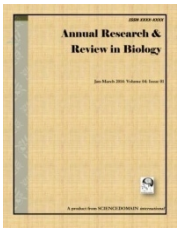

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Laccase Isoenzymes of *Pleurotus ostreatus* Grown at Different pH in Solid-State Fermentation Using Polyurethane Foam as Support

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Authors' contributions

This work was carried out in collaboration between all authors. Author GDG designed the study, prepared the protocol and supervised the work in all its aspects. Author ALV carried out trials. Author MTT managed the literature searches and wrote the first draft of the manuscript. Author RD analyzed the results. Authors MDBM, OL, CS and STB followed and supervised this study in the experimental part. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Effect of culture medium pH (3.5, 4.5, 6.5, 7.5 and 8.5) on the activity and number of laccase isoenzymes of *Pleurotus ostreatus* grown in solid-state fermentation using polyurethane foam as a support was evaluated.
Methodology: *Pleurotus ostreatus* was grown in solid-state fermentation using polyurethane foam as inert support at different initial pH of the culture medium. The

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enzymatic extracts were obtained by pressing the polyurethane foam every day of culture. The laccase activity was measured in each enzymatic extract using 2,6-dimethoxyphenol as substrate and the isoenzymes number were detected through zymograms.
Results: In general, the fungus showed high values of specific growth rate grow that all pH tested, and the higher were at pH 3.5 and 8.5 (0.78 and 0.82h^{-1}, respectively), whereas at pH6.5 was 0.034h^{-1} and pHs of 4.5 and 7.5 was 0.047h^{-1}. Furthermore, the maximum biomass values were low, about 3.7g/L in all cases. The maximum values of laccase activity were observed in fermentations development at pH4.5 and 6.5 (approximately 40000 U/L). The largest number of isoenzymes was observed in fermentations carried out at pH 7.5 and 8.5.
Conclusion: In solid-state fermentation on polyurethane foam, the pH of the culture medium did not affect the growth of the fungus, however, there were differences in the production and activity of laccases.

Keywords: Laccases; Pleurotus ostreatus; solid-state fermentation; zymogram.

1. INTRODUCTION

The genus *Pleurotus* is a cosmopolitan group of ligninolytic fungi, which includes mushrooms with a high nutritional value, therapeutic properties, and several environmental and biotechnological applications [1]. *Pleurotus* produces a range of enzymes, most notably laccases and peroxidases, which enable it to grow on a variety of substrates [2,3]. It adapts easily to different growth conditions, grows relatively quickly in submerged cultures, reduces the content of phenolic compounds originated in the paper industry in parallel with laccase production and can carry out the discoloration of different dyes used in the textile industry, which also are associated with laccase activities [4].

Biotechnology applications developed are associated with microbial enzymes having unusual physicochemical properties in certain processes [5]. The combination of pH and temperature for optimum growth can differently affect each fungal strain and has practical significance for the understanding of fungal physiology [6]. In general, white-rot fungi are able to produce ligninolytic enzymes, which include phenoloxidases such as manganese peroxidase, lignin peroxidase, and laccase [7]. Laccases are widely distributed in nature, occurring in fungi as well as in plants, insects, bacteria and archaea. Laccases are multicopper oxidases that catalyze the one-electron oxidation of a variety of phenolic compounds, aromatic amines and low-redox potential substrates with the concomitant reduction of O_2 to H_2O [8-11]. The production of laccases and peroxidases depends on the species of fungi, the conditions of cultivation, and the sources and concentration of carbon and nitrogen [12]. In addition, laccases are also responsible for various physiological functions in fungi [13]. In most of fungi, laccases have been found to be encoded by multigene families [14]. This gene redundancy, which usually involves differences in their physico-chemical and kinetic properties, regulatory mechanisms and localization [15,16], suggest differential physiological roles such as ones related to nutrition, morphogenesis and inter-reaction [17].

The broad substrate specificity of laccases permits their use in multiple biotechnological and industrial applications as inexpensive biologically and environmentally friendly tools for the pretreatment of lignocellulose for bioethanol production, pulp bleaching, dye degradation, and xenobiotic transformation and detoxification [18].

