added to the corresponding cultures (iSmF) by the ninth day of culturing.

The laccase gene family transcription profiles in the iSmF and SSF cultures were different from those seen under the control conditions (SmF). A greater number of *Lacc* genes were expressed differentially ($P < 0.05$) in the iSmF cultures (65%) than in the SSF cultures (42%); notably, whereas most of the differentially expressed genes in iSmF were upregulated (46% upregulated versus 19% downregulated), the majority were downregulated in SSF (4% upregulated versus 38% downregulated).

The high percentage of genes with altered transcriptional responses in iSmF reveals a complex regulation mechanism that could be related to the sensitivity of the laccase gene family to the phenolic compounds and sugars present in the inducer extract. The genes showing the highest upregulation among all of the strains were *Lacc2* (4.2-fold average upregulation) and *Lacc10* (3.5-fold average upregulation) (Fig. 1; see also Table S2 in the supplemental material). The promoters of these genes contain a higher number of motifs that are sensitive to components present in the wheat straw extract, such as XRE (xenobiotic response elements) and MRE (metal-responsive elements), than the promoters of other genes in this family (39). The presence of these elements was confirmed in the strains used in this work (data not shown), and consequently, the expression of these genes is expected to be sensitive to these inducers. The only gene for which a small downregulation was observed under these conditions was *Lacc7* (Fig. 1; see also Table S2 in the supplemental material).

The laccase transcriptional profile of the dikaryotic strains appeared more stable (i.e., they showed a lower number of differentially expressed genes) than that of the monokaryotic strains, and slow-growing strains also showed a more stable laccase expression pattern than fast-growing strains (Table 2). Careful evaluation of the underlying cause of these differences revealed that the monokaryotic PC9 strain (fast-growing phenotype) was more sensitive to the induction and culture conditions than the other strains (see Tables S2 and S3 in the supplemental material).

In the SSF cultures, there was an overall downregulation of the laccase gene expression (Table 2) that was notably robust in certain cases (*Lacc2* in strains N001, PC15, and SS; see Table S3 in the supplemental information). The complexity of the culture conditions (pH, moisture content, air transfer, wheat straw composition) hinders efforts to determine the elements responsible for these changes. However, we believe that the water content of the culture could be one of the most relevant factors because it could affect the diffusion of the phenolic compounds into the substrate, thereby affecting laccase gene transcription. Moreover, Economou et al. proposed that an increase in water content contributes

to increased sugar availability (7), which is an additional critical factor for laccase production in most fungi (28). Similarly, Sharma and Arora (47) reported that laccase activity in *Phlebia floridensis* cultured on wheat straw increased with water content and reached a 34-fold increase at the maximum moisture content, and studies performed with *Aspergillus oryzae* cultured on wheat bran have indicated that expression of genes encoding other secreted enzymes was dependent on the water content in the substrate (19).

Laccase enzymatic activity. The laccase inducer effect of the wheat straw extract was assayed in SmF cultures following 2 days of induction. Whereas the laccase activity was negligible in the control SmF cultures (Fig. 2), as reported by other authors on the basis of studies of submerged uninduced cultures (39), a significant ($P < 0.05$) increase in extracellular laccase activity was observed in the fast-growing strains following the addition of wheat straw extract. This increase reached a maximum of 94.67 U liter^{-1} (PC9) and occurred in the same samples that showed transcriptional upregulation of *Lacc2* and *Lacc10* (Fig. 2). In contrast, no laccase activity was induced in the slow-growing strains. These results demonstrate that the increase in laccase activity was independent of biomass increase, because the PC15 strain showed a significant increase in biomass (upon the addition of the inducer) but no laccase activity. The induction of laccase activity was evident in the fast-growing strains (N001, PC9, and FF) that are homozygous for the PC9 variant of chromosome VIII and that contain at least one copy of the PC9 alleles for the *Lacc2* and *Lacc10* genes. The transcriptional response of *Lacc2* and *Lacc10* discussed above, together with the strong induction of the extracellular enzymatic activity, suggests an important role for these two enzymes in the lignin-degrading machinery of *P. ostreatus*.

