LACCASE GENE EXPRESSION OF *PLEUROTUS OSTREATUS* GROWN AT DIFFERENT pH OF THE LIQUID CULTURE MEDIUM

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ABSTRACT

Pleurotus ostreatus is a white rot fungus capable of degrading many xenobiotic and recalcitrant compounds due to their ability to produce a nonspecific enzyme system able to catalyze the oxidation of many types of organic compounds including textile dyes. Expression profile of 5 genes of laccase of *P. ostreatus* developed in media at different pH in submerged fermentation was determined. 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. Several studies reported that the number of isoenzymes depend on the conditions of development of *P. ostreatus*; expression profile also is conditioned to various dependent transcription factors of carbon source, nitrogen source and pH, etc.

Key words: *Pleurotus ostreatus*, laccases, expression, genes, submerged fermentation

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 [1]. The white rot fungi have been widely used for production of laccases production [2, 3]. 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 [4 - 7]. These fungi are easily adapted to different growth conditions including submerged cultures. It has been reported to degrade pollutants and phenolic compounds produced in different industries such as textiles and paper, among others. This degradation of xenobiotic compounds is attributed mainly to enzymes phenoloxidases [8]. *P. ostreatus* produces laccase isoenzymes both constitutive and inducible, depending on growth conditions [4, 9]. Palmieri *et al.*[10] studied the expression of two genes of laccases (*Lacc* 6 and *Lacc* 10) in *P. ostreatus* and observed that in the presence of copper, the *Lacc* 6 gene expression was increased. Castanera *et al.* [11] evaluated the expression profile of laccase genes from different strains of *Pleurotus ostreatus* and they observed that the activity and expression level of these enzymes were dependent on the strain and the growth medium either solid or submerged fermentation. Diaz *et al.* [9] evaluated the expression profile of five genes of *P. ostreatus* laccases in submerged fermentation developed different initial pH, where they observed that the expression of genes depends on the initial conditions of development, suggesting the presence of transcription factors activated by pH change in the culture medium. In this study, the expression profile of five genes of laccase (*Lacc*1, *Lacc*4, *Lacc*6, *Lacc*9 and *Lacc*10) of *P. ostreatus* developed in media of different pH (3.5, 4.5, 6.5 and 8.5) in SmF was determined.

MATERIALS AND METHODS

Microorganism

A strain of *Pleurotus 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 25ºC 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; KH_2PO4, 0.6; MgSO4-

$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4.5H_2O$, 0.25; $FeSO_4.7H_2O$, 0.05; $MnSO_4$, 0.05; $ZnSO_4.7H_2O$, 0.001 [4]. 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. Flask were inoculated with three mycelial plugs and incubated at 25ºC for 23 days on a rotary shaker at 120 rpm [4]. Evaluations of biomass and laccases activity were carried out on samples taken every 24 h after the third day of growth. Four fermentations were performed in triplicate. The zymogram and expression patterns were obtained at 144, 168, 264, 312, 408, 504 and 528 h of fermentation.

Biomass evaluation

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,

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

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;

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

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) [12].

Total RNA isolation and RT-PCR

A semi-quantitative RT-PCR was used to study genes expression of laccasses 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 resuspended in 20 ìl of diethylpyrocarbonate-treated water,

Table 1. Specific primers of laccases genes of *P. ostreatus* grown at different pH initial in SmF.

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 CGCTTGTTTCACTCGCATTA Rv ATAGTGTCGAGTCGGGATGG
Z34847 Z22591.1	Lacc9	Fw GTCTCTCCTGACGGCTTCAC Rv ATACTGGGTGGAAAGATGCG
Z34848	Lacc10	Fw ACGAGCTGGAGGCTAACAAA Rv TCACGAAGCGAATAGTGACG
GU062704.1 AB690874.1	gpd	Fw TCTGCGGTGTTAACCTTGAGTCGT Rv TGGTAGCGTGGATGGTGCTCATTA