The information on effect of pH and temperature on laccase production is scarce, but most reports indicate initial pH between 4.5 and 6.0 that is suitable for enzyme production [19]. Diaz et al. [20] studied the production of laccases of *Pleurotus ostreatus* grown at different pH in submerged fermentation (SmF). The activity and isoenzymes number were evaluated in buffer solutions of different pH values. 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. However, at initial pH of 6.5, the isoenzymes number was higher. It was found that the pH of growth, the pH of laccases production and pH of laccases activities were different.

On the other hand, solid-state fermentation (SSF) is defined as fermentation process occurring in absence or near absence of free liquid, employing an inert compound (synthetic materials) or a natural substrate (organic materials) as a support [21]. SSF is shown to be particularly suitable for the production of enzymes by filamentous fungi because they mimic the conditions under which the fungi grow naturally [21,22].

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

The SSF was carried out in a flask of 250 ml containing 0.5 g of polyurethane foam of low density (PUF; 17 kg/m^3) cubes (0.5×0.5×0.5 cm) as an inert support [23] impregnated with 15 mL of sterile culture medium with composition (in g/L): 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; $MnSO_4$, 0.05; $ZnSO_4$-$7H_2O$, 0.001.The water activity value with this medium on PUF was 0.995. The actual water activity value was determined with AquaLab 4TE Water Activity Meter. Three mycelia plugs (4 mm diameter) taken from the periphery of colonies of *Pleurotus ostreatus* grown for 7 d at 25ºC in Petri dishes containing potato dextrose agar (DIFCO^{TM}) were used as inoculum for each flask. 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. All inoculated flasks were incubated at 25°C and samples were taken every 24 h after third day of growth.

2.3 Enzymatic Extract 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).

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

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

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 $X = X_0$; 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) [23].

2.4 Laccase Activity Assay

Laccase activity was determined in each EE of cultures grown at different pH values (3.5, 4.5, 6.5, 7.5 and 8.5) incubated in different buffer solution pH values (3.5, 4.5, 6.5, 7.5 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 μL substrate (2 mM DMP in either 0.1 M citrate buffer pH 3.5, acetate buffer pH 4.5, phosphate buffer pHs 6.5, 7.5 or Tris-base buffer pH 8.5) and 50 μL EE, which were incubated at 40 ºC for 1 min [24]. 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.

2.5 Laccases Zymogram

The laccase activity was detected by zymograms, using the modified technique SDS-PAGE [25]. 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 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 solutions at different initial pH values of 3.5, 4.5, 6.5, 7.5 and 8.5). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 30 min.

3. RESULTS AND DISCUSSION

3.1 Growth of *Pleurotus ostreatus* at Different Initial pH of the Growing Medium in SSF

In all fermentations studied in this work, the growth stationary phase was observed at around 200 h of culture (Fig. 1), whereas the same strain developed at different pH in SmF, their growth stationary phases were reported after 400 h of fermentation [20]. Tellez-Tellez et al. [25] also reported that the stationary phase showed much earlier in SSF than in SmF. The *μ* values observed in this study were 0.078, 0.047, 0.034, 0.047 and 0.082 h^{-1} for fermentations at pH 3.5, 4.5, 6.5, 7.5 and 8.5 respectively, which are significantly greater

than those observed by Díaz et al. [20] in this strain developed at different pH in SmF (the μvalues 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). The μ values were reported around 0.02 h^{-1} for this fungus grown in SmF at initial pH of 6.0 [25], and at pH of 6.5 [26].

However, although the growth of *Pleurotus ostreatus* ATCC 32783 is much more rapid for the SSF, in all cases previously reported and the observed in this study, the X_{max} values were higher in SmF. The X_{max} was around the 3.7 g/L in cultures at pH values of 3.5, 6.5, 7.5 and 8.5 and near to 4.4 g/L in culture at pH value of 4.5 (Fig. 1). Díaz et al. [20] reported that the X_{max} values were of 5.2, 5.5, 9.6 and 8.3 g/L for this same strain in SmF grown at initial pH of 3.5, 4.5, 6.5 and 8.5, respectively. In general, reports of X_{max} for fungus as *Aspergillus niger* indicate that SSF using PUF as support is more efficient than the SmF, which has been explained mainly by increased aeration coupled with a lower catabolic repression by the gradient formation of carbon sources in the solid medium [23,27]. However, in the case of *Pleurotus ostreatus*, has already been reported atypical behavior in the case of the production of laccases [25].