The patterns of evolution of laccase activity seen during growth of SSF cultures (Fig. 3) differed between strains. The PC15 strain did not release any detectable laccase activity into the solid substrate during the entire culturing period, similar to iSmF culture results (Fig. 2). These data suggest that most (if not all) laccase activity secreted by N001 was due to the presence of the PC9 nucleus in this dikaryon. However, we can rule out the possibility of an overall deficiency in laccase production associated with the PC15 nucleus because this activity was observed in the SS strain, which bears the PC15 haplotype for chromosome VIII and a slow-growing phenotype.

The dikaryotic FF and SS strains exhibited different laccase activity patterns over time (Fig. 3) that could have been caused by their different efficiencies in substrate colonization. Each of the two strains is homozygous for one of the two variants of N001 chromosome VIII. This chromosome carries a QTL for dikaryotic growth rate ($R^2 = 20.3$) and the gene *Lacc2*. The differences in the laccase patterns of these dikaryotic strains in SSF cultures seen over time suggest that other factors beyond the laccase family genotype control the production of these enzymes in natural environments. Several of these factors have been genetically mapped as activity QTLs (44). Laccase activity values recorded for the SSF cultures were similar to others reported for *Pleurotus* species growing in similar substrates (3) but lower than those reported for SSF cultures made using agricultural wastes (16, 45).

Isozyme patterns in SmF and SSF. The zymograms of the intra- and extracellular laccase fractions revealed three isozymes: Lacc2, Lacc10, and Unk1 (Fig. 4). Unk1 had been recently described in a study of SSF cultures performed using tomato pomace (16), and it appeared to be abundant under the SSF conditions used in our study, which were more similar to the natural environmental conditions under which *P. ostreatus* grows than the conditions used for the submerged cultures. Interestingly, expression of this isoform does not appear to be induced by the wheat straw extract and consequently could be associated with the physical culture conditions, rather than with the presence of phenolic inducers. Thus far, Unk1 has not been associated with any laccase gene, no PCR-specific primers are available for it, and its transcription profile is unknown. These facts could explain the apparent discrepancy between the SSF transcription profile and the enzymatic activity data that were obtained.

Laccase upregulation by aromatic compounds (such as ferulic, p-coumaric, vanillic, and p-hydroxybenzoic acids), which occur widely in cell walls of gramineous plants (54), has been theorized to constitute a protective response to potentially toxic lignin-related compounds (51). Our results suggest that this type of water-extractable inducer, which causes transcriptional upregulation of most of *Lacc* genes in submerged fermentation, can be used to produce enzyme cocktails that can be further used in lignin-degrading industrial processes.

In summary, the results presented in this paper show that (i) laccase gene transcription is upregulated in iSmF cultures but is downregulated in SSF cultures, (ii) *Lacc2* and *Lacc10* genes are primarily responsible for the laccase activity in SmF upon induction with water-soluble wheat straw extracts, and (iii) an additional, as-yet-uncharacterized activity (Unk1) is specifically induced in SSF cultures that complements the activity of Lacc2 and Lacc10. Moreover, the patterns of enzymatic laccase activity and the *Lacc* gene family transcription profiles differ markedly between closely related strains. These differences could be exploited by biotechnological breeding programs optimizing enzyme production in submerged fermentation reactors.

ACKNOWLEDGMENTS

This work was supported by funds from project AGL2008-05608-C02-01 of the Spanish National Research Plan, from Bioethanol-Euroinnova, and from the Autonomous Government of Navarre and by additional institutional support from the Public University of Navarre.

R.C. and G.P. carried out the qPCR experiments. A.O. carried out the enzymatic determinations and set up the straw extraction protocol. M.A., R.C., A.A., and V.F. carried out the protein analyses. A.G.P. revised and edited the manuscript. L.R. led and coordinated the project. The manuscript was written by R.C. and L.R.

