

Influence of initial pH of the growing medium on the activity, production and genes expression profiles of laccase of *Pleurotus ostreatus* in submerged fermentations

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Abstract

Background: Enzymatic activity and laccase isoenzymes number of *Pleurotus ostreatus* grown in different pH values of the growing medium in submerged fermentation and incubated in buffer solutions of different initial pH values were determined. The expression profiles of five laccase genes (Lacc1, Lacc4, Lacc6, Lacc9 and Lacc10) in these cultures were also studied.

Results: The highest laccases activity was obtained in cultures grown at initial pH of 4.5 and the lowest in cultures grown at initial pH of 8.5. Isoenzyme profiles were different in all the cases. Lacc1, Lacc4, Lacc6 and Lacc10 were expressed in all the cultures.

Conclusions: The initial pH of the growing medium is an important factor for regulating the expression of laccase genes, having an effect on the activity and on the laccase isoenzymes number produced by *P. ostreatus* in SmF. This is the first report on the influence of different initial pH values of the growing medium on the laccases activity, laccase isoenzymes number and laccases expression profiles of *P. ostreatus* grown in submerged fermentation.

Keywords: laccases, *Pleurotus ostreatus*, submerged fermentation, zymogram.

INTRODUCTION

Laccases (EC 1.10.3.2) are glycoproteins classified as multi-copper oxidases, that catalyze one-electron oxidation of a wide range of inorganic and organic compounds with the reduction of oxygen to water (Gianfreda et al. 1999). The white rot fungi have been the most used for laccases production (Guillen et al. 2000; Galhaup et al. 2002). It has been reported that in fungi the activity and laccase isoenzymes number depend on environmental conditions, temperature, pH, inductors, culture conditions and medium composition (Durán et al. 2002; Téllez-Téllez et al. 2008; Piscitelli et al. 2011; Pezzella et al. 2012). *Pleurotus ostreatus* showed the maximal laccase production at pH value of 5.5 (Krishna-Prasad et al. 2005). Wang et al. (2006) observed that *Monotospora* sp had the maximal laccase production at an initial pH of 8.5 in submerged fermentation (SmF). It was found that *Trametes versicolor* showed activity of laccases at pH of 5.2 (Tavares et al. 2006). *Botryosphaeria rhodina* MAMB-05 did not show significant differences in laccases production in a wide pH values range from 3.5 to 8.0, however, it enhanced fifteen times when the pH increased from 4.0 to 5.5 in the presence of inductor (veratryl alcohol) in SmF (Dekker et al. 2007). Adejoye and Fasidi (2009), reported that the

initial pH of 5.5 is an optimum value to obtain maximal biomass and laccases activity by *Schizophyllum commune* (Fr.) in SmF. Moreira-Neto et al. (2009), reported that *Lentinus crinitus* and *Psilocybe castanella* showed the greatest laccases activity at pH values of 3.5 and 4.5, respectively. In *P. ostreatus*, the presence of one and two laccase isoenzymes at initial pH values of 3.5 and 6.5, respectively were observed Díaz et al. (2011b). Palmieri et al. (2000) studied the expression of two laccase genes (Lacc6 and Lacc10) in *P. ostreatus* and found that the expression of Lacc6 increased in the presence of copper. Luis et al. (2005) found different expression levels of laccase genes in basidiomycetes isolated from soil. Castanera et al. (2012) examined the expression profiling of laccases of different strains of *P. ostreatus* and found that the activity and expression of these enzymes depend on the strain used in solid state fermentation and in SmF. Several studies have been carried out in order to know the enzymatic inductor type and the enzymatic inductor concentration as well as the composition of the culture medium to increase the laccases activity, however, there is very little information on the effect of the initial pH of the growing medium on the molecular and enzymatic aspects of laccase of *P. ostreatus*. In this work, the effect of different initial pH values (3.5, 4.5, 6.5 and 8.5) of the growing medium on the production and number of laccases of *P. ostreatus* incubated in buffer solutions of different initial pH values (3.5, 4.5, 6.5 and 8.5) in SmF were determined. The expression of five laccase genes (Lacc1, Lacc4, Lacc6, Lacc9 and Lacc10) in the *P. ostreatus* cultures was also studied.

MATERIALS AND METHODS

Microorganism

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

Culture conditions

Mycelial plugs (4 mm diameter) taken from the periphery of colonies of *P. ostreatus* grown for 7 days at 25°C in Petri dishes containing potato dextrose agar were used as inoculum. A liquid medium, previously optimized for the production of laccases by this fungus in SmF, was prepared containing (in g/L): glucose, 10; yeast extract, 5; KH₂PO₄, 0.6; MgSO₄·7H₂O, 0.5; K₂HPO₄, 0.4; CuSO₄·5H₂O, 0.25; FeSO₄·7H₂O, 0.05; MnSO₄, 0.05; ZnSO₄·7H₂O, 0.001 (Téllez-Téllez et al. 2008). Flasks of 250 mL containing 50 mL of culture medium adjusted by separated at different pH values (3.5, 4.5, 6.5 and 8.5) using either 0.1 M HCl or NaOH were prepared. Flasks were inoculated with three mycelial plugs and incubated at 25°C for 23 days on a rotary shaker at 120 rpm (Téllez-Téllez et al. 2008). Evaluations of biomass and laccases activity were carried out on samples taken every 24 hrs after the third day of growth. Four fermentations were performed by triplicate. The zymogram and expression patterns were obtained at 144, 168, 264, 312, 408, 504 and 528 hrs of fermentation.

