CHARACTERIZATION OF THE GROWTH AND LACCASE ACTIVITY OF STRAINS OF *PLEUROTUS OSTREATUS* IN SUBMERGED FERMENTATION

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Kinetic parameters of growth and laccase activity of five ATCC strains of *Pleurotus ostreatus* in submerged fermentation were evaluated. The best strain for laccase production and the time of maximum laccase activity were also determined. The greatest laccase activity (37490 U/L), laccase productivity (78 U/L h), specific growth rate (0.026/h), and specific rate of laccase production (119 U/gX h) were observed with the strain of *P. ostreatus* ATCC 32783. In general, the isoenzyme patterns were different in all the cases; however, all the strains showed two laccase bands in the same position in the gel. Not all strains responded in the same way to the addition of Cu in the culture medium. In general, the sensitivity to Cu could be used to select strains having high laccase activity for commercial exploitation.

Keywords: Laccase; Fermentation; Pleurotus ostreatus; Zymogram

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INTRODUCTION

Laccases (p-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are glycoproteins that belong to the group of blue multi-copper oxidases, which use oxygen as an electron acceptor to remove hydrogen radicals from phenolic hydroxyl groups (Gianfreda et al. 1999; Thurston 1994). They catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, *ortho*- and *para*-diphenols, and also can oxidize other substrates such as aromatic amines, syringaldazine, and non-phenolic compounds, to form free radicals (Bourbonnais et al. 1997; Li et al. 1999; Robles et al. 2000). After long reaction times, there can be coupling reactions between the reaction products and even polymerization. It is known that laccases can catalyze the polymerization of various phenols and halogen, alkyl-, and alkoxy-substituted anilines (Kobayashi et al. 2001, 2003).

Due the catalytic action of laccases, these enzymes can be used for various biotechnological and environmental applications such as textile dye decolouration, delignification, pulp bleaching, effluent detoxification, biosensing, and bioremediation (Thurston 1994; Hublik and Schinner 2000; Mayer and Staples 2002). Laccases have been found mainly in white rot fungi, in other fungi, insects, some plants, and bacteria (Guillen et al. 2000; Galhaup et al. 2002). However, the successful use of laccases in bioremediation processes is based both on obtaining an organism that produces enzymes with the best catalytic properties, and on establishment of the conditions for development

of strains that produce high levels of these enzymes.

It has been suggested that laccase activity and the number of laccase isoenzymes is influenced by environmental factors such as temperature, pH, inducers, culture conditions, and medium composition (Giardina et al. 1999; Téllez-Téllez et al. 2008). It was observed that the addition of 150 µM $CuSO_4$ to culture broth increases considerably the total laccase activity of *Pleurotus ostreatus* (Giardina et al. 1999; Palmieri et al. 2000). Several studies to increase laccase production have been carried out. For example, laccase production of *Ganoderma lucidum* (D'Souza et al. 1999), *P. ostreatus* (Mikiashvili et al. 2006), and *P. sajor-caju* (Bettin et al. 2009) has been examined after changing and increasing the type and concentration of carbon and nitrogen sources. In other studies, productivity has been evaluated using different inducers with *Coriolus hirsutus* (Koroljova-Skorobogat'ko et al. 1998), *P. ostreatus* (Baldrian and Gabriel 2002), and *Streptomyces psammoticus* (Niladevi et al. 2008). Others have evaluated the production of laccasse by *P.ostreatus* (Téllez-Téllez et al. 2008; Ramírez et al. 2003) and the expression of a heterologe laccase in *Aspergillus niger*, in solid-state (SSF) and submerged fermentation (SMF) (Téllez-Jurado et al. 2006). Tlecuitl-Beristain et al. (2008) reported that *P.ostreatus* (strain Po83) has a laccase activity of 12200 U/L at 432 h of growth and shows four isoenzymes at its stationary phase of growth in a culture medium containing $(NH_4)_2SO_4$ in SMF. On the other hand, Téllez-Téllez et al. (2008) observed that such a strain produced four isoenzymes of laccase and a laccase activity from 8000 to13000 U/L at 408-456 h after growing in a culture medium containing yeast extract in SMF.

