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ISOENZYMES AND ACTIVITY OF LACCASES PRODUCED BY *Pleurotus ostreatus* GROWN AT DIFFERENT TEMPERATURES

ISOENZIMAS Y ACTIVIDAD DE LACASAS PRODUCIDAS POR *Pleurotus ostreatus* CRECIDO A DIFERENTES TEMPERATURAS

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Abstract

Pleurotus ostreatus produces laccase isoenzymes depending on the conditions in which it grows, it has been suggested that the presence of inducers, the pH of the culture medium as well as the type and concentration of carbon and nitrogen sources promote the production of these isoenzymes, however, the effect of temperature of *Pleurotus ostreatus* growth on its production of laccase isoenzymes has not been evaluated. In this study, *Pleurotus ostreatus* was grown in solid-state fermentation at different temperatures (10, 15, 20, 25, 30 and 35 °C) and the activity and isoenzymes number of laccases produced in each bioprocess was determined. At 10 and 35 °C there was no growth of the fungus. The activity of laccases was different in each fermentation, being at 25 ºC where the highest value was observed (20000 U L^{-1}). The laccase isoenzymes pattern was also different depending on the incubation temperature of the fungus, it was observed in some times of the culture, 4 isoenzymes in the fermentations incubated at 15 and 20 ºC, 3 isoenzymes at 25 ºC and 2 isoenzymes at 30 ºC. These results show that the growth temperature of *Pleurotus ostreatus* modifies its activity profiles and its isoenzyme patterns of laccases.

Keywords: laccases, *Pleurotus*, solid-state fermentation, temperature, zymograms.

Resumen

Pleurotus ostreatus produce isoenzimas de lacasa dependiendo de las condiciones de crecimiento, se ha sugerido que inductores, el pH del medio de cultivo y el tipo y concentración de fuentes de carbono y nitrógeno promueven la producción de estas isoenzimas, sin embargo, el efecto de la temperatura de crecimiento de *Pleurotus ostreatus* sobre su producción de isoenzimas de lacasa no se ha evaluado. En este estudio, *Pleurotus ostreatus* se cultivó en fermentación sólida a diferentes temperaturas (10, 15, 20, 25, 30 y 35 ° C), se determinó la actividad y el número de isoenzimas de lacasas producidas en cada bioproceso. A 10 y 35 °C no hubo crecimiento del hongo. La actividad fue diferente en cada fermentación, siendo a 25 ºC donde se observó el valor más alto (20000 U L^{-1}). El patrón de isoenzimas de lacasas también fue diferente según la temperatura de incubación del hongo, se observó en algunos momentos del cultivo, 4 isoenzimas en las fermentaciones incubadas a 15 y 20 ºC, 3 isoenzimas a 25 ºC y 2 isoenzimas a 30 ºC. Estos resultados muestran que la temperatura de crecimiento de *Pleurotus ostreatus* modifica sus perfiles de actividad y sus patrones de isoenzimas de lacasas.

Palabras clave: lacasas, *Pleurotus*, fermentación sólida, temperatura, zimogramas.

1 Introduction

Laccase (E.C.1.10.3.2, benzenediol: oxygen oxidoreductase) is a copper-protein belonging to the group of blue oxidases (Baldrian, 2006). Laccase catalyzes the oxidation of various phenolic compounds and aromatic amines with molecular oxygen as the electron acceptor (Palmieri *et al*., 1993). It is widely distributed among plants, insects, bacteria, and fungi, especially from white rot fungi (Baldrian, 2006). The natural function of these fungal enzymes is degradation of lignin, morphogenesis of fungi, sporulation, pigment production and fruiting body formation (Bourbonnais and Paice, 1990; Thurston, 1994; Langfelder *et al*., 2003; Fang *et al*., 2010). Extracellular fungal laccases play an important role in the natural degradation of lignin because are part of a very efficient system, involving both enzymatic and nonenzymatic processes