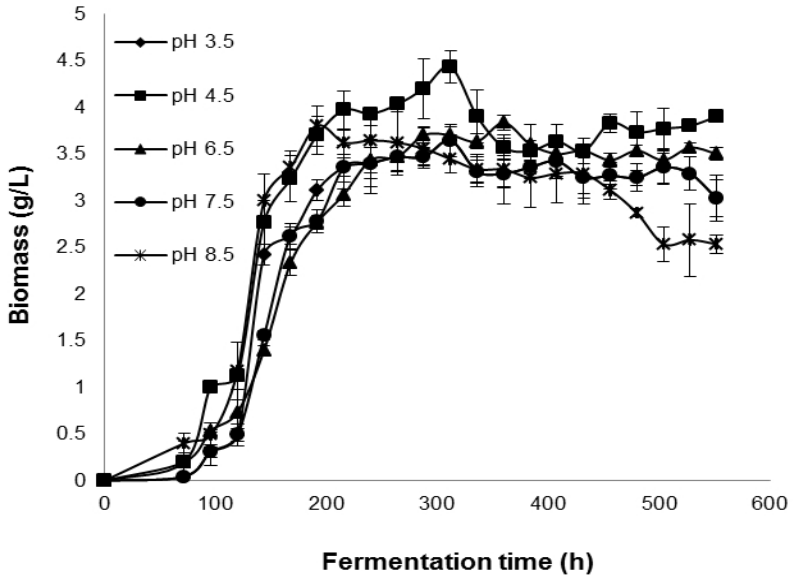

Fig. 1. Growth of *Pleurotus ostreatus* at different initial pH values in SSF

3.2 LaccasesActivity

The highest laccases activity was produced in the stationary growth phase of the fungi in all the cases and this phenomenon was reported in SmF using the same strain [20]. In cultures grown at initial pH 4.5 and 6.5, was observed higher laccase activities than those produced at pH 3.5, 7.5 and 8.5 (Fig. 2). The maximum laccases activity (E_{max}) of *Pleurotus ostreatus* grown at initial pH of 3.5 was of 1453 U/L and 2123 U/L in those EE incubated at pH 3.5 and 4.5, respectively. On the other hand, E_{max} of 375 U/L, 390 U/L and 323 U/L were observed in those EE incubated in buffer solution pH 6.5, 7.5 and 8.5, respectively. The highest value of E_{max} in culture developed at pH 4.5 was observed at activity pH of 6.5 (40856 U/L).

