

Full Length Research Paper

Temporal profiles of intra- and extracellular laccase isoenzymes from *Pleurotus ostreatus* produced in submerged and solid-state cultures

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Laccases are enzymes that have a great potential for use in breaking down toxic compounds. Fungal laccases show high enzymatic activity, especially those produced by basidiomycetes. Depending on the culture conditions and the strain used, a variety of isoenzymes and/or enzymatic activities can be obtained. In this study, extracellular laccase enzymes produced by *Pleurotus ostreatus* was identified in a submerged culture (SmF), with and without copper sulphate as a chemical inducer, and in a solid-state culture (SSF), using wheat straw as natural inducer. This study was conducted to observe the expression of the enzymes produced under the culture conditions tested and their persistence during the culture, as well as the extracellular activity produced and the correspondence that the isoenzymes presented between the intracellular and extracellular media. A positive effect of the inducers on the specific laccase activity was observed either in SmF with copper sulphate or SSF (41.11 and 40.43 UI/mg protein, respectively), compared with that obtained in SmF without copper sulphate (2.87 UI/mg protein). This effect was different only at the time when the highest activity appeared (360 and 120 h, respectively), showing advantages in SSF. The same three isoenzymes were observed in the three kinds of cultures. The main differences among the laccase profiles reside in the time when they appeared in each culture and only an additional form of lower molecular weight was observed in SSF. The laccase enzymes in the intracellular extracts were equal to those in the extracellular ones. The laccase isoenzymes profiles suggest that the presence of inducers helps in maintaining the activity through the culture time.

Key words: Phenol oxidases, basidiomycete, enzymatic activity, copper, wheat straw, solid-state culture (SSF), submerged culture (SMF).

INTRODUCTION

Pleurotus ostreatus is one of the most widely studied fungal species, because it is edible, has medicinal

properties, and can produce enzymes used at experimental and industrial levels in food, medicine, and

recently, in bioremediation processes (Piscitelli et al., 2010). The fungus belongs to the white rot basidiomycetes and some of the enzymes they produce (and of the greatest interest) present laccase activity (Eggert et al., 1997; Baldrian, 2006).

Laccases (benzenediol: oxygen oxide-reductases EC 1.10.3.2) are glycoproteins known as blue multi copper oxidases. In general, they are monomeric enzymes, although some of them are multimeric, intracellular or extracellular, and belong to the family of phenoloxydases, which act on *p*-diphenols (Palmieri et al., 2000). They have a catalytic site, characterized by four copper atoms linked to three REDOX sites (T1, T2, and T3), through which it binds to four electrons to reduce oxygen to water, while oxidizing a wide spectrum of substrates (Kunamneni et al., 2008). The different forms in which they occur are called isoforms or isoenzymes. The term "isoenzyme" has been used as an operational definition that brings together multiple enzyme forms with the same substrate specificity (Marker and Moller, 1959). Nevertheless, the nomenclature established by the IUPAC-IUB (1981) recommends to restrict the use of the term "isoenzyme" to enzymatic products with the same catalytic activity but different due to genetic causes.

The laccase activity and the number of isoenzymes vary depending on the culture conditions (including the use of inducers), the fungus species, and even the specific strain used (D'Souza et al., 1999; Giardina et al., 1999; Mansur et al., 2003; Palmieri et al., 2000, 2003).

Nevertheless, it is difficult to compare the results from different reports because the laccase activity and the number of isoenzymes are measured using different substrates. Additionally, each strain of a particular fungal species has been tested in one specific condition at a time, making difficult to know the difference between enzymatic activities and isoenzyme patterns of the same strain under different culture systems, which would help to improve comparisons and to reach more accurate conclusions.

It has been reported that the *P. ostreatus* genome encodes around eleven genes of laccases, and six different laccases have been isolated and characterized showing that they are encoded by different genes. For this reason, the different forms of the laccase enzymes produced by *P. ostreatus* are referred as isoenzymes in several reports (Pezzella et al., 2013; Velázquez-Cedeño et al., 2007).

On the other hand, it has been reported that laccases have different functions, some of them are carried out in the intracellular environment (defense under stressful condition, formation of conidia) and others extracellularly (lignin degradation, detoxification of pollutants), without making it clear whether the isoenzymes operating in each

environment are different or the different functions are performed by the same isoenzymes (Mansur et al., 1997; Galhaup et al., 2002; Velázquez-Cedeño et al., 2007).