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implicated in degradation of lignocelluloses and, as a result of fungal transformation, the complex of lignocellulose becomes the main source of elements for many different organisms (Eriksson *et al*., 1990; Leonowicz *et al*., 1999; Leonowicz *et al*., 2001). Fungal laccases demonstrate great applications in lignin degradation, environmental detoxification, and a variety of industries including paper, textile, bioremediation (detoxification of polluted water), biocatalysis, diagnostic, and food industries (Widsten and Kandelbauer, 2008; Rajeeva and Lele, 2010; Zhang *et al*., 2011; Martínez-Berra *et al*., 2018; Kuss *et al*., 2018).

Several studies have shown that the activity and number of laccase isoenzymes produced in fungi depend on environmental conditions such as temperature, pH, inductors, culture conditions and medium composition (Duran *et al*., 2002; Téllez-Téllez *et al*., 2008; Piscitelli *et al*., 2011; Pezzella *et al*., 2012). Heat treatment has been proven to be a useful stimulus for biological cells from bacteria to plant cell; for example, the application of heat stress during microbial fermentation has been found to be effective for the increased production of biological targets such as gentamicin, red pigments, and glycerol (Berovic and Herga, 2007; Himabindu *et al*., 2007; Babitha *et al*., 2007). Heat shock treatment is also known to affect the transcription and synthesis of many proteins (Chen *et al*., 2010). The expression genes encoding heat shock proteins (HSPs) increase in response to elevated temperature. The majority of HSPs functions as either part of the protease for degrading misfolded proteins and/or as chaperones for preventing misfolded proteins (Chou, 2007). Mushrooms have drawn attention of investigators since they constitute a source of important compounds comprising lectins, laccases, proteases, ribonucleases, ribosome inactivating proteins, antibacterial proteins, antifungal proteins, and polysaccharides (Ng, 2004). Recently, the mycelial growth and exo-polysaccharide production by various fungal strains have been extensively researched for potential applications using environmental parameters as well as the nutritional requirements and factors of the environment for the cultures for the mycelial growth by *Pleurotus* (Choi *et al*., 2006). With respect to ligninolytic enzymes, effects of temperature on the stability and activity was almost exclusively studied with purified molecules and on the enzymatic extracts (Hofrichter, 2002; Baldrian, 2006). Although the effect of temperature on enzyme production is important for the understanding of fungal physiology.

2 Materials and methods

2.1 Microorganism

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

2.2 Culture conditions

Solid-state fermentation (SSF) by triplicate was the culture system for this study. Polyurethane foam (PUF) was used as inert support. 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) and 15 mL of sterile liquid 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. 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 (PDA) were used as inoculum for each flask. The liquid medium contained (in g L^{-1}): glucose, 10; yeast extract, 5.0; KH_2PO_4, 0.6; $MgSO_4{\cdot}7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4{\cdot}5H_2O$, 0.25; $FeSO_4{\cdot}7H_2O$, 0.05; $MnSO_4{\cdot}H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 0.001. The C/N ratio was approximately of 8.0, which was calculated with the amount of C in the glucose and considering the 10% of N present in the yeast extract. The pH was adjusted to 6.5 using 0.1 M NaOH (Téllez-Téllez *et al*., 2008). The fermentations were incubated independently at 15, 20, 25 and 30 °C for 576 h using refrigerated incubator (Prendo®, Mod. INO 650V-7). Three flasks were taken as samples each 24 h after the third day of the fermentation process.

2.3 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^{-1}).

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

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

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 $X = X_0$; 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) (Diaz-Godínez *et al*., 2001; Téllez-Téllez *et al*., 2008; Alvarez-Cervantes *et al*., 2016). The coefficient $Y_{E/X}$ was calculated as the relation between the maximal enzymatic activity and the biomass observed at the same time (U gX^{-1}), the productivity (Pro, U L^{-1} h^{-1}) was obtained as the relation between the maximal enzymatic activity and its time of production.