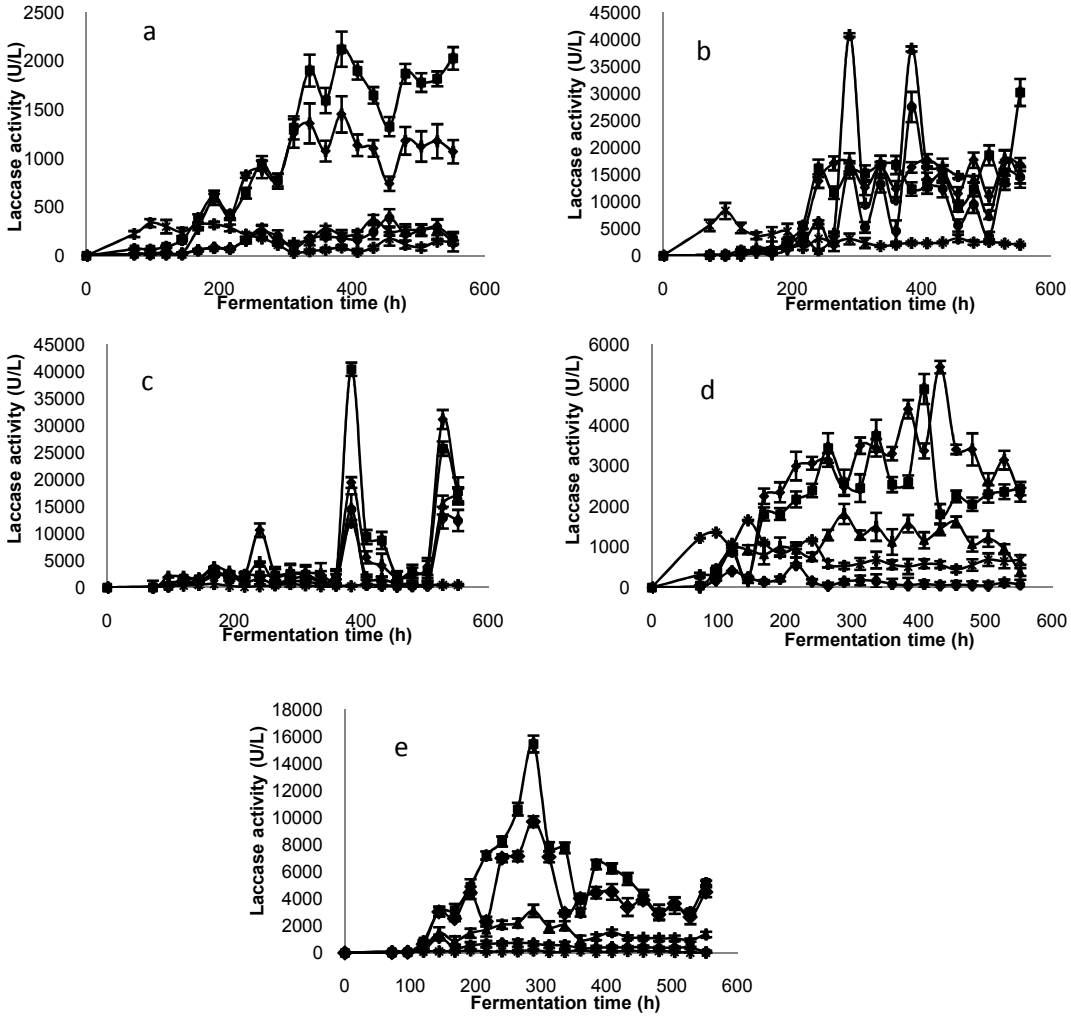

Fig. 2. Laccase activity of *Pleurotus ostreatus* grown in SSF on PUF at initial pH of (a) 3.5, (b) 4.5, (c) 6.5, (d) 7.5 and (e) 8.5, and incubated at different pH values. The error bars represent the standard deviation of three different fermentation runs. The symbols are the same than those in Fig. 1

E_{max} around of 30000 U/L was observed in those EE incubated at pHs of 4.5 and 7.5. 17720 U/L and 8290 U/L were observed in EE incubated at pHs of 3.5 and 8.5, respectively. The laccase activity produced at pH 6.5 showed E_{max} values of 31100, 40380, 17015, 14460 and 680 U/L at pHs of 3.5, 4.5, 6.5, 7.5 and 8.5, respectively. The activity obtained at initial pH of the medium of 7.5, shows that the E_{max} values observed at pH 3.5 and 4.5 were around of 5000 U/L. E_{max} evaluated at pH 3.5 and 4.5 reached values of 1700 U/L. E_{max} of 557 U/L was observed in EE incubated at pH 4.5. The laccases activity produced at pH of 3.5. The E_{max} were of 9227 U/L and 15473 U/L in those EE incubated at pH 3.5 and 4.5, respectively. E_{max} of 3127 U/L, 1144 U/L and 204 U/L were observed in those EE incubated in buffer solution pH 6.5, 7.5 and 8.5, respectively.

In this study, the activity values at initial pHs of 4.5 and 6.5 incubated in buffer solution pH 6.5 add 4.5, respectively (around 40000 U/L) were approximately 3.3 times (12200 U/L) and 8 % (37000 U/L) higher than that laccases activity reported by Tlecuitl-Beristain et al. [26] and Díaz et al. [20] respectively. However, that activity value is almost half of that reported (77500 U/L) for the same strain developed in SmF at initial pH of 4.5 incubated in buffer solution pH 4.5 [20,28]. Patel et al. [29] reported that pH is one the important parameters in the production of laccases by an indigenous strain of *Pleurotus ostreatus* developed at various pH (3.0 to 9.0) in SSF. Optimal pH for maximum laccases production (3356 U/g of dry substrate) was observed at pH 5.0. Exponential increase in laccase activity was observed from pH 3.0 to 5.0, but thereafter laccase production was decreased with increase in pH. This may be attributed to the fact that change in pH may alter the three-dimensional structure of the enzymes [30].