P. ostreatus produces higher enzymatic activities during submerged culture (SmF) than those produced during solid-state culture (SSF) using inert supports (e.g., polyurethane foam) (Téllez-Téllez et al., 2008). However, the differences between laccase activities produced during SSF on natural and biodegradable supports, and those produced during SmF are not so evident due to the nature of the systems, the units used to report these activities, the substrate kind and the different parameters used for their evaluation. There are reports showing higher laccase activities in SSF than in SmF (Ramírez et al., 2003; Stajić et al., 2004). In another study (Teixeira et al., 2010), enzymatic extracts (EE) obtained from this species, cultured on waste materials from agricultural processes, yielded better laccase activities when compared with those obtained from commercial enzymes applied to the degradation of AZO pigments, in spite of their lower activity (5.4 U/g of dry substrate (DS)). All of this has increased the interest in the study of SSF with the goal of applying them to these processes at different levels.

Few studies have assessed whether these variations are due mainly to the type of isoenzymes produced in both types of cultures: some studies show more isoenzymes in SmF than in SSF and, in other works, opposite results have been obtained. In the field of gene manipulation, this information is very useful because of the interest in working with those isoenzymes, which express themselves efficiently under particular conditions for a better handling of organisms focusing on improved production. So far, this has not been achieved in existing improved strains (Piscitelli et al., 2010; Record et al., 2002; Rodgers et al., 2010), due to the lack of studies on the regulatory processes of the species used.

The aim of this study was to evaluate changes in the laccase isoenzymes produced by *P. ostreatus* and the time when these isoenzymes appear in three culture conditions: SmF (without and with CuSO₄ as a chemical inducer) and SSF (assuming that the lignocellulosic components of wheat straw could act as natural inducers), as well as to determine the correspondence between the intra and extracellular isoenzymes observed.

MATERIALS AND METHODS

Strain and culture conditions

The *P. ostreatus* strain ATCC 32783 was used. The fungus was prepared in a medium of malt agar extract (MAE) (DIBICO, Mexico)

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and incubated at 25°C for 168 h. The mycelium was conserved and refrigerated at 4°C.

Production of laccase enzymes

For SmF, the fungus was inoculated into 125 ml Erlenmeyer flasks, in 50 ml of a medium for laccase production by *P. ostreatus*, containing (in grams per liter): glucose, 10; yeast extract, 5; KH_2PO_4 , 0.6; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; K_2HPO_4 , 0.4; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001. For SmF with chemical inducer, 0.25 g/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added to the aforementioned composition. The pH was adjusted to 6.0 with 0.1 M NaOH (Télez-Télez et al., 2008). For SSF, wheat straw was used as substrate and natural inducer. As a pre-treatment, the wheat straw was washed three times with distilled water and then pasteurized and adjusted to 70% humidity. Previously, the laccase activity was tested in the extracts of the pre-treated wheat straw without fungus and laccase activity was not observed. The fungus was inoculated in crystallizers of $9 \times 5 \text{ cm}^2$, containing 35 g of pre-treated wheat straw.

The experimental units were inoculated with 2 ml of a suspension of the fungal mycelium (0.05 g approximately), obtained after mechanic homogenization of the mycelium in physiological solution, with the help of an electric mixer (Aerolatte®, aerolatte® Ltd, United Kingdom). The experiment was replicated three times and conducted during 20 days, sampling three experimental units from each experimental condition every 24 h.

The enzymatic extract (EE) and biomass were obtained from each experimental unit. In the case of the SmF with and without CuSO_4 , EE was obtained by centrifugation at 5000 rpm for 10 min and stored at -20°C.

For the SSF on wheat straw, the EE was recovered through lixiviation of extracellular products with 75 ml of distilled water and orbital shaking at 300 rpm for 30 min and subsequent extrusion of the substrate with the mycelium through a gauze. This straw residue with mycelium was used for the extraction of the intracellular enzymatic extract (IEE).

The IEE was obtained through maceration of the frozen straw residue with mycelium with liquid nitrogen and lixiviation with 50 ml of 0.1 M phosphates buffer, pH 6.5, and recovered by centrifugation at 10000 rpm for 10 min.