Enzymatic extract and biomass evaluation

The enzymatic extract (EE) was obtained by filtration of the cultures using filter paper (Whatman No. 4), and the biomass (X) was determined as difference of dry weight (g/L).

Assay of biomass $X = X(t)$ was done using the Velhurst-Pearl or logistic equation,

$$\frac{dX}{dt} = \mu \left[1 - \frac{X}{X_{max}} \right] X$$

[Equation 1]

Where μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. The solution of Equation 1 is as follows:

$$X = \frac{X_{max}}{1 + Ce^{-\mu t}}$$

[Equation 2]

Where, $C = (X_{max} - X_0)/X_0$, and $X = X_0$; the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using a non-linear least square-fitting program "Solver" (Excel, Microsoft) (Díaz-Godínez et al. 2001).

Laccase activity assays

Laccase activity was determined in each EE of cultures grown at different pH values (3.5, 4.5, 6.5 and 8.5) incubated in different buffer solution pH values (3.5, 4.5, 6.5 and 8.5) by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol (DMP) as substrate. It was carried out in order to observe the relationship between pH of production of laccases and maximum pH values of laccase activity. The assay mixture contained 950 μ L substrate (2 mM DMP in either 0.1 M citrate buffer pH 3.5, acetate buffer pH 4.5, phosphate buffer pH 6.5 or Tris-base buffer pH 8.5) and 50 μ L EE, which were incubated at 40°C for 1 min (Téllez-Téllez et al. 2005). One enzymatic unit (U) of laccase activity is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The activity was expressed in U/L of EE.

Laccases zymogram

The laccase activity was detected by zymography, using the modified technique SDS-PAGE (Téllez-Téllez et al. 2008). The running gel contained 100 g acrylamide/L and 27 g bis-acrylamide/L. The stacking gel contained 40 g acrylamide/L and 27 g bis-acrylamide/L. Each EE (20 μ L approx.) was mixed with sample buffer without a reducing agent for the disulfide bonds. The samples were placed in gels (thickness 0.75 mm) of Mini-Protean III electrophoresis system (BioRad) and then 150 V was applied for 1 to 1.25 hrs. After the electrophoresis, gels were washed with deionised water on an orbital shaker (20 to 30 rpm) for 30 min, and the water was changed every 10 min to remove SDS. Finally, the gels were incubated at room temperature in substrate solutions (2 mM DMP in buffer solutions at different initial pH values of 3.5, 4.5, 6.5 and 8.5). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 30 min. Molecular weight marker 10-250 kDa (Precision Plus Protein™ Standards, BioRad) was used.

Total RNA isolation and RT-PCR

A RT-PCR semi-quantitative was used to study genes expression of laccase of *P. ostreatus*. RNA was isolated from frozen mycelium produced at different fermentation times (see Culture Conditions Section), using the TRIZOL (Invitrogen) extraction and was spectrophotometrically quantified by determining the optical density at OD_{260/280}. RNA was treated with DNase I free of RNase (Invitrogen) and then suspended in 20 μ L of diethylpyrocarbonate-treated water.

cDNA synthesis was performed using oligos dT and Moloney murine Leukemia virus reverse transcriptase (M-MuLV-RT; Fermentas) according to the manufacturer's instructions. The RT reaction mixture product (1 μ g) and 10 μ mol of specific primers were mixed for specific PCR amplification using the Kit AccsesQuick™ (PROMEGA®). Denaturation conditions were 94°C for 3 min. The program included 35 cycles of 94°C for 40 sec, 56°C for 40 sec and 72°C for 50 sec. Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (gpd) was tested as housekeeping. For the design of laccase-isoenzyme-specific primer (Table 1), the open *primer-blast* software was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

The complete sequences of laccase mRNAs reported in the Gene bank were used (<http://www.ncbi.nlm.nih.gov/nucleotide>).

Table 1. Oligonucleotides sequences used for PCR.