2.4 *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 6.5) 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. The activity was expressed in U L^{-1} of EE.

2.5 *Zymograms*

The laccase activity was also detected through zymograms in the selected EE, using the modified technique SDS-PAGE (Téllez-Téllez *et al*., 2008). The running gel contained 100 g acrylamide L^{-1} and 27 g bis-acrylamide L^{-1}. The stacking gel contained 40 g acrylamide L^{-1} and 27 g bis-acrylamide L^{-1}. Each EE (20 μL 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 h. After the electrophoresis, gels were washed with deionized 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 6.5). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 30 min.

2.6 *Statistical analysis*

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

3 Results and discussion

The growth of *Pleurotus ostreatus* was affected by temperature; It is worth mentioning that in this study, fermentations were also carried out at temperatures of 10 ºC and 35ºC; at 10 °C the growth was so slow that the bioprocess was not feasible to be carried out, so it could not be characterized and at 35 °C, *Pleurotus ostreatus* did not grow. Figure 1 shows the curves adjusted with the mathematical model of the logistic equation of growth of *Pleurotus ostreatus* at temperatures of 15, 20, 25 and 30 ºC. It was observed that within the temperatures evaluated, 25 ºC was the one that favored the growth since it showed the highest values of μ and $X_{\max}$. At temperatures below 25 ºC, an effect directly proportional to μ and $X_{\max}$ was observed since increasing the temperature also increased the values of μ and $X_{\max}$ until reaching 25 ºC and at 30 ºC the opposite effect was observed, because these parameters showed lower values and at 35 ºC the fungus did not grow. The values of μ were 0.018, 0.035, 0.049 and 0.036 h^{-1} for the temperatures of 15, 20, 25 and 30 ºC, respectively, the values of $X_{\max}$ were 4.61, 4.93, 5.13 and 4.33 g L^{-1} for the same temperatures, respectively. Although in all cases the fermentations were carried out until 624 h, it was observed that the time in which the bioprocesses reached the stationary phase was inversely proportional to the temperature, being 408, 288, 240 and 216 h for the temperatures of 15, 20, 25 and 30 ºC, respectively (Figure 1).

The activity of laccases did not show a predictable behavior, since the values observed in all the fermentations were different, considering the temperature of 25ºC as the one that reported the highest value of laccase activity. It is important to mention that in all cases the highest activity of each bioprocess was observed in the stationary phase of growth of the fungus at 400 h or later (Figure 2), which could be due to a state of stress of the organism, since it has been reported that the promoter region of the lacP83 gene (present in the strain used in this study) contains putative binding transcription factors including a stress response element (Téllez-Téllez *et al*., 2012).