Determination of laccase enzymatic activity

The enzymatic activities of extracellular laccases in the EE were evaluated through absorbance changes at $\lambda=468 \text{ nm}$, after 1 min of the reaction at 39°C. The reaction mixture contained 950 μl of 2 mM 2, 6-dimethoxy phenol (DMP) ($\epsilon_{\text{DMP}}= 49,600/\text{M cm}$), as a substrate in 0.1 M phosphates buffer, at pH 6.5, and 50 μl of EE. One enzymatic international unit of laccase was defined as the amount of enzyme which catalyzes the conversion of 1 μM of substrate to product per minute, in the reaction mixture. The activity was expressed as international units per liter in SmF and as units per kilogram of dry substrate (DS) in SSF on wheat straw. The specific activity was reported as international units per milligram of extracellular protein calculated according to the relationship between the maximum activity of the laccase obtained and the extracellular protein produced at the same culture time. The extracellular protein was determined in the EE through the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Laccase isoenzymes profiles

The profiles of isoenzymes with laccase activities were evaluated based on both the EE (for the extracellular forms) and the IEE (for

the intracellular forms), through the modified zymography method of electrophoresis in polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970), following the procedure using electrophoresis in semi-denaturalizing polyacrylamide gels (Télez-Télez et al., 2005). The activity was detected with 50 ml of 2 mM of DMP as substrate in 0.1 M phosphate buffer, at pH of 6.5, and using the Precision Plus Protein Standard (BIO-RAD. Hercules, CA) marker for the partial characterization of the size of the bands observed. Finally, the size of the isoenzymes was estimated using the band analysis software Quantity One (BioRad).

To compare the maximum specific laccase activities found among the three kinds of cultures (SmF with and without CuSO_4 and SSF), the one-way analysis of variance (ANOVA) test was applied. The differences among the treatments were determined by the pos-hoc Tukey test.

RESULTS

Laccase enzymatic activity

Enzymatic activities were higher in SmF using CuSO_4 as inducer ($9000 \pm 622 \text{ UI/L}$) compared to the activities of SmF without CuSO_4 ($498 \pm 35 \text{ UI/L}$); these peak activities were observed at 360 and 264 h, respectively. Furthermore, the maximal enzymatic activity observed in SSF was $4821 \pm 30.41 \text{ UI/kg DS}$ at 120 h of culture (Figure 1A and B), a shorter time than those in SmF with or without CuSO_4 . To assess the effect of the induction on both cultures, laccase specific activities relative to the amount of protein produced was compared: the effect of the inducer yielded around 14 times more activity (41.11 ± 1.00 and $40.43 \pm 2.21 \text{ UI/mg protein}$ for SmF and SSF, respectively) compared to that obtained without induction in SmF ($2.87 \pm 0.23 \text{ UI/mg protein}$). The activities are shown in Table 1.

Significantly, one of the most important differences is the time when the maximum specific activities were achieved, with the shortest time to obtain the maximum specific activity in SSF compared to SmF.

Variance analysis revealed highly significant differences among treatments ($F_{(2,6)} = 723.9$; $p < 0.001$). Tukey test indicated differences in the maximum specific laccase activities between SmF and both SSF and SmF without CuSO_4 ($p < 0.001$) but not between the last two. However, the times when maximum activities were attained, were different from one treatment to another.

Intracellular and extracellular laccase isoenzymes profiles

In the three conditions for growing this *P. ostreatus* strain, three bands corresponding to isoenzymes were observed with laccase activity over DMP. These three isoenzymes were observed in both EE and IEE. However, there were differences in the profile for each culture condition and in the time elapsed. The isoenzymes size was estimated by the bands analyzer software, considering the sizes of the band of weight marker as referring. Figures 2 and 3 show