Access number	Gene (mRNA)	Sequence (5' - 3')
FM202673 FM202670.1	Lacc1	Fw ATGGACCAATCCCTACTCCC Rv GGCATTGGGTGCTAAGATG
FM202672	Lacc4	Fw TGCGCCTGTACTCTCATTTG Rv GGTAGAGACCGTGCCAATGT
AJ005018 AJ005017.2	Lacc6	Fw CGCTTGTTTCACTCGCATT Rv ATAGTGTGAGTCGGGATGG
Z34847 Z22591.1	Lacc9	Fw GTCTCTCTGACGGCTTAC Rv ATACTGGGTGGAAAGATGCG
Z34848	Lacc10	Fw ACGAGCTGGAGGCTAACAAA Rv TCACGAAGCGAATAGTGACG
GU062704.1 AB690874.1	gpd	Fw TCTGCGGTGTTAACCTTGAGTCGT Rv TGGTAGCGTGGATGGTGCTCATT

<http://www.ncbi.nlm.nih.gov/nucore/?term=laccases%20Pleurotus%ostreatus>

Statistical analysis

The enzymatic activity and biomass values were plotted using the mean and the error bars represent the standard error of data obtained from triplicate experiments.

RESULTS AND DISCUSSION

Growth of the cultures of *P. ostreatus* at different initial pH of the growing medium in SmF

The growth of *P. ostreatus* at different initial pH is shown in Figure 1. The X_{max} values were of 5.2, 5.5, 9.6 and 8.3 g/L, and the μ values were of 0.006, 0.014, 0.018 and 0.02 h⁻¹ in cultures grown at initial pH of 3.5, 4.5, 6.5 and 8.5, respectively. In this study, the μ values were around 0.02 h⁻¹ at initial pH values of 6.5 and 8.5. These values were similar to those reported for this fungus grown at initial pH of 6.0 (Télez-Télez et al. 2008), and to those obtained when *P. ostreatus* grew in the presence of ammonium sulfate as nitrogen source at pH of 6.5 (Tlecuil-Beristain et al. 2008). The μ values decreased 1.5-fold and 3-fold approx. at initial pH values of 4.5 and 3.5, respectively. The X_{max} obtained at initial pH values of 6.5 and 8.5 was around 10% higher than that reported for *P. ostreatus* grown under similar conditions (Tlecuil-Beristain et al. 2008). The X_{max} obtained at pH values of 3.5 and 4.5 was 30% lower than that obtained at initial pH values of 6.5 and 8.5. It has been reported that the initial pH values of *P. ostreatus* growing media are between 6.0 and 6.5 (Télez-Télez et al. 2008; Tlecuil-Beristain et al. 2008). Diaz et al. (2011b) reported that *P. ostreatus* produced high yields of biomass in a short time, producing metabolites that modified the pH from 3.5 to 6.0-6.5 in a non-buffered medium. In this research, this fungus grew between initial pH values of 3.5 and 8.5.

Laccases activity

The highest laccases activity was produced in the stationary growth phase of the fungi in all the cases.

Figure 2 shows the laccases activity of *P. ostreatus* grown at initial pH of 3.5. The maximum laccases activity was of 21360 U/L and 29490 U/L in those EE incubated in buffer solutions pH 3.5 and pH 4.5, respectively. On the other hand, a maximum laccase activity of 1595 U/L and 3535 U/L was observed in those EE incubated in buffer solutions pH 6.5 and 8.5, respectively.

The EE of cultures grown at initial pH of 4.5 and incubated in buffer solution pH 3.5 had a maximum laccases activity of around 70000 U/L. The maximum laccases activity was of 6195 U/L and 16310 U/L in the EE incubated in buffer solutions pH 6.5 and 8.5, respectively (Figure 3).

Figure 4 shows profiles of laccases activity obtained at initial pH of the growing medium of 6.5. Laccase activity patterns showed a maximum laccase activity of around 25000 U/L in buffer solutions pH 3.5, 4.5 and 6.5. The maximum laccase activity was of 5075 U/L in buffer solution pH 8.5.

Figure 5 shows profiles of laccases activity obtained at initial pH of the medium of 8.5. Maximum values of laccase activity observed at incubation buffer solutions pH 6.5 and 8.5 were of 2420 U/L and 3715 U/L, respectively. Maximum laccase activity at buffer solutions pH 3.5 and 4.5 reached values of 2420 U/L.

In this study, the activity values at initial pH of 4.5 incubated in buffer solution pH 4.5 (77500 U/L) were around six-fold (12200 U/L) and twice (37000 U/L) higher than that laccases activity reported by Tlecuitl-Beristain et al. (2008) and Díaz et al. (2011b), respectively. These results show that the optimal initial pH for the biomass production and laccase production are between 6.5 and 8.5, and between 3.5 and 4.5, respectively. In general, there is little information about the initial pH of the culture medium on the laccases activity. Mouso et al. (2003) reported that *Stereum hirsutum* did not produce laccases at alkaline initial pH. Díaz et al. (2011b) observed that the laccases activity of *P. ostreatus* depends on the pH of the culture medium.

Laccase isoenzymes patterns

Figure 6 shows zymograms of laccases obtained at initial pH of 3.5. Two isoenzymes of 29 and 65 kDa approx. were observed in gels incubated in buffer solutions pH 3.5, 4.5 and 6.5 (Figure 6a, 6b and 6c, respectively). Only the isoenzyme of 29 kDa was observed at the end of the fermentation (528 hrs) (Figure 6d) in gel incubated in buffer solution pH 8.5.