Palmieri et al. (1997) found two laccase isoforms from *P. ostreatus* named POXA1 and POXA2. POXA1 had a molecular weight of 61 kDa, an isoelectric point (pI) of 6.7, a high stability at different values of pH and T, one atom of Cu, two atoms of Zn, and one atom of Fe per molecule. On the other hand, POXA2 had a molecular weight of 67 kDa, a pI of 4, low stability between 25 and 35 ºC, and four Cu atoms per molecule. Giardina et al. (1999) purified another laccase isoform from *P. ostreatus* that they named POXA1b. It had a molecular weight of 62 kDa, a pI of 6.9, high stability at an alkaline pH, and four atoms of Cu per molecule. A laccase isoform produced by *P. ostreatus* named RK 36 was purified from a culture containing ferulic acid. This isoform had a molecular weight of 67, a pI of 3.6, and an optimum temperature of 50 ºC (Hublik and Schinner, 2000). Palmieri et al. (2003) found another two laccase isoforms in a culture containg Cu that were named POXA3a and POXA3b. Those isoforms had a subunit of 67 kDa, and a small subunit (18kDa and 16 kDa, respectively), and a pI of 4.1 and 4.3, respectively. Such heterogeneity might be given by the presence or absence of glycosylation.

P. ostreatus is the second most cultivable edible mushroom worldwide and has medicinal and nutritional properties. It is cultivated on straw, sawdust, waste of cereals, etc. Such substrates do not require sterilization, only pasteurization, which is less expensive. *P. otreatus* requires a shorter growth time in comparison to other edible mushrooms. All this makes *P. ostreatus* cultivation an excellent choice for production of mushrooms. The lignocellulolic ability of this organism is due to the enzymes that they produce, including laccases, manganese peroxidase, and veratryl alcohol oxidase (Sánchez 2009, 2010). Laccase can have biotechnological applications in fields such as pulping, textile dyes, polluted water detoxification, and others. In this research, kinetic parameters of growth and production of laccases by five ATCC strains of *P. ostreatus* grown in SMF were studied. The best strain and time for maximum laccase activity were also determined.

EXPERIMENTAL

Materials

Five strains of *P. ostreatus* were studied: *P. ostreatus* 32783 (Po83), *P. ostreatus* 201216 (Po3), *P. ostreatus* 201218 (Po7), *P. ostreatus* 38537 (Po37), and *P. ostreatus* 58052 (Po52) from the American Type Culture Collection (ATCC) (Manassas, Virginia, U.S.A.).

Methods

Culture conditions

A liquid culture medium previously optimized for producing laccases by this fungus in SMF was prepared containing (in gram per liter): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$-$5H_2O$, 0.25; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$-H_2O, 0.05; and $ZnSO_4$-$7H_2O$, 0.001 (Téllez-Téllez et al. 2008). The pH was adjusted to 6.0 using 0.1 M NaOH. Flasks of 250 mL containing 50 mL of culture medium were inoculated with three mycelial plugs (4 mm diam) taken from the periphery of a colony grown on PDA at 25°C for 7 days. The cultures were incubated at 25°C for 23 days on a rotary shaker at 120 rpm (Téllez-Téllez et al. 2008). Samples were taken every 24 h after the third day of growth.

Enzymatic extract preparation 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) (Díaz-Godínez et al. 2001).

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

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

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,

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \quad (2),$$

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

Estimation of kinetic parameters in the above equations was performed using the non-linear least square-fitting program "Solver" (Excel, Microsoft) (Téllez-Téllez et al. 2008; Díaz-Godínez et al. 2001; Viniegra-González et al. 2003). $Y_{E/X}$ is the yield of laccase per unit of biomass produced, estimated as the relation between maximal laccase activity (E_{max}) and X_{max}. Laccase productivity ($P = E_{max}/t$) was evaluated by using the time of E_{max}. The specific rate of laccase production was calculated by the equation; $qP = (\mu)(Y_{E/X})$.

Enzyme assays

Laccase activity was determined by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol as substrate (DMP). The assay mixture contained 950 µL of substrate (2 mM DMP in 0.1 M phosphate buffer at pH 6.0) and 50 µL EE, which was incubated at 40 ºC 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. All the experiments were carried out in triplicate.