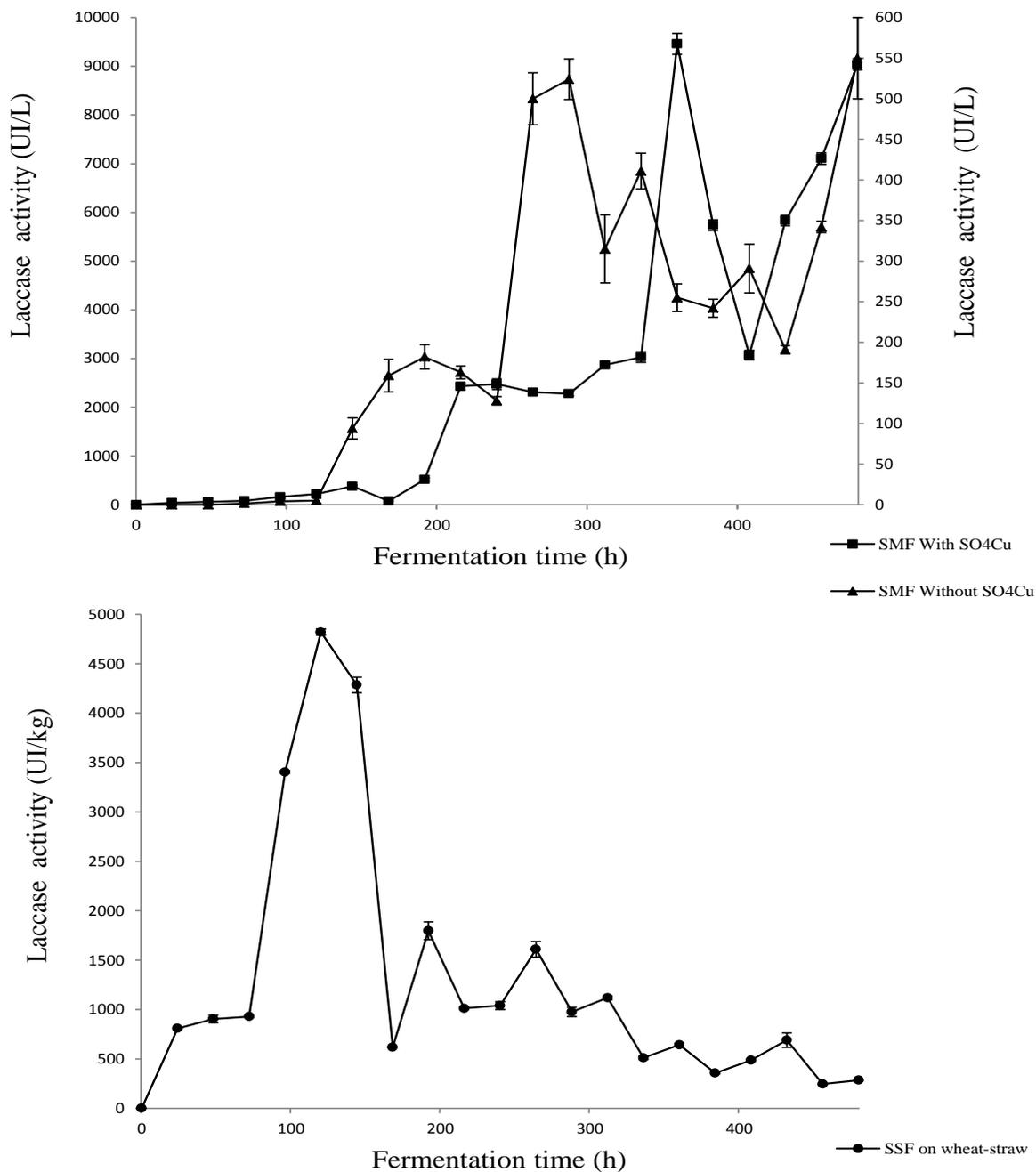


Figure 1. Laccase production by *P. ostreatus* (A) in SMF with CuSO₄ (rhombus, left axis), without CuSO₄ (triangle, right axis), and (B) in SSF (circle). The error bars represent the standard deviation of three separated replicates of each experiment.

Table 1. Activities of extracellular laccase produced in SMF with and without CuSO₄ and in SSF.

Type of fermentation	Maximum enzymatic activity (UI/L y UI/kg DS)	Specific activity (UI/mg protein)
SMF without CuSO ₄	498 ± 35 (264 h)*	2.87 ± 0.23
SMF with CuSO ₄	9000 ± 622 (360 h)*	41.11 ± 1.00
SSF	4821 ± 30.41 (120 h)*	40.43 ± 2.21

*Time of culture when the maximum values of enzymatic activity were obtained.