3.3 Laccases Zymograms

Fig. 3 shows zymograms of laccases produced at pH of 3.5. One isoenzyme was observed in gels incubated at pH 3.5 (Fig. 3a). Similar laccases patterns were observed in gels incubated at pHs of 4.5 and 6.5 with one isoenzyme observed during all fermentation times and a second lower molecular weight isoenzyme produced after 336 h of culture (Figs. 3b and 3c). Zymograms incubated at pH of 7.5 and 8.5 showed two possible isoenzymes in some fermentation times (Figs. 3d and 3e); the poor resolution could be due to little enzyme stability at alkaline pH, because the same phenomenon was observed in all cases when the gels were incubated at this pH.

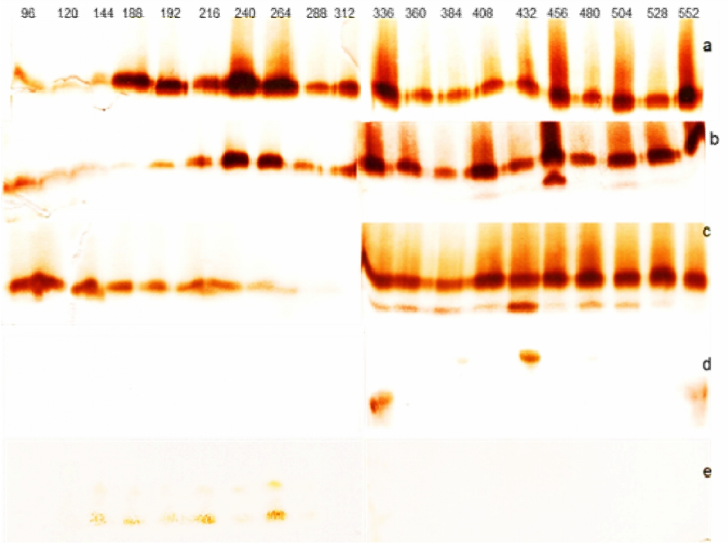

Fig. 3. Zymogram of laccases of *Pleurotus ostreatus* grown at initial pH of 3.5 and incubated at different pH values; 3.5 (a), 4.5 (b), 6.5 (c), 7.5 (d) and 8.5 (e). The numbers at the top of the figure indicate the time (h) of sampling

Fig. 4 shows the isoenzymes pattern produced at pH of 4.5. One isoenzyme was observed during all fermentation times and one more isoenzyme of higher molecular weight at the final of the culture in gels incubated at pH 3.5 (Fig. 4a). Zymogram incubated at pH 4.5 shows two isoenzymes, one from 144 h to 552 h of culture and other observed from 96 h to 188h (Fig. 4b). In Figs. 4c and 4d were observed one isoenzyme in almost all fermentation times

and another in the early times of culture. Zymograms incubated at pH of 8.5 showed one possible isoenzyme at the final of fermentation (Fig. 4e).

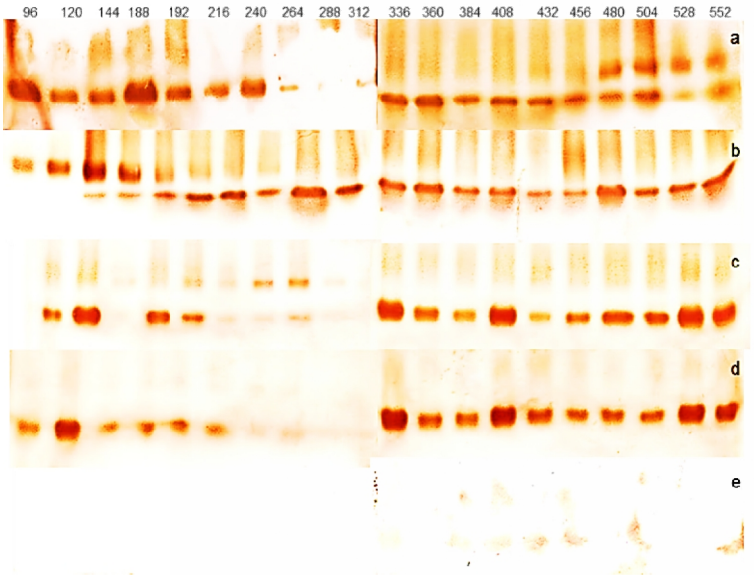

Fig. 4. Zymogram of laccases of *Pleurotus ostreatus* grown at initial pH of 4.5 and incubated at different pH values; 3.5 (a), 4.5 (b), 6.5 (c), 7.5 (d) and 8.5 (e).The numbers at the top of the figure indicate the time (h) of sampling