Zymogram analysis

The laccase activity was also detected through zymograms, using a non-denaturing system (Téllez-Téllez et al. 2005, 2008), in which a molecular marker cannot be used. The non-denaturing system can only show the isoform number. 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 (30 µL approx.) was mixed with sample buffer without a reducing agent for the disulfide bonds. Without heating, the samples were placed in gels (thickness 1.5 mm) of the Mini-Protean III electrophoresis system (BioRad) and then 150 V was applied for 1 to 1.25 h. After electrophoresis, gels were washed with deionized water on an orbital shaker (20 to 30 rpm) for 2 to 2.5 h, and the water was changed every 30 min to remove SDS. Finally, the gels were incubated at room temperature in substrate solutions (2 mM DMP). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 2 h.

RESULTS AND DISCUSSION

Figure 1 shows the growth of five strains of *P. ostreatus* monitored for 23 days every 24 h in SMF. Their curves of growth were adjusted (R^2>0.98) using the respective mathematic model (see Materials and Methods).

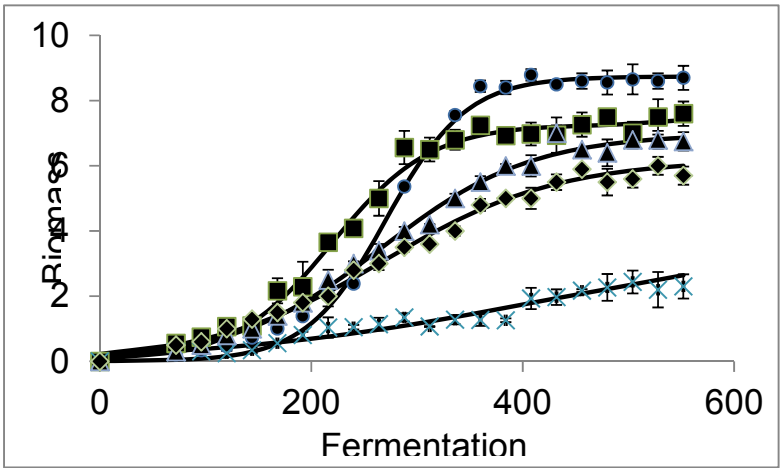

Fig. 1. Growth of *P. ostreatus*; Po83 (●), Po3 (×), Po7 (■), Po37 (▲), and Po52 (♦) in SMF. The error bars represent the standard deviation of three separate replicates from each experiment.

In general, the laccase activities of the five strains were observed at the end of the rapid growth phase and during the stationary phase of growth (Fig. 2). All of the strains showed a well-defined curve of growth, and the total glucose consumption occurred at the beginning of the stationary phase, except for the strain Po3 (data not shown). Biomass production and enzyme activity were different for all the strains.

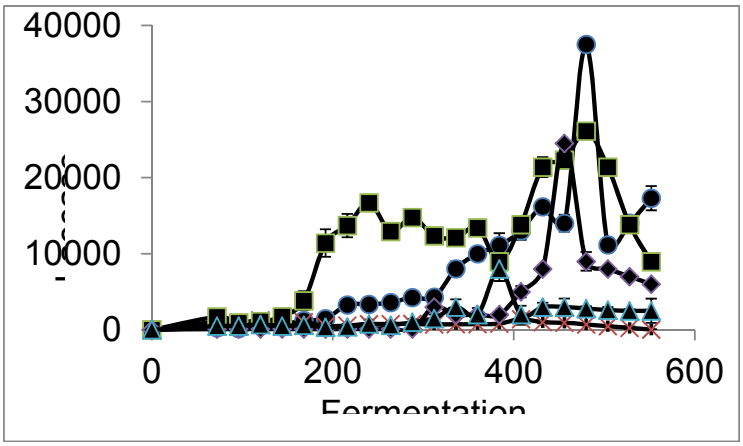

Fig. 2. Laccase activity of *P. ostreatus*; Po83 (●), Po3 (×), Po7 (■), Po37 (▲), and Po52 (♦) in SMF. The error bars represent the standard deviation of three separate replicates from each experiment.

