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CHARACTERIZATION OF PRODUCTION OF LACCASES, CELLULASES AND XYLANASES OF *Pleurotus ostreaus* GROWN ON SOLID-STATE FERMENTATION USING AN INERT SUPPORT
CARACTERIZACIÓN DE LA PRODUCCIÓN DE LACASAS, CELULASAS Y XILANASAS DE *Pleurotus ostreaus* CULTIVADO EN FERMENTACIÓN SÓLIDA UTILIZANDO UN SOPORTE INERTE

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Abstract

Activities of laccase, cellulase and xylanase produced by *Pleurotus ostreatus* grown in solid-state fermentation using polyurethane foam as inert support were evaluated, and the growth kinetic parameters of fungus were obtained. In general, all enzymatic activities were observed in the fermentation broth. Laccases showed the highest activity (18,030 U L^{-1}) in the medium with glucose as unique carbon source. The cellulose activity was 216 U L^{-1} in the medium with three carbon sources (glucose, carboxymethylcellulose and xylan), while xylanase activity was observed around the 200 h of fermentation and the highest value was 240 U L^{-1} in presence of the three carbon sources. Two isoforms of cellulase and xylanase and four laccase isoforms were observed in the zymograms. Most studies on the production of fungal enzymes in solid-state fermentation systems have been made using agroindustrial residues which acting as a support but also as a substrate so the quantification of biomass produced is complicated due to its complex composition, on the other hand is impossible determine whether enzymes of interest are constitutive or inducible. In this study, physiological parameters of *Pleurotus ostreatus* developed in solid culture could be obtained by using an inert support, also it was determined that in this fungus, laccases are constitutive enzymes, although activity values were changed depending on the carbon sources used, on the other hand, cellulases were inducible and susceptible to catabolite repression, while xylanase enzymes were observed in the three culture media, however, the highest activity value was produced in the culture medium with the three carbon sources.

Keywords: *Pleurotus ostreatus*, fermentation, laccases, cellulases, xylanases.

Resumen

Se evaluaron las actividades de lacasas, celulasa y xilanasa producidas por *Pleurotus ostreaus* cultivado en fermentación sólida utilizando espuma de poliuretano como soporte inerte, se obtuvieron los parámetros cinéticos de crecimiento del hongo. En general, se observó la actividad de todas las enzimas en el caldo de fermentación. Las lacasas mostraron la actividad más alta (18,030 U L^{-1}) en el medio con glucosa como única fuente de carbono. La actividad de celulasa fue de 216 U L^{-1} en el medio con tres fuentes de carbono (glucosa, carboximetilcelulosa y xilano), mientras que la actividad xilanasa alcanzó 240 U L^{-1} a las 200 h de cultivo en el medio con las tres fuentes de carbono. Se observaron dos isoformas de celulasa y xilanasa y cuatro isoformas de lacasa en los zimogramas. La mayoría de estudios sobre la producción de enzimas de hongos en sistemas de fermentación sólida se han realizado utilizando residuos agroindustriales que actúan como soporte pero también como sustrato por lo que la cuantificación de biomasa producida se complica y dada su composición compleja es imposible determinar si las enzimas de interés son constitutivas o inducibles. En este estudio se pudieron obtener parámetros fisiológicos de *Pleurotus ostreatus* desarrollado en cultivo sólido gracias al uso de un soporte inerte, además se pudo determinar que en este hongo, las lacasas son enzimas constitutivas, aunque los valores de actividad cambiaron dependiendo de las fuentes de carbono utilizadas, por otro lado, las celulasas fueron inducibles y susceptibles a la represión catabólica, mientras que las enzimas xilanasas se observaron en los tres medios de cultivo, sin embargo, el valor más alto de actividad se produce en el medio de cultivo con las tres fuentes de carbono.

Palabras clave: *Pleurotus ostreatus*, fermentación, lacasas, celulasas, xilanasas.