Fig. 5 shows the zymograms of laccases produced at pH 6.5. In gels incubated at pH 3.5 were observed two isoenzymes, one was present throughout the culture and the other only in some fermentation times (Fig. 5a). Three isoenzymes were perceived in gels incubated at pH 4.5 (Fig. 5b). In Fig. 5c were observed two bands, one was observed during the entire culture. Zymograms incubated at pHs of 7.5 and 8.5 showed poor resolution, but there are two possible isoenzymes at pH 7.5 and no isoenzymes were observed in gels incubated at pH 8.5 (Figs. 5d and 5e).

Zymograms of laccases produced at pH 7.5 are shown in Fig. 6. In gels incubated at pH 3.5, one isoenzyme was observed in all fermentation times and another that can only be found from 216-380 h of culture (Fig. 6a). Up to four isoenzymes were observed in gels incubated at pHs of 4.5, 6.5 and 7.5. The isoenzyme with higher molecular weight was observed throughout the fermentation one present in all fermentation times (Figs. 6b, 6c and 6d). No isoenzymes were observed in gels incubated at pH 8.5 (Fig. 6e).

Fig. 7 shows the zymograms of laccases produced at pH 8.5. In gels incubated at pH 3.5 were observed three isoenzymes, two of which were observed throughout the fermentation (Fig. 7a). Zymogram incubated at pH 4.5 shows two isoenzymes (Fig. 7b) and were observed three and four isoenzymes in the gels incubated at pH 6.5 and 7.5 respectively (Figs. 7c and 7d), and at pH 8.5 showed no isoenzymes (Fig. 7e).

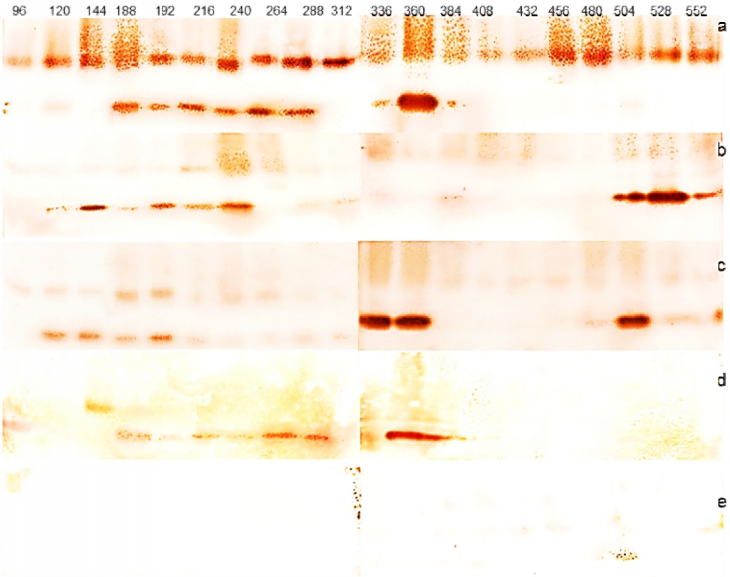

Fig. 5. Zymogram of laccases of *Pleurotus ostreatus* grown at initial pH of 6.5 and incubated at different pH values; 3.5 (a), 4.5 (b), 6.5 (c), 7.5 (d) and 8.5 (e). The numbers at the top of the figure indicate the time (h) of sampling