Table 1 shows the kinetic parameters of the growth and laccase activity of the strains of *Pleurotus ostreatus* grown with Cu in the culture medium in SMF. Strain Po83 showed the greatest μ (0.026/h). Strains Po7 and Po37 showed similar values of μ (approx. 0.020/h), which were slightly lower than those shown by the strain Po83. Strains Po52 and Po3 had lower μ values (54% and 73%, respectively) than that of Po83. There was a similar tendency in the increase or decrease between μ and X_{max} in the strains (i.e. the variation of the values of μ and X_{max} were positively correlated in all the strains). Strain Po83 had the highest X_{max} (8.2 g/L), followed by strains Po7 and Po37 (approx. 7 g/L). Strains Po52 and Po3 had the lowest X_{max} (6.1 g/L and 2.3 g/L, respectively). Strain Po83 also showed the highest E_{max} (37490 U/L), followed by strains Po7, Po52, Po37, and Po3, which corresponded to 69%, 66%, 21%, and 3.7%, respectively. These percentage evaluations were obtained on the basis of the E_{max} from strain Po83 (E_{max}=37490 U/L). As a result, strains Po83 and Po52 had the highest $Y_{E/X}$ (4573 and 4016 U/gX, respectively), followed by strains Po7, Po37, and Po3, which corresponded to 79%, 75%, and 87%, respectively. These percentages were evaluated on the basis of the $Y_{E/X}$ from Po83. Strain Po83 had the greatest q_p and P values. In general, both parameters showed the same tendency as the $Y_{E/X}$.

All the strains showed different behavior after growing under the same growth conditions and using the same culture medium. This could be due, in part, to species-specific temperature sensitivity. The optimum temperature for the growth of *P. ostreatus* varies between 18 and 28 ºC (Sánchez 2009, 2010). However, the concentration of Cu is an important component in the culture medium, affecting μ, X_{max}, and E_{max}. In this research, the concentration of Cu used as inducer of laccase activity of *P. ostreatus* was around 6.7-fold higher than that amount of Cu used for the same organism in previous studies (Giardina et al. 1999; Palmieri et al. 2000).

Table 1. Kinetics Parameters of Growth and Laccase Activity by Different Strains of *P. ostreatus* Grown with Cu in Submerged Fermentation

Strains	X_{max} (g l^{-1})	μ (h^{-1})	E_{max} (U l^{-1})	$Y_{E/X}$ (U $g^{-1}X$)	q_p (U $g^{-1}Xh^{-1}$)	P (U $l^{-1}h^{-1}$)
Po83	8.2^{a}±0.2	0.026^{a}±0.001	37490^{a}±309 480 h Ft*	4573	119	78
Po3	2.3^{d}±0.1	0.007^{d}±0.000	1400^{d}±112 408 h Ft	609	4	3
Po7	7.2^{b}±0.4	0.020^{b}±0.002	26000^{b}±830 480 h Ft	3611	72	54
Po37	7.1^{b}±0.3	0.018^{b}±0.001	8000^{c}±451 384 h Ft	1126	20	21
Po52	6.1^{c}±0.1	0.012^{c}±0.001	24500^{b}±1031 456 h Ft	4016	48	54

Means ± standard error from three separate experiments. In the same column, results with the same letter are not significantly different ($p < 0.01$).
Ft*= Fermentation time at the E_{max}.

In all strains, the E_{max} was lower in those media containing Cu than without Cu (Table 2). In particular, the strain Po3 was much more affected that the rest of the strains, since the X_{max} and μ of that strain were lower than these parameters obtained in a medium without the presence of Cu (Table 2). These results show the effect of Cu induction of laccase and that some strains were more sensitive to this metal than others. In some strains, Cu can inhibit the growth or can be toxic for the organism, which might cause the low laccase production. In this research, E_{max} observed for the strain Po83 was three times higher than E_{max} reported previously by Tlecuitl-Beristain et al. (2008) and Téllez-Téllez et al. (2008). In those studies $(NH_4)_2SO_4$ was used as the nitrogen source. Téllez-Téllez et al. (2008) studied that growth of the strain Po83 in SMF and did not observe a decrease of laccase activity.