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1 Introduction

Pleurotus ostreatus is a white-rot fungus due to its ability to produce extracellular lignocellulolytic enzymes, such as laccases, cellulases and xylanases (Stajic *et al*., 2006). The enzyme secretion system in filamentous fungi is mainly in the hyphal apex (Papinutti *et al*., 2003). Lignocellulolytic enzymes exist in multiple forms and their synthesis may be due to products of different genes or changes post translational as proteolysis and glycosylation. Differences in the composition of culture media induce the synthesis of different enzymes with the same activity, despite having similar catalytic properties, they differ significantly in their physicochemical characteristics (Manssur *et al*., 2003; Téllez-Téllez *et al*., 2005; Castro *et al*., 2013). Laccases (EC 1.10.3.2) belong to the group of polyphenol oxidases containing copper as cofactor, commonly called multicopper oxidases (Baldrian, 2006), they are able to oxidize polyphenols substituted and diamines, turning them into nontoxic compounds (quinones) or degrading them to carbon dioxide and water such is the case of anthracene and phenanthrene. The number and types of laccase isoforms that are produced depends on the species of fungus, culture medium and growth conditions (Suzuki *et al*., 2003, Téllez-Téllez *et al*., 2008). Furthermore, the endocellulases (E.C 3.2.1.4) are the cellulases most widely found that hydrolyze amorphous cellulose (cellulose pretreated with phosphoric acid) or soluble derivatives such as carboxymethylcellulose (CMC). The endocellulases are also called endo-β 1,4-glucanases, 1,4 β-D-glucan 4-glucan-hydrolases and CM-cellulases. These enzymes are strongly inhibited by the presence of cellobiose (Massadeh *et al*., 2001). Meanwhile xylanases are the enzymes responsible for the degradation of xylan, these are produced by algae, crustaceans, insects, yeasts, bacteria and fungi (Howard *et al*., 2003). These enzymes catalyze the random hydrolysis of xylan to xylo-oligosaccharides, while the β-xylosidases act on non-reducing ends of xylo-oligosaccharides (Polizeli *et al*., 2005). Although there have isolated a variety of bacteria and fungi able to utilize cellulose, not everyone can produce high levels of extracellular enzymes that degrade "in vitro" the insoluble cellulose, hence the importance of continuing the search for better new species (Ali *et al*., 1991). On the other hand, the solid-state fermentation (SSF) is a process that involves the growth of microorganisms (typically fungi, but also can be used bacteria and yeasts) on a solid material in the absence or near absence of free-flowing water. There are a wide range of solid materials used in SSF and can be classified into two great categories: inert (synthetic materials), used only as support impregnated with a synthetic culture medium and non-inert (organic materials) such as agro-industrial wastes with moisture adjusted, in this case the solid material acts as support and substrate (Viniegra-González and Favela-Torres, 2006). The SSF can be applied to produce metabolites with biotechnological uses including enzymes such as phytases, pectinases, amylases, invertases, inulinases, cellulases, xylanases, proteases, lipases, tannases, laccases etc. (Díaz-Godínez *et al*., 2001; Viniegra-González *et al*., 2003; Téllez-Téllez *et al*., 2008; Álvarez-Cervantes *et al*., 2013; Hernández-Domínguez *et al*., 2014; Ramos-Sánchez *et al*., 2015). In this study the activities of laccase, cellulase and xylanase of *Pleurotus ostreatus* developed in solid-state fermentation using polyurethane foam (PUF) as an inert support were characterized.

2 Material and methods

2.1 Microorganism and culture conditions

A strain of *Pleurotus ostreatus* from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used. The SSF was carried out in flasks of 250 mL, each containing 0.5 g of PUF cubes (0.5 cm per side and low density; 17 kg m^{-3}) as inert support and 15 mL of sterile culture medium (Díaz-Godínez *et al*., 2001). The cubes were washed twice with hot distilled water, oven-dried at 60 °C for 24 h, and then autoclaved at 120 °C for 15 min, before the culture medium was added. A mineral medium (MM) previously reported (Téllez-Téllez *et al*., 2008) but with modifications was prepared. All cultures were carry out at pH 6.5. The MM contained (in g L^{-1}): KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K2HPO_4, 0.4; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$-H_2O, 0.05; $ZnSO_4$-$7H_2O$, 0.001 y $CuSO_4$-$5H_2O$, 0.25 (all chemical compounds were from J.T. Baker®). Three fermentations using MM were developed in triplicate. Fermentation media were supplemented with (in g L^{-1}) glucose and yeast extract (10 and 5, respectively) (M1); glucose, birch xylan, carboxymethylcellulose (5 each) and yeast extract (9) (M2); birch xylan, carboxymethylcellulose (7.5 each) and yeast extract (9) (M3). Three mycelial plugs (4 mm diameter) taken from the periphery of colonies of

Pleurotus ostreatus grown for 7 d at 25 °C in Petri dishes containing potato dextrose agar were used as inoculum for each flask. All inoculated flasks were incubated for 25 d at 25°C and samples were taken every 24 h after third day of growth.