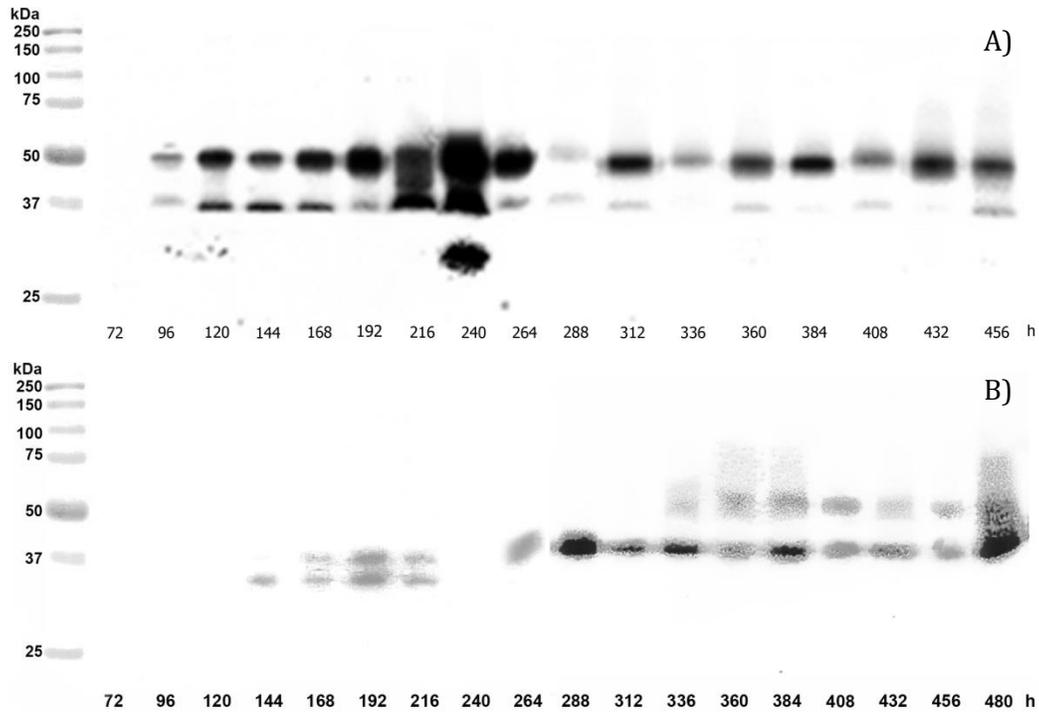


Figure 2. Profiles of laccase isoenzymes produced by *P. ostreatus* in SMF without CuSO_4 , A) intracellular and B) extracellular.

the profiles of laccase isoenzymes produced by *P. ostreatus* in SmF without and with CuSO_4 , respectively.

For the two conditions of liquid culture, the isoenzyme with constant activity is found around 50 kDa molecular weight markers, and it is present intracellularly during the culture period. For the intracellular profile, the intensity of the bands is higher between 96 and 200 h in the culture without CuSO_4 , while in the culture with CuSO_4 , the higher intensity of the bands is found between 240 and 456 h. This same isoenzyme is present in the extracellular extracts but it can be seen only from the 336 h of culture, not showing differences in the intensity of the bands due to the presence of the chemical inducer. This isoenzyme was followed by the presence of another isoenzyme around 37 kDa molecular weight markers. For the intracellular profile of the culture without CuSO_4 , its activity begins at 96 h, but it decreased from the 264 h. In the case of the culture with CuSO_4 , its activity begins at 72 h and shows a slight increase in intensity until the end of the culture. For the extracellular profile, this isoenzyme showed that the activity beginning at 168 and 192 h for the cultures with and without CuSO_4 , respectively. In this isoenzyme, no differences were found in the intensity of the bands due to the presence of chemical inducer.

The largest difference between the two conditions of SmF was found in the isoenzyme with estimated size of 34 kDa, showing activity only at 240 h, in the intracellular profile for the culture without CuSO_4 . Nevertheless, for

the culture with CuSO_4 , the isoenzyme showed activity beginning at 72 h and its activity increased gradually until the end of the culture. For the extracellular profile, in the culture without CuSO_4 , this isoenzyme showed activity only between 144 and 216 h, while for the culture with CuSO_4 , its activity appeared at 192 h and shows an increase in intensity of the band, until the end of the culture. Both profiles suggest a positive effect of the chemical inducer.