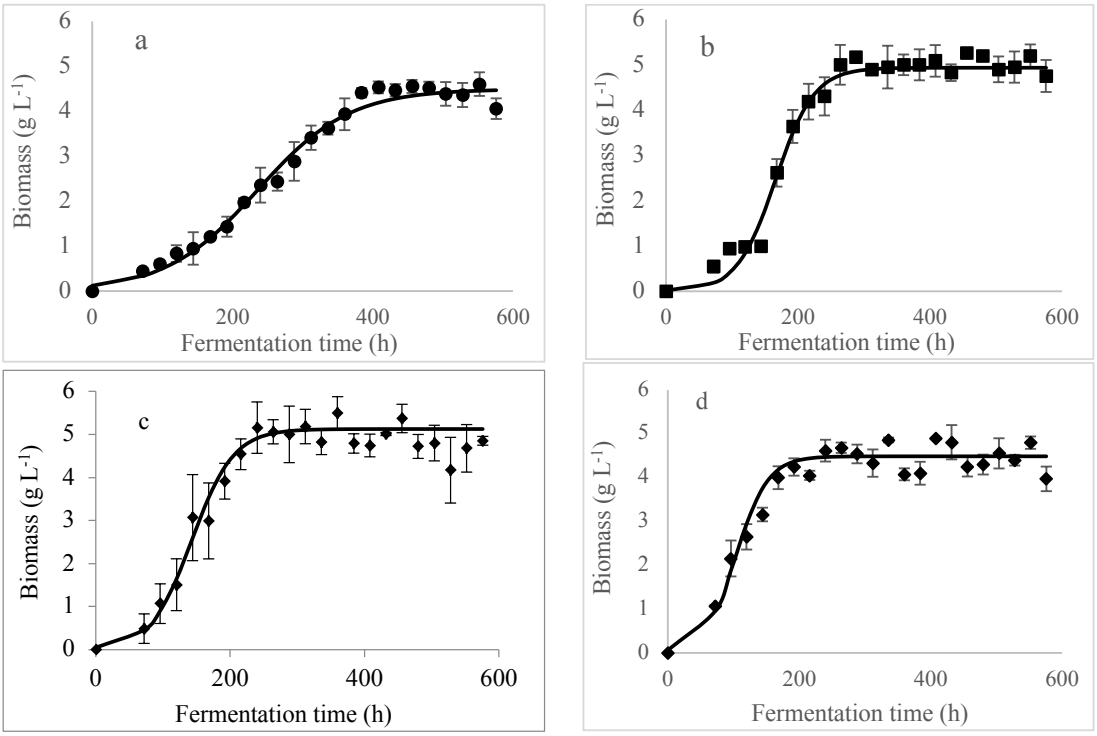

Fig. 1. Biomass evolution of *Pleurotus ostreatus* grown in SSF at 15 ºC (a), 20 ºC (b), 25 ºC (c) and 30 ºC (d).