2.2 Enzymatic extract recuperation and biomass evaluation

The enzymatic extract (EE) was obtained by soft pressing the PUF cubes and the broth was filtrated using filter paper (Whatman No. 4), and the biomass (X) immobilized on PUF cubes was determined as difference of dry weight (g L^{-1}) (Díaz-Godínez *et al*., 2001).

Evolution of biomass $X = X(t)$ was followed by the Velhurst-Pearl or 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 the following:

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

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

Estimation of kinetic parameters in the previous equation was done using a non-linear least square-fitting program, called "Solver" present in Excel electronic sheet (Microsoft) (Díaz-Godínez *et al*., 2001).

2.3 Consumption of carbon sources

The consumption of carbon sources was determined in each EE. To measure reducing sugars, the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) was used and for quantification of total carbohydrate the Anthrone method (Loewus, 1952) was used. Calibration curves were prepared: glucose, glucose-xylose (2:1) and glucose-xylose (1:1) depending on the case. All the carbohydrates used in this research were from Sigma-Aldrich Co.

2.4 Enzyme assays

2.4.1 Laccase activity

- 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 6.5) and 50 μL EE, which was incubated at 40 ºC for 1 min (Téllez-Téllez *et al*., 2008). One enzymatic unit (U) of laccase was defined as the amount of enzyme, which gives an increase of 1 unit of absorbance per min in the reaction mixture.

2.4.2 Hydrolytic enzyme activity

- Xylanase and cellulase activities in EEs were assayed by quantifying reducing sugars using the DNS method. The assay mixture contained 950 μL of substrate (0.5% birch xylan in 0.1 M acetate buffer at pH 5.3 and 1.0% carboxymethylcellulose in 0.1 M acetate buffer at pH 5.0, for xylanase and cellulase activities, respectively) and 50 μL of EE and was incubated at 50 °C. One enzymatic unit (U) of xylanase or cellulase was defined as the amount of enzyme that liberated 1 μmol of xylose or glucose, respectively, per minute under assay conditions. The activity was expressed in U L^{-1} of EE. All the experiments were carried out in triplicate.

2.4.3 Zymography analysis

- Laccase, xylanase and cellulase activities were detected *in situ* by zymograms (Raghukumar *et al*., 2004; Téllez-Téllez *et al*., 2012; Álvarez-Cervantes *et al*., 2013).

3 Results and discussion

Figure 1 shows the growth of *Pleurotus ostreatus*, it was similar in the three culture media, with X_{max} at 300 h of about 5.03-5.2 g L^{-1} and a μ of 0.022-0.026 h^{-1}.