In the EE of the cultures grown at initial pH of 4.5, three isoenzymes were observed in gels incubated in buffer solutions pH 3.5, 4.5 and 6.5 (Figure 7a, 7b and 7c, respectively), two of them were similar to those perceived at initial pH of 3.5 and the other had a molecular weight of 47 kDa approx. The isoenzyme of the lowest molecular weight (29 kDa approx.) had the highest intensely-stained band. Only the isoenzyme of 29 kDa was shown in gel incubated in buffer solution pH 8.5 (Figure 7d).

Figure 8 shows zymograms of laccases of *P. ostreatus* grown at initial pH of 6.5. Three isoenzymes of approx. 29, 47 and 65 kDa were observed in the gels incubated in buffer solutions pH 3.5 and 4.5 (Figure 8a and 8b, respectively). Four isoenzymes (molecular weights; 65, 47, 38 and 29 kDa) were perceived in gel incubated in buffer solution pH 6.5 (Figure 8c). One isoenzyme (29 kDa) with low activity was observed in the gel incubated in buffer solution pH 8.5 (Figure 8d).

Zymogram of laccases of *P. ostreatus* grown at initial pH of 8.5 are shown in Figure 9. Two isoenzymes of 47 and 29 kDa were observed in gels incubated in buffer solution pH 3.5. From those, the isoenzyme of lower molecular weight had the highest intensely-stained band and was presented in all the fermentation (Figure 9a).

Three isoenzymes (molecular weights; 65, 47 and 29 kDa) were perceived in gels incubated in buffer solutions pH 4.5 and 6.5 (Figure 9b and 9c, respectively). One laccase isoenzyme (of 29 kDa molecular weight) was observed in the gel incubated in buffer solution pH 8.5 (Figure 9d), having the lowest intensely-stained band.

In different family of fungi, laccase isoenzymes can be present due of either the expression of different genes or the postransductional modifications. This is the first study on the initial pH of the growing medium on the laccase isoenzymes patterns. It was observed that the isoenzymes production is regulated by this parameter. In this research, the zymogram patterns showed four laccase isoenzymes with molecular weights of 65, 47, 38 and 29 kDa. Téllez-Téllez et al. (2008) observed that *P. ostreatus* grown in submerged culture, presented four isoenzymes of with laccase activity (molecular weights were not reported). Laccases isoenzymes of *P. ostreatus* of a molecular weight (29 kDa) as low as the reported in this work have never been found. In previous research, laccases of 43.7 kDa (Tlecuitl-Beristain et al. 2008), 67 kDa (Hublik and Schinner, 2000), 61 kDa (laccase poxA1) and 67 kDa (laccase poxA2) (Palmieri et al. 1997), 62 kDa (Lacc6) (Giardina et al. 1999), poxA3a and poxA3b consisting of a large (67 kDa) and a small (18 or 16 kDa, respectively) subunits were reported (Giardina et al. 2007). None study have been carried out to determine the molecular weights of Lacc1, Lacc4, Lacc9 and Lacc10. Téllez-Téllez et al. (2012) found a laccase isoenzyme of 56 kDa (SDS-PAGE) of *P. ostreatus* grown in submerged culture. It showed a gene with 98.2 percent sequence similarity to Lacc10.

Laccase gene expression from *P. ostreatus*

Figure 10a shows PCR products obtained at initial pH of 3.5 at different fermentation times (144, 168, 264, 312, 408, 504 and 528 hrs). Five laccase genes were examined; Lacc10, Lacc4, Lacc1 and Lacc6 that were expressed in nearly all the fermentation times, however, Lacc9 was not expressed at any time. Lacc10 and Lacc4 were not expressed at 312 hrs and 264 hrs, respectively. Lacc1 was expressed at low level at 264 and 312 hrs. Lacc6 showed very little expression levels at 264 and 408 hrs of fermentation.

The expression of the laccase genes in the cultures at initial pH of 4.5 is shown in Figure 10b. Lacc9 was only expressed at 144 hrs of fermentation. Lacc10, Lacc4, Lacc1 and Lacc6 were expressed at different levels during the fermentation, however, Lacc6 showed more intensely-stained bands than the rest of the genes.

Figure 10c shows genes expression profiles of laccase of cultures grown at initial pH of 6.5. In this case, Lacc10, Lacc4, Lacc1 and Lacc6 were expressed in all the fermentation times, however, Lacc9 was not expressed. The expression of Lacc10, Lacc4, Lacc1 and Lacc6 increased during the fermentation.

Figure 10d shows gene expression profiling of laccases at initial pH of 8.5 at different fermentation times. Lacc4, Lacc1 and Lacc6 were expressed during the fermentation. Lacc10 was expressed at low level during the fermentation. Lacc6 showed more intensely-stained bands than the rest of the genes. Lacc9 was not expressed.