http://www.ncbi.nlm.nih.gov/nuccore/?term=laccases%20*Pleurotus*%*ostreatus*

cDNA synthesis was performed using oligos dT and Moloney *Murine Leukaemia* virus reverse transcriptase (M-MuLV-RT; Fermentas) according to the manufacturer's instructions. The RT reaction mixture product (1 µg) and 10 ρmol of specific primers were mixed for specific PCR amplification using the Kit Accses Quick™ (PROMEGA^{MR}). Denaturation conditions were 94 °C for 3 min. The program included 35 cycles of 94°C for 40 s, 56°C for 40 s and 72°C for 50 s. Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (gpd) was tested as housekeeping. For the design of isoenzyme laccase specific primers (Table 1), the open *primer-blast* software was used (http://www.ncbi.nlm.nim.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).

RESULTS AND DISCUSSION

Growth of *P. ostreatus* at different initial pH in submerged fermentation

Fig. 1 shows the growth of *P. ostreatus* at different initial pH. X_{max} values of 5.2, 5.5, 9.6 and 8.3 g / l, and ì values of 0.006, 0.014, 0.018 and 0.02 h^{-1} at initial pH of 3.5, 4.5, 6.5 and 8.5, respectively were observed. In this study, the ì values were around 0.02 h^{-1} at initial pH values of 6.5 and 8.5. These values were similar to those reported by Téllez-Téllez *et al*. [4] for this same fungus at pH 6.0, and to those obtained when *P. ostreatus* grew in the presence of ammonium sulfate as nitrogen source at pH of 6.5 [13]. The ì 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 [13]. 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 [4, 13]. Díaz *et al*. [7] 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 to 8.5.

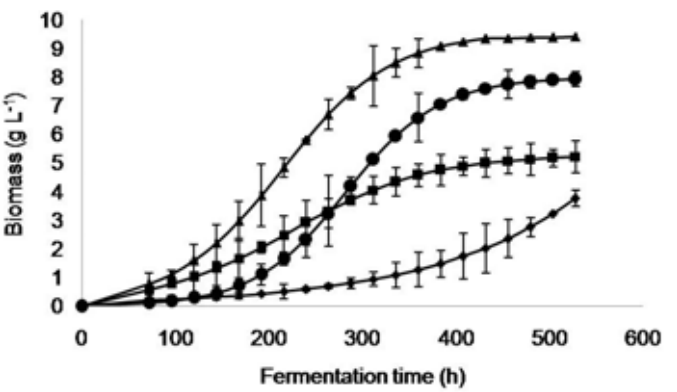

Figure 1. Growth of *P. ostreatus* at different initial pH; 3.5 (♦), 4.5(■), 6.5 (▲), and 8.5 (●) in SmF. Error bars show media ± SD of three replicate samples

Laccase genes expression of *P. ostreatus*

Fig. 2 shows the PCR products obtained at pH 3.5 at different times of fermentation (144, 168, 264, 312, 408, 504 and 528 h). Five laccase genes were examined. Lacc10, Lacc4, Lacc1 and Lacc6 were expressed in nearly all the fermentation times, however, Lacc9 was not expressed at any time. Lacc10 and Lacc4 were not expressed at 312 and 264 h, respectively. Lacc1 was expressed at low level at 264 and 312 h. Lacc6 showed very little expression at 264 and 408 h of fermentation.

The expression profile of the laccase genes in culture to initial pH of 4.5 is shown in Fig. 3. Lacc9 was only expressed at 144 h 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.

Fig. 4 shows the profile of laccase gene expression in culture at initial pH 6.5. In this case, Lacc9 was not expressed, however, Lacc10, Lacc4, Lacc1 and Lacc6 were expressed in all the fermentation times. The expression of Lacc10, Lacc4, Lacc1 and Lacc6 increased through the fermentation. The Fig 4 shows the profile of laccase gene expression in culture at initial pH 8.5. Lacc9 was not expressed. Lacc10 was expressed at low level during the fermentation. Lacc4, Lacc1 and Lacc6 were expressed during all the fermentation times. Lacc6 showed more intensely-stained bands than the rest of the genes.