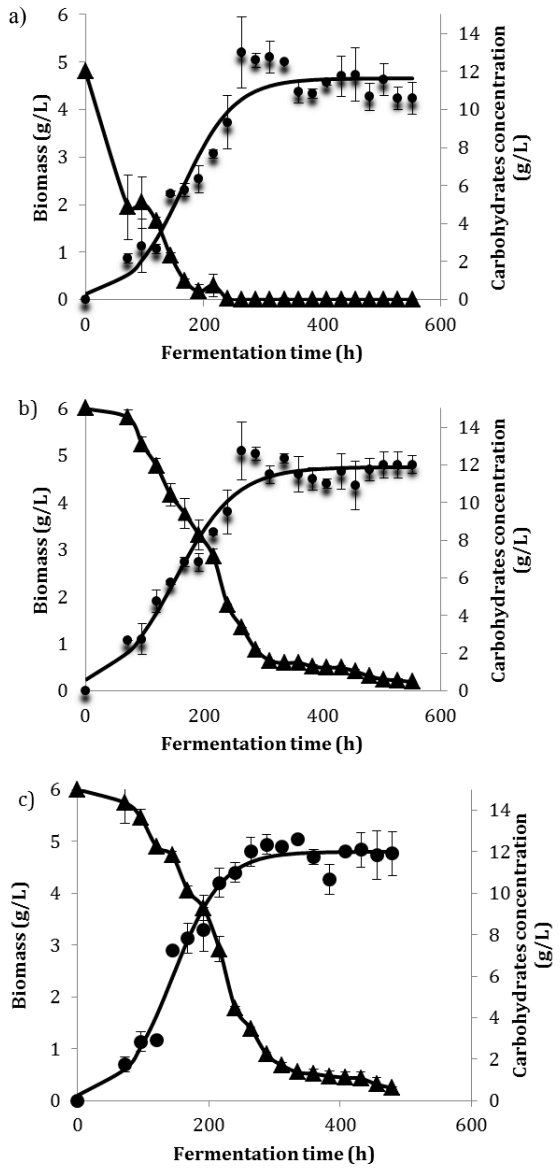

Fig. 1. Biomass evolution () and carbohydrates uptake (▲) by *Pleurotus ostreatus* grown in SSF using the M1 (a), M2 (b) and M3 (c) media.

However, the fungus had a faster uptake of glucose than of carbohydrate mixture (Figure 1), this could be because in fungi, the carbon sources are incorporated and stored inside the hyphae, as carbon and energy reserve, so it the carbohydrates disappearance to the culture media has a different speed than the carbohydrates metabolism. Figure 2 shows the enzymatic activities. The highest laccase activity was obtained in M1 (18,030 U L^{-1}). In M2 and M3, the laccase activity was 42.7% and 10.1% less than M1, respectively (Figure 2a).

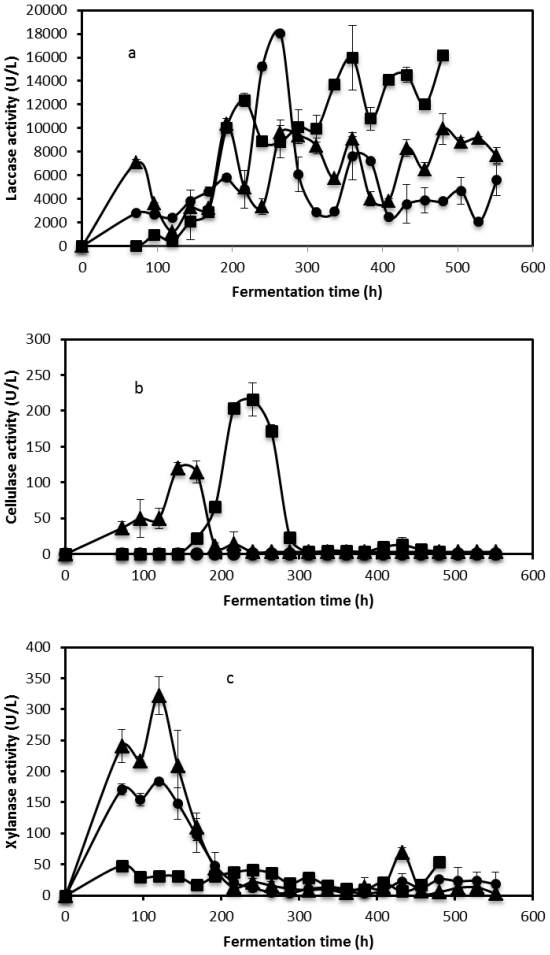

Fig. 2. Enzymatic activities of laccase (a), cellulase (b) and xylanase (c) of *Pleurotus ostreatus* grown in SSF using the M1 (), M2 (▲) and M3 (■) media.

The cellulase activity was observed only in M2 (120 U L^{-1}) and M3 (216 U L^{-1}) media and just during the exponential growth phase (Figure 2b), this suggest that this enzyme is inducible and susceptible to catabolite repression by glucose, since M1 contains only glucose, M2 contains inductor and glucose, while M3 is glucose free. It was observed a constitutive basal level for xylanase activity. The xylanase activity was observed around the 200 h of fermentation and the highest values were 184, 240 and 54 U L^{-1} for M1, M2 and M3, respectively (Figure 2c). Two laccase isoforms were observed during all fermentation times in the three media and two isoforms more were produced in some times, mainly in the exponential growth phase (Figure 3). Zymograms showed two isoforms either xylanase or cellulase in EEs with the highest activity in each media (Figures 4 and 5, respectively).