Several studies have been carried out on the effect of metallic ion, aromatic compounds and carbon and/or nitrogen sources on the laccases expression, however, there is no report on the effect of the initial pH of the growing medium on the laccases expression profiles of *P. ostreatus*. Collins and Dobson (1997) reported that the laccase expression of the white-rot fungus *T. versicolor* is regulated at level of gene transcription by copper and nitrogen. RT-PCR studies showed that the addition of copper and nitrogen to the medium increased the growth of the fungus and enhanced the laccases activity, increasing gene transcription levels of the enzyme. Díaz et al. (2011a) studied the effect of the copper on the laccase activity of several strains of *P. ostreatus* grown in SmF. In general, the cultures added with copper had the lowest maximal biomass and showed very high laccases activity. The phenolic and phenolic structure compounds either related or derived from the lignin are frequently used to increase the production of fungal laccases (De Souza et al. 2004). The induction of laccases by phenolic compounds might represent a stress response to the toxic aromatic compounds (Piscitelli et al. 2011). Castanera et al. (2012) studied the expression laccase profiles of different strains of *P. ostreatus* in solid and submerged condition and found that the genes Lacc2 and Lacc10 were the main sources of laccase activity in SmF. Five laccase genes evaluated in this research were monomeric. On the other hand, Lacc2 has been reported as an atypical heterodimeric laccase, constituted by a large subunit, clearly homologous to other fungal laccases, and a small subunit, probably involved in the stabilization of such complex (Palmieri et al. 2003; Giardina et al. 2007). Lacc12 is a laccase recently isolated from *P. ostreatus* fruiting body (Lettera et al. 2010). The analysis of *P. ostreatus* genome disclosed the existence of new laccase genes, putatively coding for previously uncharacterized laccases (Lacc3, Lacc5, Lacc7 and Lacc8) (Pezzella et al. 2009).

In this study, four laccase isoenzymes were observed, and four laccase genes (Lacc1, Lacc4, Lacc6, and Lacc10) were expressed. It could indicate correspondence between genes. These results suggest that Lacc6 and Lacc10 are isoenzymes of 65 and 47 kDa, respectively, and Lacc4 and Lacc1 could express laccase isoenzymes of molecular weights of 38 and 29 kDa, respectively. One laccase gene (Lacc9) was not expressed. Results of this research show that the expression of laccase isoenzymes of *P. ostreatus* is regulated by the pH. This is an important finding since the pH values of growth, laccase production and isoenzymes activity were different.

CONCLUDING REMARKS

The initial pH of the growing medium is an important factor for regulating the expression of laccase genes that have an effect on the activity and number of laccase isoenzymes produced of *P. ostreatus* in SmF. This is the first report about the effect of the initial pH of the growing medium on the production

and expression of laccases profiles of *P. ostreatus* grown in SmF. These results contribute to the knowledge of the regulation of laccase gene expression.

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Figures

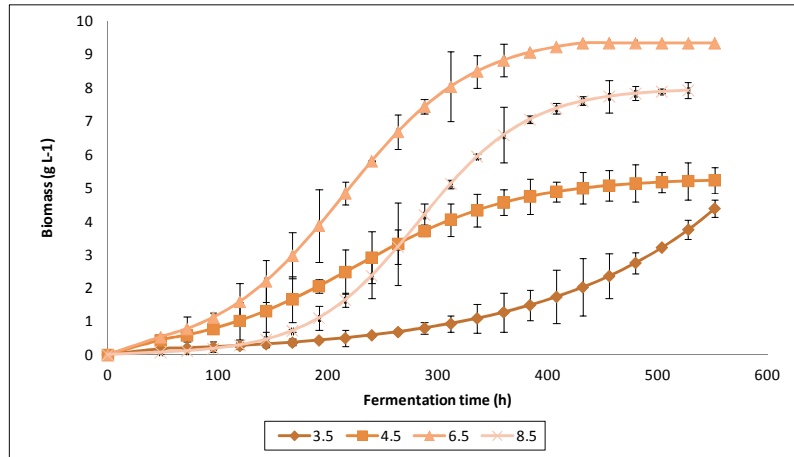


Fig. 1 Growth of *P. ostreatus* at different initial pH values in SmF.

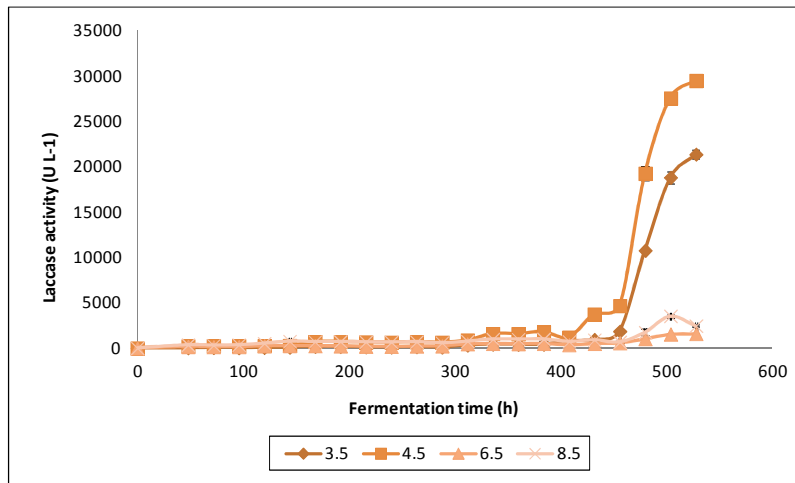


Fig. 2 Laccases activity of *P. ostreatus* grown at initial pH of 3.5 and incubated in buffer solutions at different initial pH values.