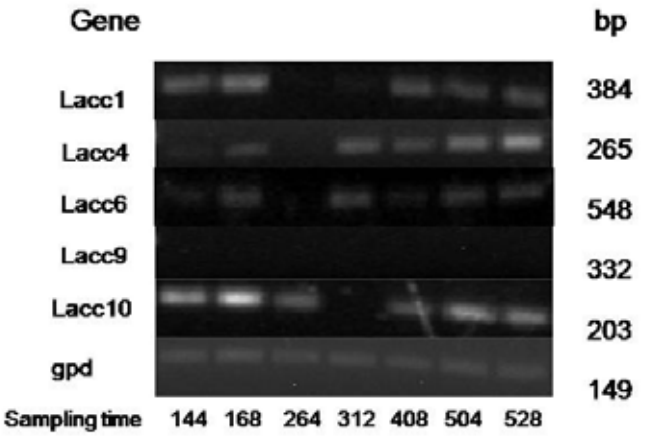

Figure 2. Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 3.5

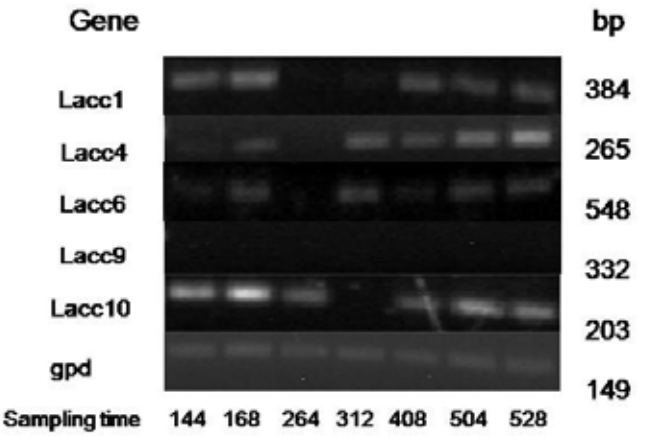

Figure 3. Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

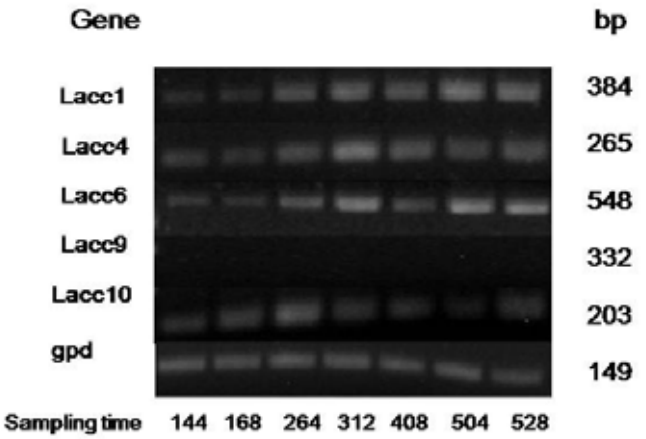

Figure 4. Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

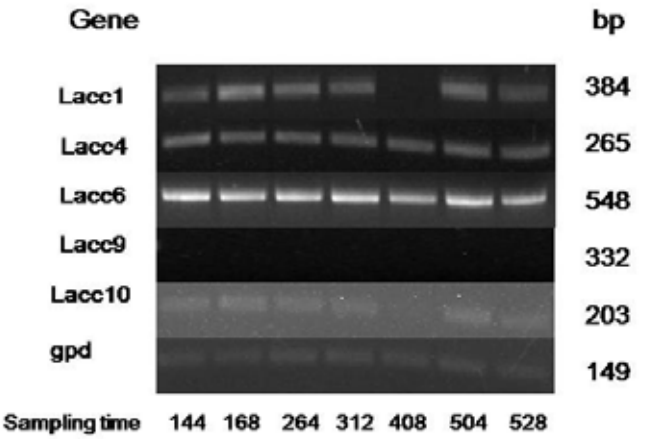

Figure 5. Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

CONCLUSION

The initial pH of the culture medium is an important factor which regulates the expression of the laccase genes in addition to having an effect on the activity and number of isoenzymes produced. These results contribute to the understanding of the regulation of the expression of the laccase genes.

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