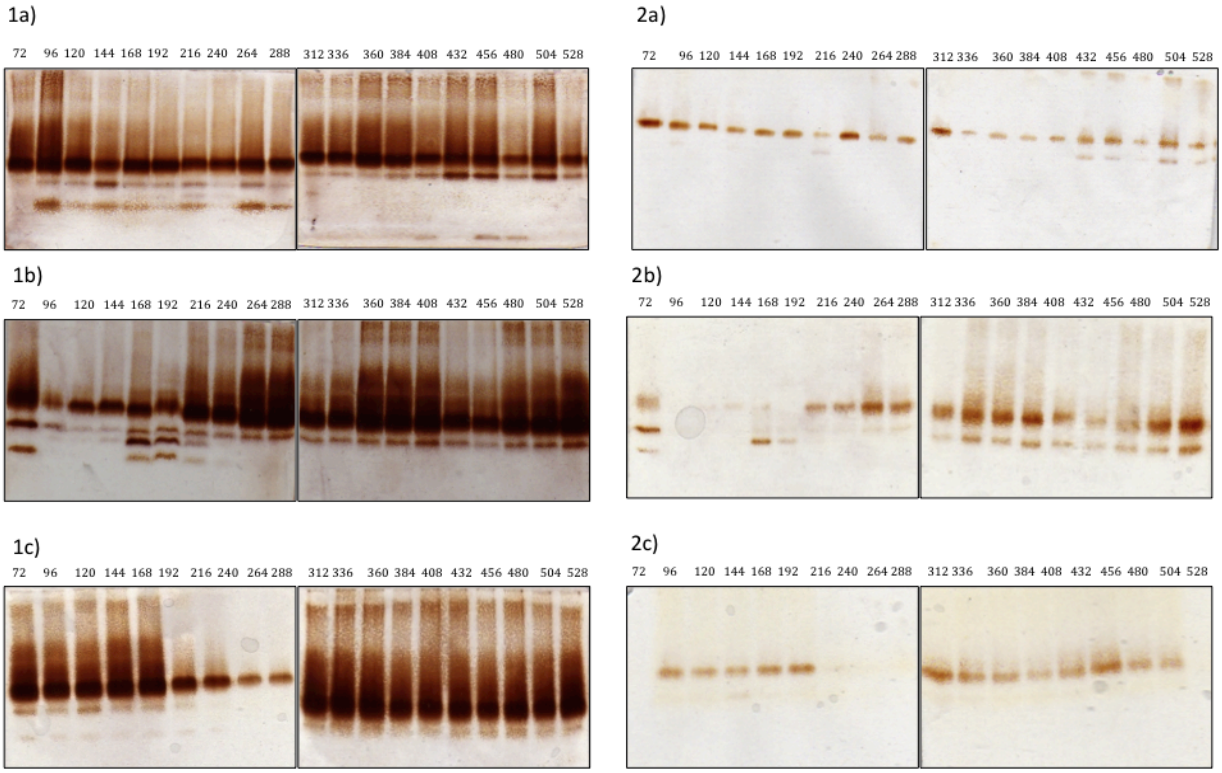

Fig. 3. Xylanases zymograms of *Pleurotus ostreatus* grown in SSF. M1 (a), M2 (b) and M3 (c) media. The numbers on the gels are the sampling time.

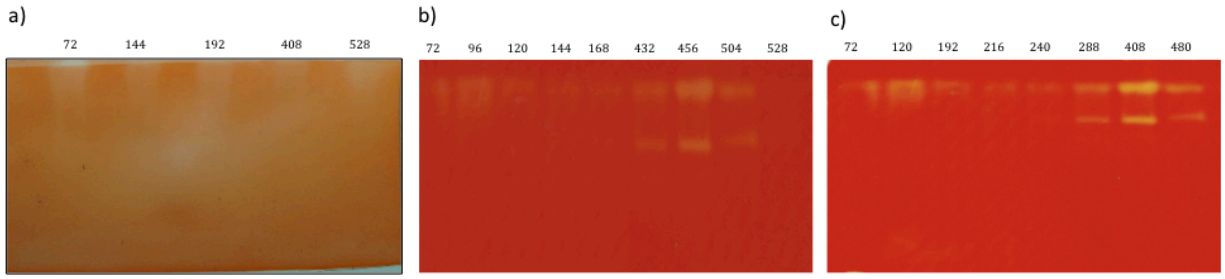

Fig. 4. Xylanases zymograms of *Pleurotus ostreatus* grown in SSF. M1 (a), M2 (b) and M3 (c) media. The numbers on the gels are the sampling time.