Figure 4A and B shows the profiles of laccase isoenzymes produced by *P. ostreatus* in the SSF on wheat straw, (A) intracellular and (B) extracellular. First, in the case of the isoenzyme of size of 50 kDa approximately, its activity is not evident from the beginning of the culture as in the SmF, it can be seen from 144 and 192 h in the intra and extracellular profiles, respectively. The isoenzyme of size of 37 kDa approximately begins its activity at 240 h in both profiles, but only showed activity until 312 and 336 h in the extra and intracellular profiles, respectively. Finally, the isoenzyme of estimated size of 34 kDa, showed activity between 120 and 240 h, in the intra and extracellular profiles, respectively. In addition to these three isoenzymes, the same isoenzymes observed in the SmF with and without CuSO_4 , another isoenzyme with lower molecular weight, estimated in 30 kDa, was identified in both intra and extracellular profiles for the SSF; its enzymatic activity is low in the beginning, but it remains

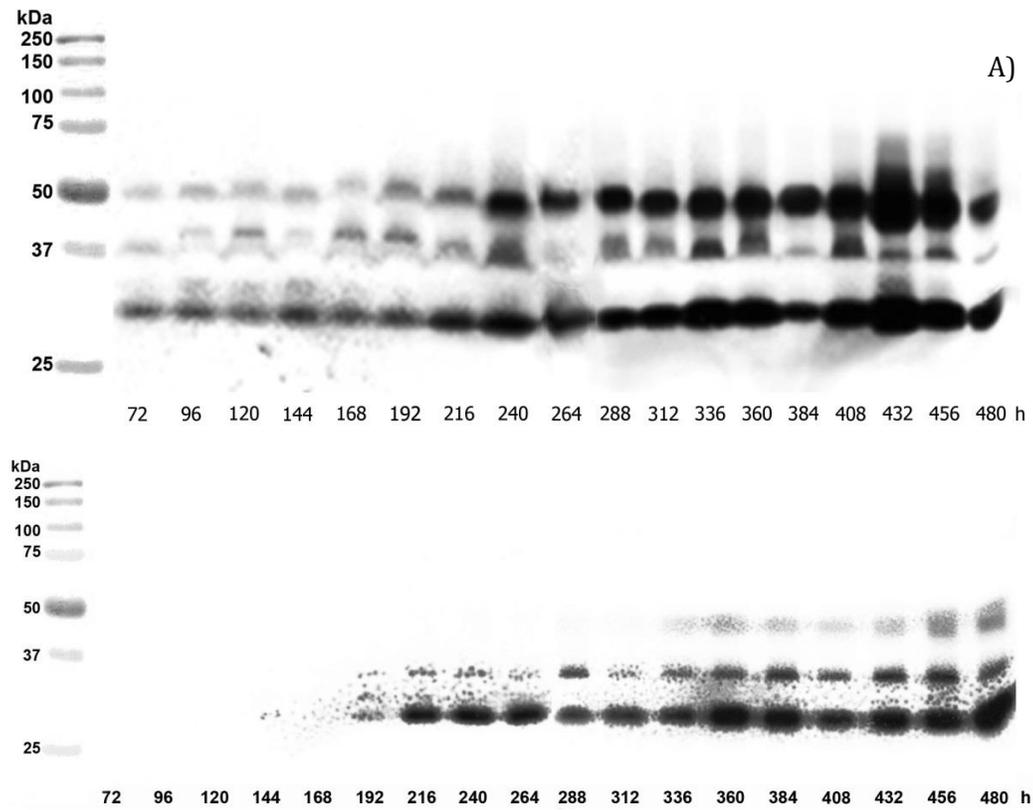


Figure 3. Profiles of laccase isoenzymes produced by *P. ostreatus* in SMF with CuSO_4 . A) intracellular and B) extracellular.