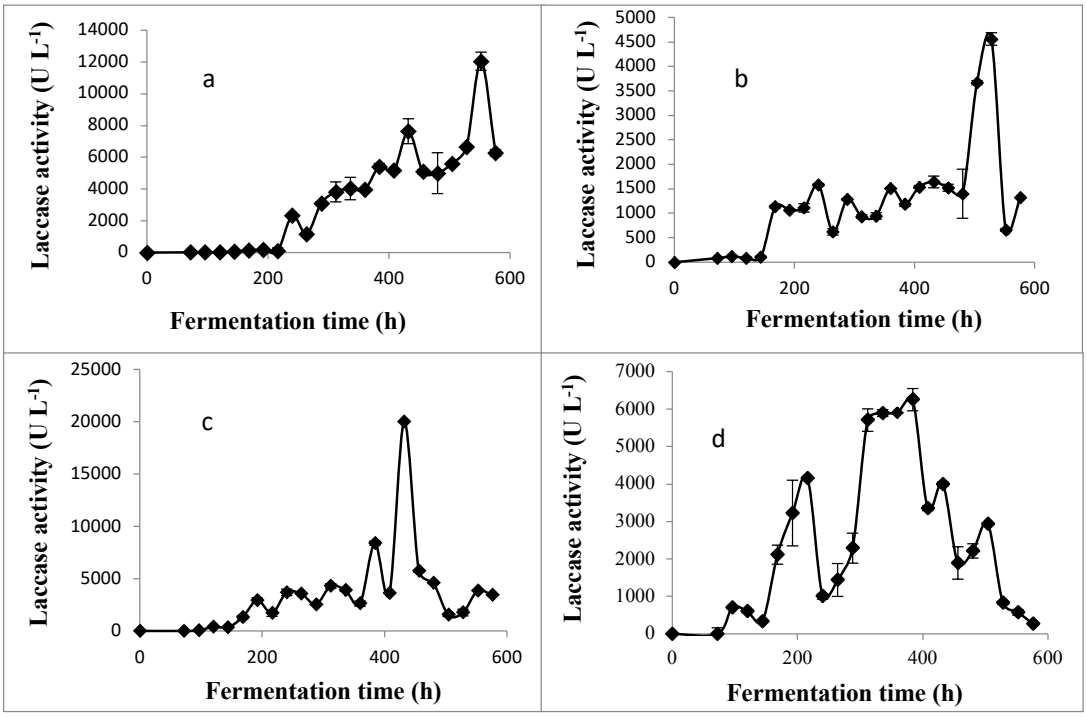

Fig. 2. Laccase activity of *Pleurotus ostreatus* grown in SSF at 15 ºC (a), 20 ºC (b), 25 ºC (c) and 30 ºC (d).

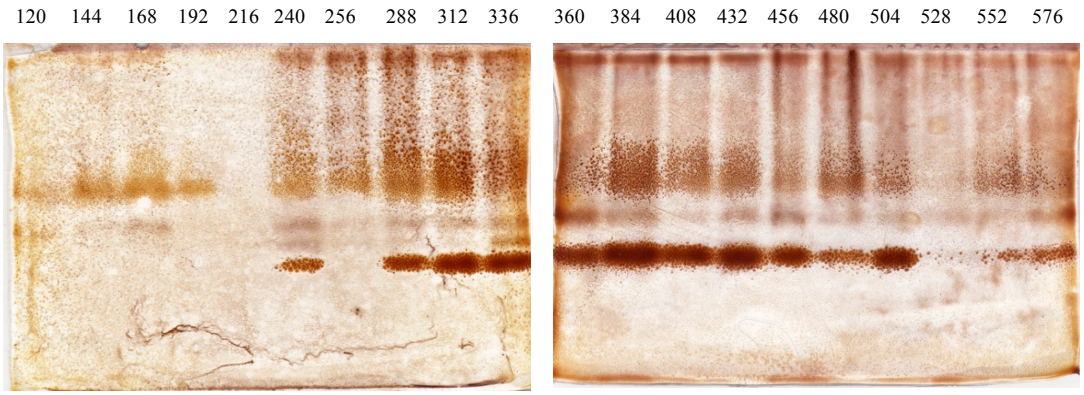

Fig. 3. Zymogram of laccases produced by *Pleurotus ostreatus* grown in SSF at 15 ºC. The numbers above the gel are the sampling times.

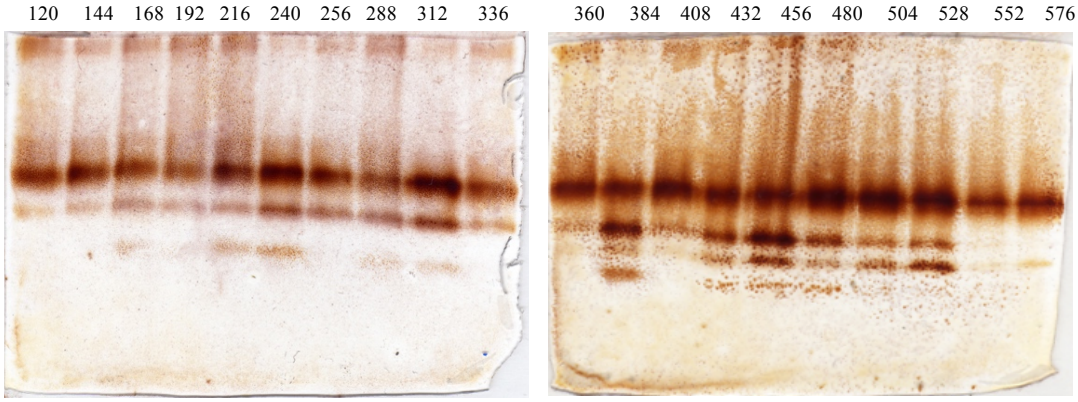

Fig. 4. Zymogram of laccases produced by *Pleurotus ostreatus* grown in SSF at 20 ºC. The numbers above the gel are the sampling times.