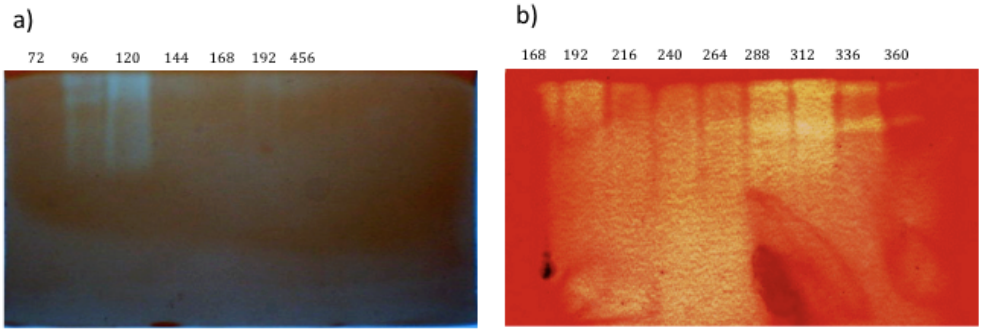

Fig. 5. Cellulases zymograms of *Pleurotus ostreatus* grown in SSF. M2 (a) and M3 (b) media. The numbers on the gels are the sampling time.

Growth kinetic parameters of *Pleurotus ostreatus* were not affected by the type and concentration of the carbon sources added to the culture media. It has been reported that, when *Pleurotus ostreatus* grew in SSF on PUF, the values of X_{max} reached 4.5 g L^{-1} with a μ of 0.033 h^{-1} and in submerged fermentation (SmF) showed values of X_{max} and μ of 5.5 g L^{-1} and 0.022 h^{-1}, respectively, at pH 6.0 and using glucose as carbon source in both culture systems (Téllez-Téllez *et al*., 2008). It has been reported that the values of μ for this fungus in SmF are about 0.02 h-1 when the initial pH of the culture medium 6.5 using ammonium sulphate as nitrogen source (Tlecuitl-Beristain *et al*., 2008). In other study, *Pleurotus ostreatus* grown in SSF on PUF showed a X_{max} value near to 4.0 g L^{-1} and μ value of 0.034 h^{-1}, however the laccase activity was 17,015 U L^{-1} (Velázquez *et al*., 2014), which is similar to reported in this study. It has been reported that *Aspergillus niger* produce more biomass and higher enzymatic activities in SSF on PUF than in SmF due to increased aeration and to a lesser catabolite repression (Díaz-Godínez *et al*., 2001; Viniegra-González *et al*., 2003). *Pleurotus ostreatus* showed that can produce several laccases isoforms, some constitutive and other inducible (Téllez-Téllez *et al*., 2008), suggesting that the physiological role of these enzymes is not exclusive for lignin degradation; on the other hand, since the natural habitat of fungi of the genus Pleurotus is on lignocellulosic substrates, the hemicellulose and cellulose act as direct inducers of the cellulases and xylanases (Beauchemin *et al*. 2003). Although *Pleurotus ostreatus* is a white-rot fungus having the capacity to produce different hydrolytic enzymes such as cellulases and xylanases among others as well as phenoloxidases mainly laccases, some of those require inducers and other are constitutive, so is very important know the requirements for optimize their production, in that sense, the type and concentration of carbon source, pH of culture medium and other factors determines which enzyme and its amount is produced (Mikiashvili *et al*., 2006; Karp *et al*., 2012). Production of extracellular enzymes involves the expenditure of energy in the cell, therefore the carbon source regulates the expression of genes of enzymes involved in the degradation of cell wall polymers of plants. Most genes are repressed by the presence of carbon sources easily assimilated, such as glucose and inducible in the presence of the substrates. However, since the polymers are too large to enter cells, induction occurs by molecules that are produced by the hydrolysis of enzyme action (Margolles *et al*., 1997; Aro *et al*., 2005).

In a study, the laccase activity of *Pleurotus ostreatus* observed in SSF on PUF at pH 6.0 was of 2,430 U L^{-1} which represent 7.4 times less than the activity observed with M1 medium of this study; these results show that pH 6.5 of culture medium is better than 6.0 for laccase production by this fungus (Téllez-Téllez *et al*., 2008). Sethuram *et al*., (1999) reported that *Cyathus stercoreus* showed laccase activities of 3.5 and 3.06 U mL^{-1} using DMP and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as substrates, respectively. On the other hand, laccase production of *Trametes versicolors* and *Aspergillus niger* was higher with glucose as carbon source (Téllez-Jurado *et al*., 2005). It has been reported that the production of these enzymes depends on the strain, substrate and composition of culture medium and growth conditions (Téllez-Téllez *et al*., 2008, 2012).