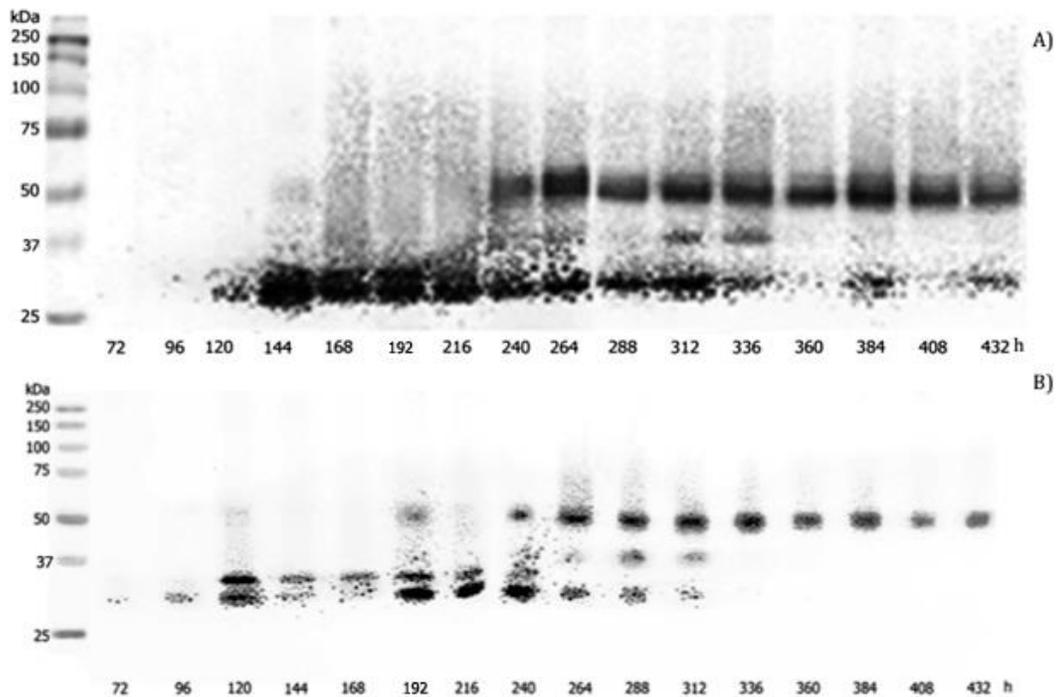


Figure 4. Profiles of laccase isoenzymes produced by *P. ostreatus* in SSF over wheat straw, A) intracellular and B) extracellular.

throughout the whole culture in the intracellular profile, while it only presents activity until 312 h in the extracellular profile.

DISCUSSION

In the SmF, with and without copper sulphate, the enzymatic activities obtained depicted the same behavior reported by others authors in previous papers (D'Souza et al., 1999; Palmieri et al., 2000) where the influence of Cu as the inducer increased the activity around 20 times.

However, the enzymatic activities produced here (9,000 UI/L) were lower than those reported previously in similar conditions (13,000 U/L) (Téllez-Téllez et al., 2008); this may be due to the way the inoculum was prepared, probably because of the extrusion of the agar pellets from the mycelium and the homogenization before the inoculation. This could have promoted the destruction of some cells and perhaps the mycelium had an additional growth recovery process, resulting in a decrease of enzymatic activity.

The modification in the preparation of the inoculum was performed to remove the mycelial agar pellets, because there are reports indicating that the composition of the medium influences the production of laccase isoenzymes (Giardina et al., 1999; Palmieri et al., 2000, 2003; Mansur et al., 2003). In this study, we did not want to interfere in the propagation medium in order to assess the actual effect of the tested substrates. Similarly, attempts were made to provide a homogeneous inoculum to the substrate to allow the mycelium to extend its contact area, primarily in the SSF on wheat straw, so it could grow in all areas of the substrate simultaneously. This cannot be achieved when complete pellets are punctually inoculated.

Moreover, no reports were found evaluating enzymatic activity of laccases produced by *P. ostreatus* on wheat straw using DMP as a substrate. However, there are reports of laccase activity produced on other solid substrates using 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as an enzyme substrate (Márquez-Araque et al., 2007; Sathishkumar et al., 2010).

In the present study, the maximal enzymatic activity observed in the SSF was around 5000 UI/kg of DS, using DMP as substrate, this is comparable to the activity obtained in *P. ostreatus* var. *florida* using banana peel, which was 5400 UI/kg of DS, with ABTS as substrate (Sathishkumar et al., 2010). The activity observed in the present study was lower when compared to that obtained by *P. ostreatus* grown on bagasse where it was observed a maximum activity evaluated on ABTS, of 15,540 UI/kg of DS of an EE (Márquez-Araque et al., 2007).