The maximum activity values of laccases were 12048, 4560, 20000, 6250 U L^{-1} for the temperatures of 15, 20, 25 and 30 ºC, respectively. The values of Pro and $Y_{E/X}$ were 21.8, 8.6, 46.3 and 16.3 U L^{-1} h^{-1} and 2613, 925, 3899 and 1443 U gX^{-1} for the temperatures of 15, 20, 25 and 30 ºC, respectively.

Figures 3, 4, 5, and 6 show the zymographic patterns of laccases produced by *Pleurotus ostreatus* at 15, 20, 25 and 30 °C, respectively. It was observed that the temperature, in addition to modifying the activity values of laccases, also caused changes in the pattern of laccase production, which could be because the growth temperature causes differences in the expression of genes encoding laccases and/or causes changes in post-translational modifications in the laccases produced. Up to four isoenzymes were observed, but depending on the growth temperature of the fungus, the number of isoenzymes and the production time were modified. In general, in all the fermentations, two isoenzymes were observed that apparently are the same due to their position in the gel, being the one with the highest and lowest weight of the four observed, however the intensity of the bands, which is related to the proportion which has within the same sample, the isoenzyme of higher molecular weight was more intense at 20 and 25 ºC, while at 15 and 30 ºC the lower molecular weight band was more intense. At temperatures of 15 and 20 ºC, two more intermediate weight bands were observed and one of those isoenzymes was observed in only a few samples at 25 ºC. In the fermentation carried out at 15 ºC, two isoenzymes were observed in the times corresponding to the adaptation phase, four in the exponential phase and three in the stationary growth phase of the fungus (Figure 3).

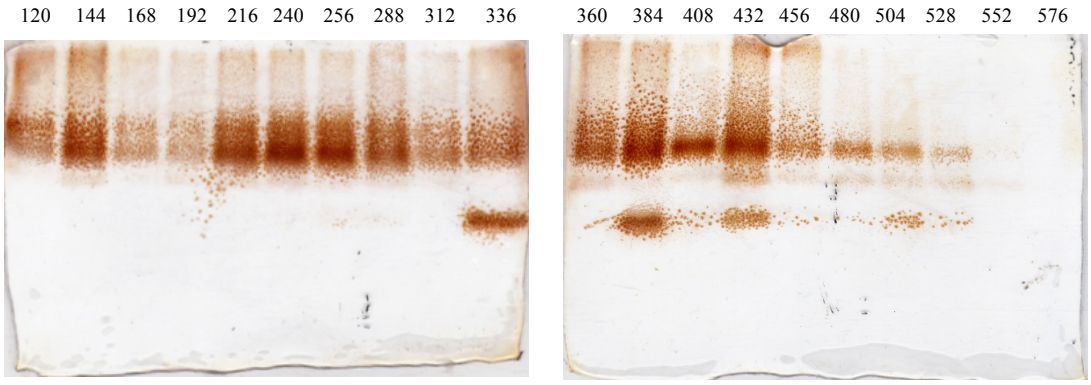

Fig. 5. Zymogram of laccases produced by *Pleurotus ostreatus* grown in SSF at 25 ºC. The numbers above the gel are the sampling times.