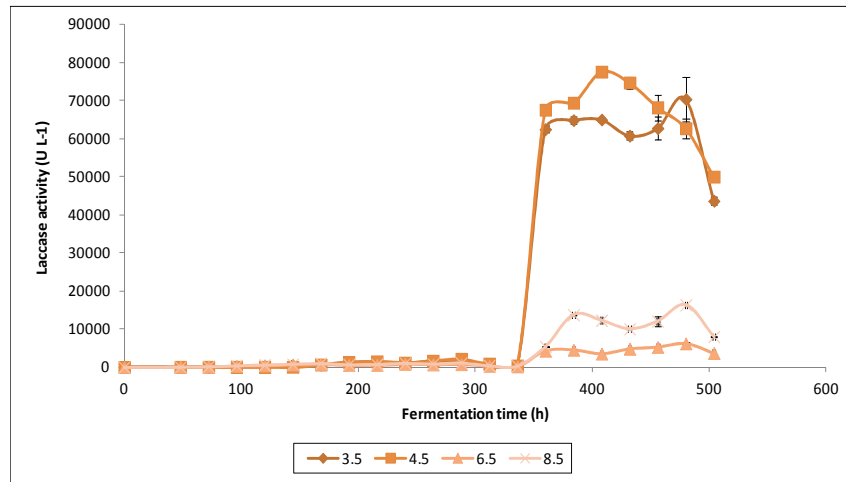


Fig. 3 Laccases activity of *P. ostreatus* grown at initial pH of 4.5 and incubated in buffer solutions at different initial pH values.

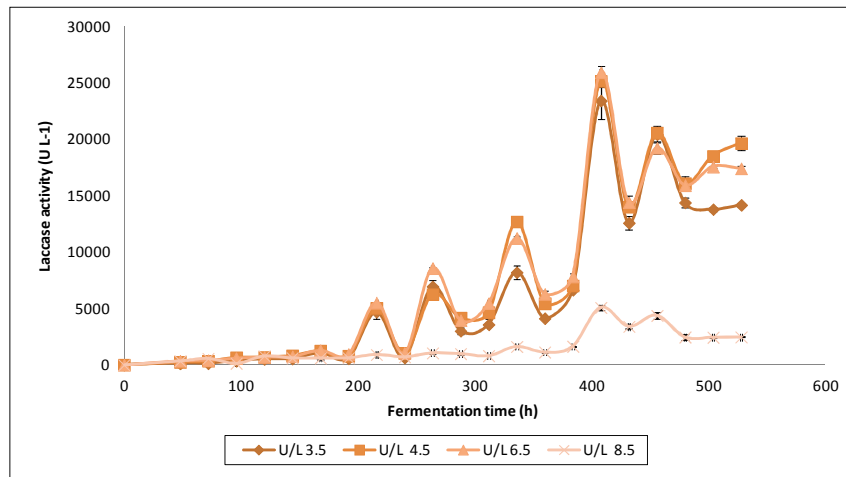


Fig. 4 Laccases activity of *P. ostreatus* grown at initial pH of 6.5 and incubated in buffer solutions at different initial pH values.

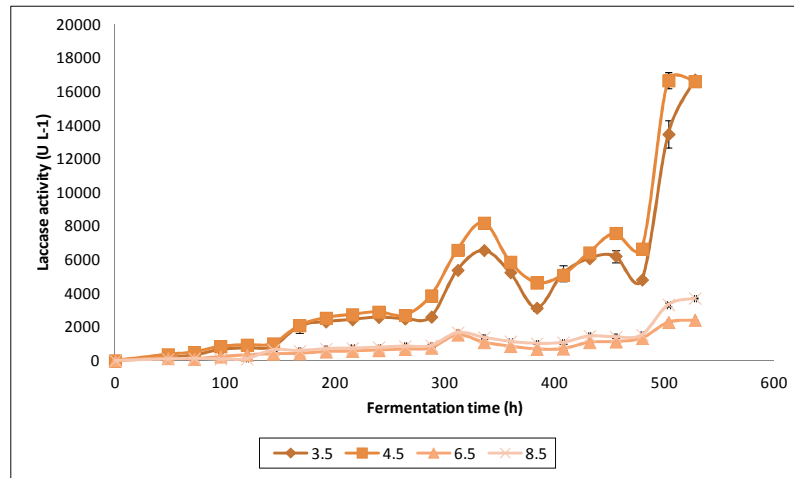


Fig. 5 Laccases activity of *P. ostreatus* grown at initial pH of 8.5 and incubated in buffer solutions at different initial pH values.