Table 2. Kinetics Parameters of Growth and E_{max} by Different Strains of *P. ostreatus* Grown in Submerged Fermentation without Cu

Strains	X_{max} (g l^{-1})	μ (h^{-1})	E_{max} (U l^{-1})	$Y_{E/X}$ (U $g^{-1}X$)	q_p (U $g^{-1}Xh^{-1}$)	P (U $l^{-1}h^{-1}$)
Po83	10.6^{a}±0.2	0.017^{b}±0.001	1086^{b}±90 456 h Ft*	102.5	1.74	2.4
Po3	5.5^{d}±0.3	0.012^{c}±0.001	1000^{b}±29 384 h Ft	181.8	2.18	2.6
Po7	8.0^{b}±0.5	0.019^{a}±0.002	4853^{a}±95 408h Ft	606.6	11.50	11.9
Po37	7.9^{b}±0.6	0.019^{a}±0.001	450^{d}±41 408 h Ft	56.9	1.08	11.0
Po52	7.2^{c}±0.1	0.017^{b}±0.001	747^{c}±31 408 h Ft	103.8	1.76	1.8

Means ± standard error from three separate experiments. In the same column, results with the same letter are not significantly different ($p < 0.01$).
Ft*= Fermentation time at the E_{max}.

Figure 3 shows the isoenzymes produced by the different strains of *P. ostreatus* in SMF, as revealed with DMP. The isoenzymes pattern was different in all the cases; however, all the strains showed two laccase bands that migrate at the same time in the gel (Fig. 3). The strain Po83 (Fig. 3a) showed four laccase isoenzymes in the E_{max} that corresponded to the stationary phase of growth. From these, the slowest migrating band showed the greatest staining intensity. Po3 (Fig. 3b) showed two isoenzymes bands. In general, the strains Po7 (Fig. 3c) and Po3 showed a similar zymogram pattern. Two bands (lane 2 and 4) from the zymogram of the strain Po7 run at the same time. The strain Po37 (Fig. 3d) showed around three isoenzymes, which were similar to those observed in the strain Po83. The strain Po52 (Fig. 3e) also showed three laccase isoenzymes. The two isoenzymes present in all the strains might be constitutive, since they were produced in a culture medium without the addition of Cu (Téllez-Téllez et al. 2005). The isoenzyme patterns of the strain Po37 and Po52 were similar. Three isoenzymes were observed in the same position of the gel for the strains Po37, Po52, and Po83.

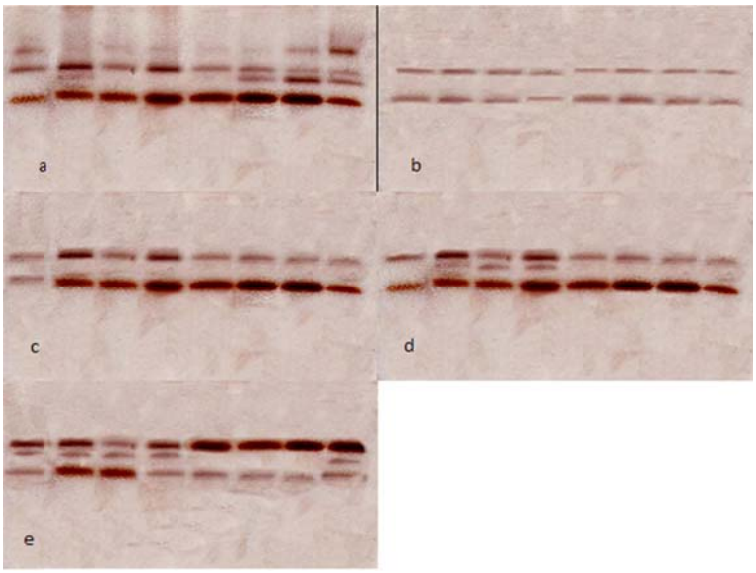

Fig. 3. Zymogram of laccase isoenzymes of Po83(a), Po3(b), Po7(c), Po37(d) and Po52(e) obtained in SMF using DMP as substrate. The samples correspond to times of fermentation between 336 and 504 h.

CONCLUSIONS

1. The maximum values for laccase activity were found.
2. Not all the strains responded in the same way to the addition of Cu in the culture medium.
3. In general, the sensitivity to Cu could be used to select strains with high laccase activity for commercial exploitation.
4. The knowledge about the physiology of the strains makes it possible to select more productive strains and establishes the optimal conditions for maximum laccase production.

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