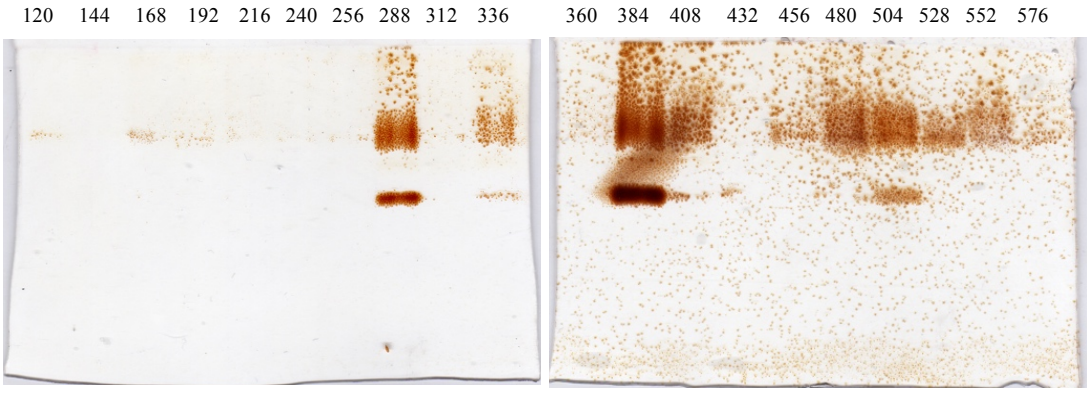

Fig. 6. Zymogram of laccases produced by *Pleurotus ostreatus* grown in SSF at 30 ºC. The numbers above the gel are the sampling times.

At 20 ºC, two laccase isoenzymes were observed in the adaptive phase of the fungus, three in the exponential phase and four in the stationary phase (Figure 4). *Pleurotus ostreatus* grown at 25 °C produced a laccase isoenzyme in the adaptation phase, two in the exponential phase and three in the stationary phase (Figure 5). In the fermentation at 30 ºC, the fungus presented a very light band in the first 256 h of fermentation, two isoenzymes in the exponential phase and in some times of the stationary phase (Figure 6).

Temperature is a factor that influences the growth of microorganisms and the formation of the product during solid culture. As the temperature increases, the rates of chemical and enzymatic reactions, which occur inside the cells of the microorganisms, become increasingly rapid, as well as the rate of growth. However, there is a temperature limit within which metabolic functions can occur and, when this is exceeded, cellular functions begin to decay drastically. In solid culture, temperature variation may accelerate or decelerate the production rate of lignocellulolytic enzymes (Tao *et al*., 1997), increase or decrease production (Jecu, 2000; Azin *et al*., 2007), and stop production altogether (Shah and Madamwar, 2005). In other cases, the culture temperature can cause excessive water losses of the supports used for the production of enzymes, seriously affecting their production (Mazutti *et al*., 2007).

It is well known that since *Pleurotus ostreatus* is a cosmopolitan fungus and depending on its origin, its optimum growth temperature may be different, since its growth has been reported in the range of 10 to 30 ºC and few studies report growth at 35 ºC or higher. In this study, at the temperature of 25 ºC, the highest biomass production was observed with a value similar to that previously reported for the same strain (Téllez-Téllez *et al*., 2008; Díaz *et al*., 2011). There are studies where they have also evaluated the growth of other strains of

Pleurotus ostreatus, as reported by Pineda-Insuasti *et al*., (2013), where they reported the growth kinetics of the fruiting body of the strain CEBA gliie-010606 in the range of 10 to 30 °C. Little change in the value of μ was observed when modifying the temperature in the range of 10 to 25 °C and at 30 °C a lower value was observed, concluding that the growth of the fruiting body of the strain studied and its parameters μ and X_{max} depend of the temperature. Hoa and Wang (2015), grew species of oyster mushroom (*Pleurotus ostreatus* and *Pleurotus cystidiosus*) on PDA medium at different temperatures (16, 20, 24, 28 and 36 ºC). The mycelium growth in all temperatures was very similar for the two species, and the best growth for both species was observed at 28 ºC followed by 32 ºC and 24 ºC. In another study, the effect of temperature on PDA growth of five *Pleurotus* species (*Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Pleurotus eryngii*, *Pleurotus columbinus* and *Pleurotus sapidus*) was evaluated and all *Pleurotus* species were found to grow better at 25 °C (Sardar *et al*., 2015). Based on the studies carried out, it can be observed that the growth in plate and for production of fruiting bodies in the majority is of 25 ºC, and with the results of this study it was observed that the growth in SSF on PUF of this strain of *Pleurotus ostreatus* it was also better at 25 ºC. It has been reported in the saprotrophic fungi, the inhibition of its growth at high temperatures could be due to, among other things, the excessive energy costs necessary for the replacement of heat-inactivated molecules mainly extracellular enzymes that are indispensable for the utilization of nutrients. Under natural conditions, short periods of very high temperature occur (in the close subsurface parts of tree trunks and dramatically increase the heat inactivation of enzymes or induces a heat-shock response (Šnajdr and Baldrian, 2007).