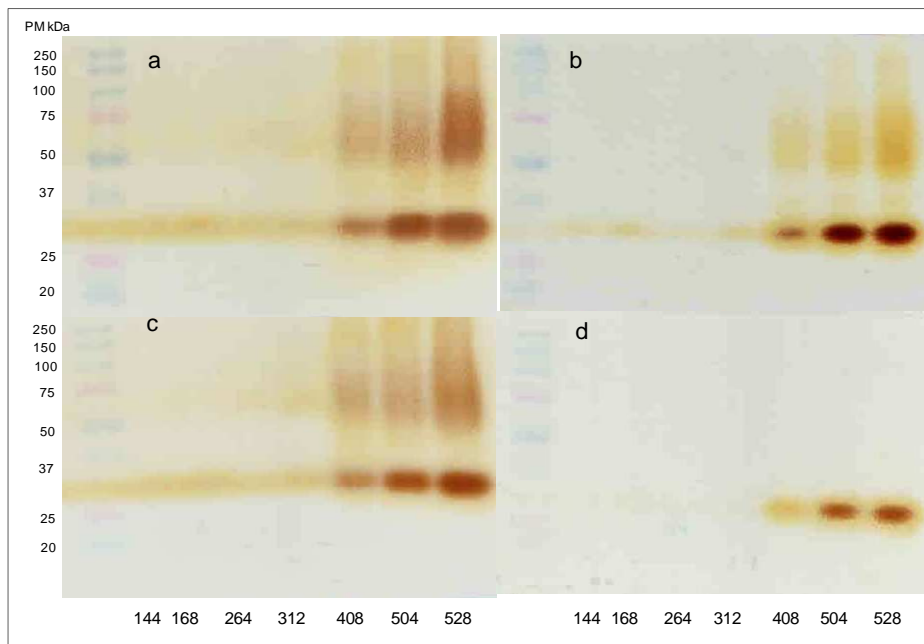


Fig. 6 Zymogram of laccases of *P. ostreatus* grown at initial pH of 3.5 and incubated in buffer solutions at different initial pH values; 3.5 (a), 4.5 (b), 6.5 (c), and 8.5 (d).

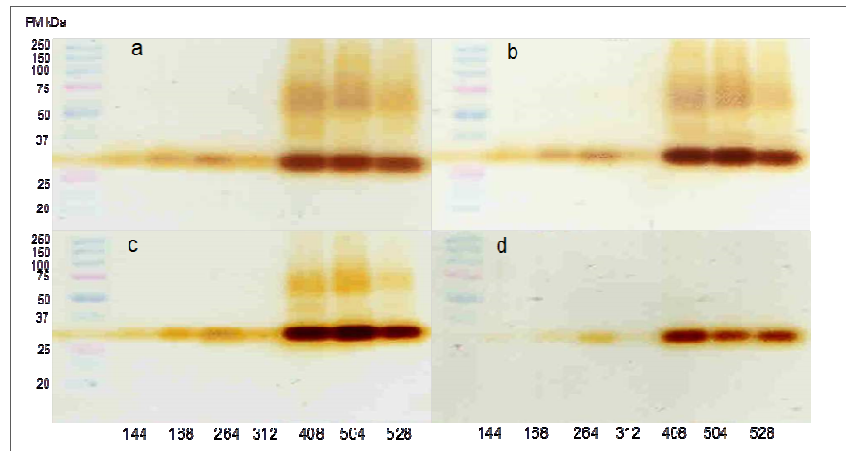


Fig. 7 Zymogram of laccases of *P. ostreatus* grown at initial pH of 4.5 and incubated in buffer solutions at different initial pH values; 3.5 (a), 4.5 (b), 6.5 (c), and 8.5 (d).

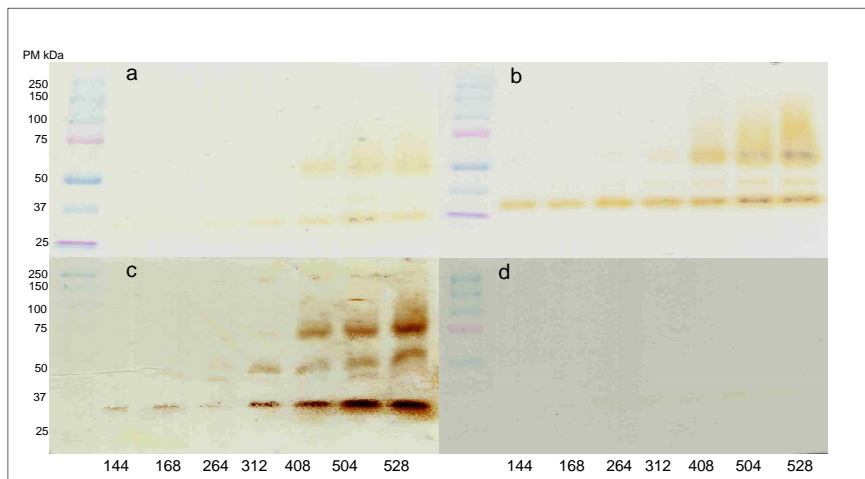


Fig. 8 Zymogram of laccases of *P. ostreatus* grown at initial pH of 6.5 and incubated in buffer solutions at different initial pH values; 3.5 (a), 4.5 (b), 6.5 (c), and 8.5 (d).