REFERENCES

1. **Bustin SA.** 2004. Meaningful quantification of mRNA using real-time PCR, p 225–233. *In* Weissensteiner T, Griffin H, Griffin A (ed), PCR technology: current innovations, 2nd ed. CRC Press, Boca Raton, FL.
2. **Bustin SA, et al.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. **55**: 611–622.
3. **Camarero S, Bockle B, Martinez MJ, Martinez AT.** 1996. Manganese-mediated lignin degradation by *Pleurotus pulmonarius*. Appl. Environ. Microbiol. **62**:1070–1072.
4. **Chandler DP, Wagnon CA, Bolton H, Jr.** 1998. Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. Appl. Environ. Microbiol. **64**:669–677.
5. **de Souza-Cruz PB, Freer J, Siika-Aho M, Ferraz A.** 2004. Extraction and determination of enzymes produced by *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. Enzyme Microb. Technol. **34**: 228–234.
6. **Dheda K, et al.** 2005. The implications of using an inappropriate refer-

ence gene for real-time reverse transcription PCR data normalization. Anal. Biochem. **344**:141–143.
7. **Economou CN, Makri A, Aggelis G, Pavlou S, Vayenas DV.** 2010. Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil. Bioresour. Technol. **101**:1385–1388.
8. **Faraco V, et al.** 2008. Heterologous expression of heterodimeric laccase from *Pleurotus ostreatus* in *Kluyveromyces lactis*. Appl. Microbiol. Biotechnol. **77**:1329–1335.
9. **Folin O, Denis W.** 1912. On phosphotungstic-phosphomolybdic compounds as color regents. J. Biol. Chem. **12**:239–243.
10. **Giardina P, et al.** 1996. The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*. Eur. J. Biochem. **235**:508–515.
11. **Giardina P, et al.** 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. Biochem. J. **341**:655–663.
12. **Goudopoulou A, Krimitzas A, Typas MA.** 2010. Differential gene expression of ligninolytic enzymes in *Pleurotus ostreatus* grown on olive oil mill wastewater. Appl. Microbiol. Biotechnol. **88**:541–551.
13. **Hammel KE, Cullen D.** 2008. Role of fungal peroxidases in biological ligninolysis. Curr. Opin. Plant Biol. **11**:349–355.
14. **Heid CA, Stevens J, Livak KJ, Williams PM.** 1996. Real time quantitative PCR. Genome Res. **6**:986–994.
15. **Higuchi R, Fockler C, Dollinger G, Watson R.** 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology **11**:1026–1030.
16. **Iandolo D, Piscitelli A, Sannia G, Faraco V.** 2011. Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace. Appl. Biochem. Biotechnol. **163**:40–51.
17. **Kainz P.** 2000. The PCR plateau phase—towards an understanding of its limitations. Biochim. Biophys. Acta **1494**:23–27.
18. **Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N.** 2007. Statistical significance of quantitative PCR. BMC Bioinformatics **8**:131.
19. **Kobayashi A, et al.** 2007. The glucoamylase-encoding gene (*glaB*) is expressed in solid-state culture with a low water content. Biosci. Biotechnol. Biochem. **71**:1797–1799.
20. **Kunamneni A, Plou FJ, Ballesteros A, Alcalde M.** 2008. Laccases and their applications: a patent review. Recent Pat. Biotechnol. **2**:10–24.
21. **Larraya L, et al.** 1999. Identification of incompatibility alleles and characterisation of molecular markers genetically linked to the *A* incompatibility locus in the white rot fungus *Pleurotus ostreatus*. Curr. Genet. **34**: 486–493.
22. **Larraya LM, et al.** 2002. Quantitative trait loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. Appl. Environ. Microbiol. **68**:1109–1114.
23. **Larraya LM, et al.** 1999. Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **65**:3413–3417.
24. **Larraya LM, Pérez G, Ritter E, Pisabarro AG, Ramírez L.** 2000. Genetic linkage map of the edible basidiomycete *Pleurotus ostreatus*. Appl. Environ. Microbiol. **66**:5290–5300.
25. **Lettera V, et al.** 2010. Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body. Fungal Biol. **114**:724–730.
26. **Ling D, Salvaterra PM.** 2011. Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. PLoS One **6**:e17762.
27. **Lyng MB, Laenkholm AV, Pallisgaard N, Ditzel HJ.** 2008. Identification of genes for normalization of real-time RT-PCR data in breast carcinomas. BMC Cancer **8**:20.