It was observed that the temperature affects differently the activity of laccases and the number of isoenzymes. The $Y_{E/X}$ parameter reflects the ratio of activity of laccases to biomass; in the case that the activity of laccases depended only on the amount of biomass, the parameter of $Y_{E/X}$ would be the same in all the fermentations, however, based on the results of this study, it is suggested that the temperature affects so different to the growth of the fungus and the activity of laccases. It has been reported that the laccases are regulated by several factors including temperature; in the region of the promoter of the laccase enzyme of *Pleurotus ostreatus* called LacP83, one stress response element was found, three potential sites of metal response elements, three possible xenobiotic response elements and one potential defense response site (Téllez-Téllez *et al*., 2012). The highest number of isoenzymes and the greater value of activity of laccases were observed at 25 °C, coinciding with the fact that at this temperature the highest values of μ and X_{max} were presented. Although there are several reports on the effect of temperature on the activity of laccases in various fungi (Hofrichter, 2002; Baldrian, 2006), there are few on *Pleurotus ostreatus*. In a study was reported that *Pleurotus ostreatus* and *Trametes versicolor* grew in static submerged fermentation with cellulose as a sole C source and high C/N ratio, produced laccase and Mn-peroxidase (MnP) at 5-35 °C, but the highest production was at 25-30 °C in *Pleurotus ostreatus* and at 35 °C in *Trametes versicolor* (Šnajdr and Baldrian, 2007). It is important to mention that this study is the first to report the activity and number of isoenzymes of laccases of *Pleurotus ostreatus* grown in solid-state fermentation using an inert support. Regarding the number of isoenzymes, it can be said that at 25 ºC a pattern similar to that previously reported was observed where the same fermentation system and the same strain were used (Téllez-Téllez *et al*., 2008). The results obtained in this work show that *Pleurotus ostreatus* has a better growth and laccase activity at 25 °C, however at the temperature of 15 and 20 °C more isoenzymes were produced.

Conclusions

- At temperatures below 25 ºC, an effect directly proportional to μ and X_{max} de *Pleurotus ostreatus* was observed, since when the temperature increased the μ and X_{max} values also increased until reaching 25 ºC and at 30 ºC the opposite effect was observed.
- It was observed that the patterns of activity and isoenzymes of laccases of *Pleurotus ostreatus* were modified by changing the growth temperature of the fungus.
- It was observed that the number of isoenzymes of laccases does not determine the activity values, it is suggested that the activity depends on the amount of each isoenzyme produced and not on the number of these.
- It is suggested that the regulation of the laccase isoenzymes production could be found in the

stress response elements in the promoters of the genes encoding the laccases.

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Nomenclature

EE	enzymatic extract
U	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, U L^{-1} of EE
X	biomass determined as difference of dry weight, g L^{-1}
X_{max}	experimental maximum biomass
C	relation between maximum biomass and initial biomass, $(X_{max} - X_0)/X_0$, and $X = X_0$; the initial biomass value
$Y_{E/X}$	relation between the maximal enzymatic activity and the biomass observed at the same time, U gX^{-1}
Pro	relation between the maximal enzymatic activity and its time of production, U L^{-1} h^{-1}
SDS	sodium dodecyl sulfate
DMP	2,6 dimetoxifenol
SSF	solid-state fermentation
PUF	polyurethane foam

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