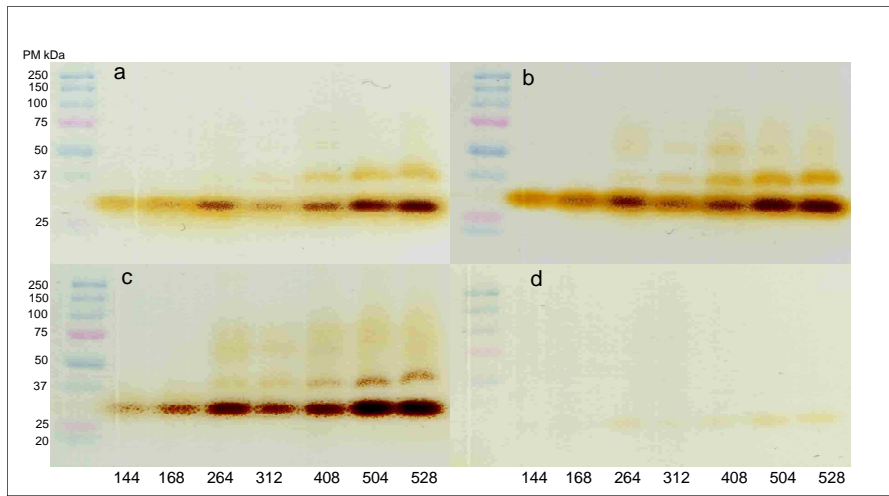


Fig. 9 Zymogram of laccases of *P. ostreatus* grown at initial pH of 8.5 and incubated in buffer solutions at different initial pH values; 3.5 (a), 4.5 (b), 6.5 (c), and 8.5 (d).

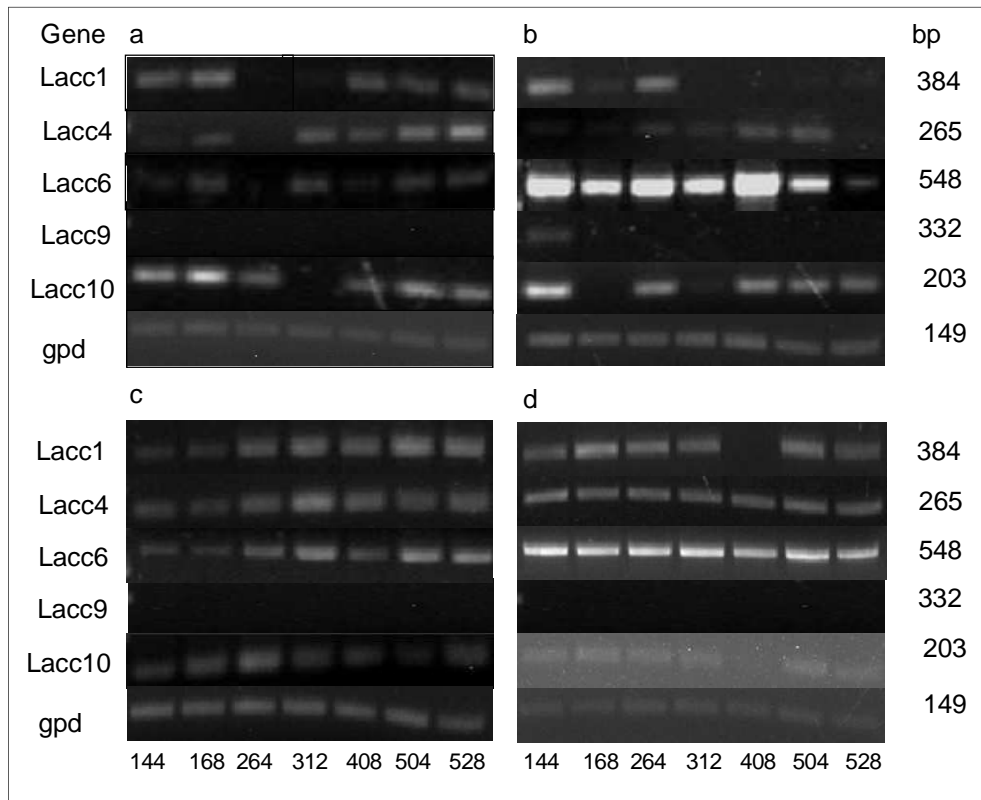


Fig. 10 Expression profiles of laccase genes of *P. ostreatus* grown at initial pH of 3.5 (a), 4.5 (b), 6.5 (c) and 8.5 (d).