In this study were identified four laccase isoforms at pH 4.5. In general, there are reported until four laccase isoforms in this fungus grown in SmF (Tlecuitl-Beristain *et al*., 2008; Díaz *et al*., 2011a, 2011b, 2013). There is a study where reported one or two laccase isoforms of *Pleurotus ostreatus* grown in SSF on PUF at pH of 3.5, 4.5 and 6.5 of culture medium and three and four at pH 7.5 and 8.5, respectively (Velázquez *et al*., 2014). In other study, was mentioned that the number of laccase isoenzymes produced by *Pleurotus ostreatus* were modified by the pH (only one isoenzyme at pH 3.5 and two isoenzymes at pH 6.5) (Díaz *et al*., 2011a). Téllez-Téllez *et al*. (2012) reported the zymogram patterns of extracellular laccases of ten strains of Pleurotus grown on agar without addition of inducers, using 2.6-dimethoxyphenol, ρ-anisidine and o-tolidine as substrates. Zymogram patterns were only similar for strains within same species, independently of the substrate used, six strains of *Pleurotus ostreatus* and one strain of *Pleurotus ostreatus* var. *florida* showed three isoenzymes, two strains of *Pleurotus pulmonarius* showed two isoenzymes and one strain of *Pleurotus cornucopiae* also showed two isoenzymes but in a different position in comparison to the other strains. These results suggest that *Pleurotus* species produce a basal level of laccase activity and the number of extracellular laccase isoenzymes is species dependent.

Furthermore, in this study, one cellulase isoform and two xylanase isoforms were observed. It has been mentioned that filamentous fungi are the best source of cellulolytic and hemicellulolytic enzymes and that

their production is regulated by the corresponding substrate (Amore *et al*., 2013). Generally xylanase production by filamentous fungi is high in SSF using lignocellulosic waste (Haltrich *et al*., 1996; Polizeli *et al*., 2005).

There are not reports about the cellulases and xylanases of *Pleurotus ostreatus* grown in SSF on PUF; recently was reported the xylanase activity of *Sporisorium reilianum* grown in SSF on PUF reaching almost 1 U mL^{-1} using glucose as sole carbon source (Álvarez-Cervantes *et al*., 2013). Hernández-Domínguez *et al*. (2014) reported the xylanase and cellulase activities of *Stenocarpella maydis* grown in SSF on PUF, when glucose was used as sole carbon source, the xylanase activity value was near to 8 U mL^{-1} but cellulase activity was not detected, and in presence of inducers, the fungus reported high activities levels of cellulase (9,439 U L^{-1}) and xylanase (19,266 U L^{-1}), observing that the regulation of xylanase enzyme production in basidiomycetes depends not only on the presence of xylan but also of its nature and concentration (Zakariashvili and Elisashvili, 1993). The use of the PUF as support in the SSF, besides being inert allowing fungal growth and quantification of biomass for obtaining their kinetic parameters, is a system that provides the control of the type and concentration of nutrients, and controlling the water activity with an uniform dispersion of the inoculum (Díaz-Godínez *et al*., 2001; John *et al*., 2007). Using PUF also allows efficient transfer of heat and oxygen (Hu *et al*., 2011).

Conclusions

This is the first report about the production of lignocellulolytic enzymes of *Pleurotus ostreatus* grown in SSF using PUF as inert support. The use of PUF as inert support allowed observe and quantified the growth of *Pleurotus ostreatus* in a system very similar to its natural habitat, obtaining the production patterns of three enzymes. In this fungus, laccases were shown as constitutive enzymes, although activity values were changed depending on the carbon sources used, on the other hand, cellulases were inducible and susceptible to catabolite repression, while xylanase enzymes were observed in the three culture media, however, the highest activity value was produced in the culture medium with the three carbon sources, this suggests that laccases break lignin and expose the fibers of cellulose and hemicellulose, and the xylanases act simultaneously, while cellulose will be attacked only if no easily assimilated sugars.

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Nomenclature

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ATCC	American Type Culture Collection
DMP	2,6-dimethoxyphenol
DNS	3,5-dinitrosalicylic acid
EE	enzymatic extract
MM	mineral medium
M1	medium 1
M2	medium 2
M3	medium 3
PUF	polyurethane foam
SSF	solid-state fermentation
U	international unit of enzymatic activity

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