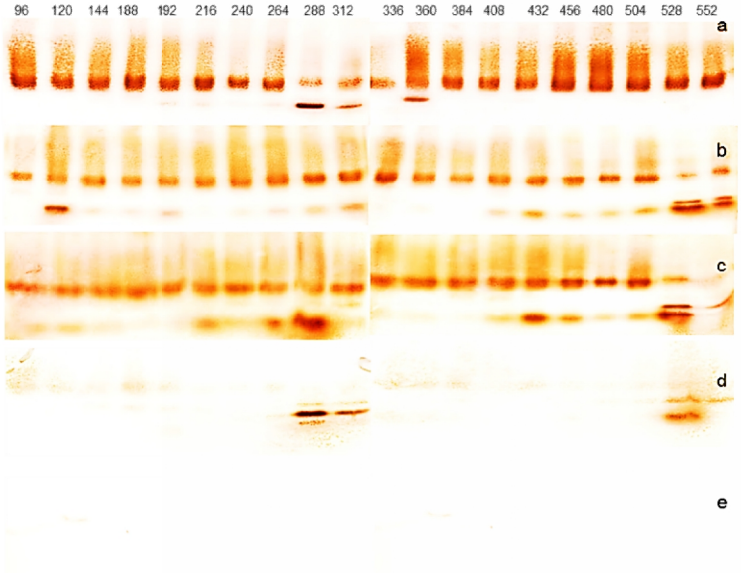

Fig. 6. Zymogram of laccases of *Pleurotus ostreatus* grown at initial pH of 7.5 and incubated at different pH values; 3.5 (a), 4.5 (b), 6.5 (c), 7.5 (d) and 8.5 (e). The numbers at the top of the figure indicate the time (h) of sampling

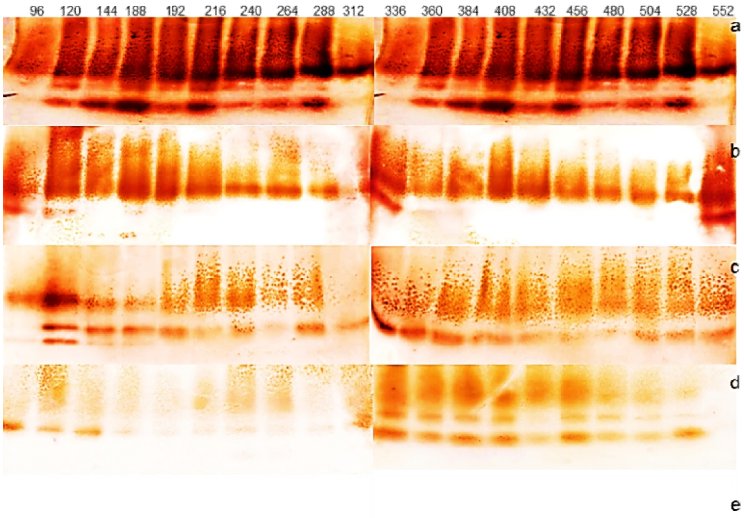

Fig. 7. Zymogram of laccases of *Pleurotus ostreatus* grown at initial pH of 8.5 and incubated at different pH values; 3.5 (a), 4.5 (b), 6.5 (c), 7.5 (d) and 8.5 (e). The numbers at the top of the figure indicate the time (h) of sampling

In this research, the zymogram patterns showed two laccaseisoenzymes in EE produced and incubated at pH 6.5, while, Téllez-Téllez et al. [25] observed that *Pleurotus ostreatus* grown at pH 6.5 in SSF presented two laccase isoenzymes and in SmF using the same culture medium were observed four isoenzymes. Diaz et al. [20] reported that *Pleurotus ostreatus* developed in SmF at different pH produced four isoenzymes. Castanera et al. [31] suggested that laccase gene transcription is upregulated in the induced SmF but down regulated in the SSF. It is suggested that, *Pleurotus ostreatus* grows faster in SSF but the values of X_{max} and E_{max} as well as number of isoenzymes are higher in SmF.

Many reports permit to conclude that fungi are able to produce several laccases and the proportion of the enzymes produced depends upon the culture composition and operational conditions employed [32,25]. Diaz et al. [20] suggested that 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 laccaseisoenzymes number produced by *Pleurotus ostreatus* in SmF, however, was observed the same phenomenon in SmF. In addition to the pH, type of culture (SSF or SmF) also has an effect on the activity and number of isoenzymes of laccases produced. SmF produced more number of isoenzymes and higher activity for the possible stress-responsive promoter in the laccase genes [33].

4. CONCLUSION

In solid-state fermentation on polyurethane foam, the pH of the culture medium did not affect the growth of the fungus, however, there were differences in the production and activity of laccases. In general, the higher activity values were observed at incubation pH of 4.5. The largest number of isoenzymes was observed at alkaline pH. This is the first report on the influence of different initial pH values of the growing medium on the laccases activity and laccase isoenzymes number of *Pleurotus ostreatus* grown in SSF on PUF.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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