These differences may be due to the composition of the waste of sugar bagasse since it is richer and more easily assimilated as compared to the wheat straw or banana residue.

Regarding the comparison of cultures, as mentioned earlier, it is difficult to make comparisons based only on the activity of the EE of the obtained laccase due to the nature of the culture systems.

However, a comparison can be made based on the specific activity of laccase; it was observed that in both media, the SmF supplemented with CuSO₄ and the SSF using wheat straw as a natural inducer, were able to increase the activity up to 14-fold relative to the activity obtained without addition of CuSO₄ in the SmF.

In this comparison, the SSF maximum activity was reached in less time (120 h). Comparing SmF without copper, a significant increase in the activity of laccase was observed in the SmF with CuSO₄; however, this was achieved at a longer culture time (360 h). These results could indicate one advantage of the SSF in the production of laccases.

In the three conditions of growing this strain of *P. ostreatus*, three isoenzymes, apparently the same isoenzymes, were observed with laccase activity evaluated with DMP. Taking into account the two conditions of the liquid culture, the isoenzymes profiles suggest a positive effect of CuSO₄ on the isoenzyme activity of less molecular weight, which maintain its activity throughout the culture time in the intracellular extracts and for the extracellular profile, they showed activity from the 192 h until the end of the culture.

In SSF, the profile was different regarding the time; these isoenzymes were active, but only during the intermediate culture times, when the vigorous growth of the mycelium began. In addition, a new isoenzyme of approximately 30 kDa was active during almost all the culture time in IEE and until 312 h in EE. The isoenzyme profile of 34 kDa approximately, is similar to the profile observed in that of 30 kDa approximately, in the first half of the culture time. It is necessary to perform other studies to determine whether these isoforms are isoenzymes or they are the same isoenzyme but with different glycosylation level. However, the isoform lower size could be related to the fungus response to the growth on a complex carbon source (lignin) because it only appears in SSF. The interplay of the other isoenzymes starts when the concentration of the less complex sugars increases, and finally keeps the isoenzyme of the highest molecular size with constant activity during the rest of the culture.

In the zymograms, one additional band was observed in the SSF with respect to SmF. These results are different, considering a previous report (Téllez-Téllez et al., 2008); this difference can be due to the fact that the solid support is inert and does not produce any inducer effect on the fungus. However, regarding the isoenzymes profile found in the SmF, the differences can be due to the influence of the residual culture medium of the agar pellets on the propagation of the inoculum producing an additional band. The results obtained in the SSF concur with other report, where three isoenzymes were also

observed with sizes of 52, 46, and 30 kDa (Ramirez et al., 2003).

With respect to size, it is not possible to arrive at a conclusion because the sizes of the isoenzymes in the present study were only estimated considering a molecular weight marker. It is worth mentioning, that the sizes of the laccase isoenzymes seem to be lesser (30 to 50 kDa) than those reported for this fungal species (60 to 80 kDa) (Palmieri et al., 2000, 2003; Tleucitl-Beristain et al., 2008).

It has been observed that at least three of the isoenzymes produced in SmF are present in the stationary phase after 408 and 456 h, whereas just one of them was observed throughout the whole culture (Tleucitl-Beristain et al., 2008). This study demonstrated that in the SmF intracellular activities of the three isoenzymes are produced from the very beginning of the culture. In the case of the SSF, however, the largest isoenzymes were observed only after the adaptation phase of the fungus.

These results suggest that the changing conditions of the culture regulate the activity of the laccase isoenzymes produced by this *P. ostreatus*, irrespective to the kind of isoenzymes being produced. Only in the case of the exclusive SSF, isoenzyme is possible to suggest its additional participation and differential action on the oxidation of the lignin of the wheat straw.

The number and apparent size of the laccase isoenzymes observed in each condition, for both intracellular and extracellular extracts were similar with differences in their expression along the culture time. These results did not show evidence of the presence of specific isoenzymes for intracellular environment, instead they appear to be the same which are secreted extracellularly according to the needs of the fungus.

Conflict of interest

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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