28. **Majeau JA, Brar SK, Tyagi RD.** 2010. Laccases for removal of recalcitrant and emerging pollutants. Bioresour. Technol. **101**:2331–2350.
29. **Mansur M, Suarez T, Gonzalez AE.** 1998. Differential gene expression in the laccase gene family from basidiomycete I-62 (CECT 20197). Appl. Environ. Microbiol. **64**:771–774.
30. **Martínez AT, et al.** 2005. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. **8**:195–204.
31. **Miller GL.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**:426–428.
32. **Palmieri G, et al.** 2003. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. Enzyme Microb. Technol. **33**:220–230.
33. **Pérez G, Pangilinan J, Pisabarro AG, Ramírez L.** 2009. Telomere organization in the ligninolytic basidiomycete *Pleurotus ostreatus*. Appl. Environ. Microbiol. **75**:1427–1436.
34. **Pezzella C, et al.** 2009. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. Curr. Genet. **55**:45–57.
35. **Pfaffl MW.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**:e5.
36. **Pfaffl MW.** 2004. Quantification strategies in real-time PCR, p 87–120. *In* Bustin SA (ed), A-Z of quantitative PCR. International University Line, La Jolla, CA.
37. **Pfaffl MW, 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.
38. **Pfaffl MW, Horgan GW, Dempfle L.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30**:e36.
39. **Piscitelli A, et al.** 2011. Induction and transcriptional regulation of laccases in fungi. Curr. Genomics **12**:104–112.
40. **Piscitelli A, Giardina P, Mazzoni C, Sannia G.** 2005. Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **69**:428–439.
41. **Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G.** 2010. Heterologous laccase production and its role in industrial applications. Bioeng. Bugs **1**:254–264.
42. **Qin L-X, et al.** 2006. Evaluation of methods for oligonucleotide array data via quantitative real-time PCR. BMC Bioinformatics **7**:23.
43. **Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM.** 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. **339**:62–66.
44. **Santoyo F, González AE, Terrón MC, Ramírez L, Pisabarro AG.** 2008. Quantitative linkage mapping of lignin-degrading enzymatic activities in *Pleurotus ostreatus*. Enzyme Microb. Technol. **43**:137–143.
45. **Sathishkumar P, Murugesan K, Palvannan T.** 2010. Production of laccase from *Pleurotus florida* using agro-wastes and efficient decolorization of Reactive blue 198. J. Basic Microbiol. **50**:360–367.
46. **Schmittgen TD, Zakrajsek BA.** 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Methods **46**:69–81.
47. **Sharma RK, Arora DS.** 2010. Production of lignocellulolytic enzymes and enhancement of *in vitro* digestibility during solid state fermentation of wheat straw by *Phlebia floridensis*. Bioresour. Technol. **101**:9248–9253.
48. **Soden DM, Dobson AD.** 2001. Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. Microbiology **147**:1755–1763.
49. **Ståhlberg A, Hakansson J, Xian X, Semb H, Kubista M.** 2004. Properties of the reverse transcription reaction in mRNA quantification. Clin. Chem. **50**:509–515.
50. **Suslov O, Steindler DA.** 2005. PCR inhibition by reverse transcriptase leads to an overestimation of amplification efficiency. Nucleic Acid Res. **33**:e181.
51. **Thurston CF.** 1994. The structure and function of fungal laccases. Microbiology **140**:19–26.
52. **Vandesompele J, et al.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3**:research0034.0031–0034.0011.
53. **Vandesompele J, Kubista M, Pfaffl M.** 2009. Reference gene validation software for improved normalization, p 47–64. *In* Logan J, Edwards K, Saunders N (ed), Real-time PCR: current technology and applications. Caister Academic Press, London, United Kingdom.
54. **Yamamoto E, Bokelman GH, Lewis NG.** 1989. Phenylpropanoid metabolism in cell walls: an overview, p 68–88. *In* Lewis NG, Paice MG (ed), Plant cell wall polymers. Biogenesis and biodegradation. American Chemical Society, Washington, DC.
55. **Zhang Q, et al.** 2006. A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. J. Appl. Phycol. **18**:445–450.