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LACCASE ACTIVITY OF *Pycnoporus cinnabarinus* GROWN IN DIFFERENT CULTURE SYSTEMS

ACTIVIDAD DE LACASA DE *Pycnoporus cinnabarinus* CRECIDO EN DIFERENTES SISTEMAS DE CULTIVO

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Abstract

Activity and zymogram patterns of laccases produced by *Pycnoporus cinnabarinus* (HEMIM-79) grown in three systems of submerged fermentation (airlift reactor, stirred tank reactor and Erlenmeyer flasks) and in solid-state fermentation using polyurethane foam as inert support, were studied. A culture medium based in glucose, yeast extract and mineral salts including copper sulfate as laccases inductor, was used in all grown systems. Very different activity levels were observed depending on the growth system and in all cases the zymogram patterns were similar. So, in this strain, the number of isoenzymes was independent of culture conditions but their amount changed, showing the highest values in the culture in shake flasks followed by the solid-state fermentation, followed by stirred tank reactor and the lowest activity was observed in airlift reactor. It is suggested that the difference in laccase activity could be due to the type of growth given in each system, the pellets by their small size are the metabolically more active compared to the big mass produced in the airlift reactor; although the stirred tank showed small pieces of mycelium, not showed an adequate growth by mechanical damage to cells. In the case of solid culture, the fungus growth was in mycelium form which is similar to its natural habitat.

Keywords: airlift reactor, Erlenmeyer flasks, isoenzymes, laccases, solid-state fermentation, stirred tank reactor.

Resumen

Se obtuvo la actividad y los patrones zimográficos de lacasas producidas por *Pycnoporus cinnabarinus* (HEMIM-79) crecido en tres sistemas de fermentación sumergida (reactor airlift, reactor de tanque agitado y matraces Erlenmeyer) y en fermentación en estado sólido usando espuma de poliuretano como soporte inerte. En todos los sistemas se utilizó un medio de cultivo con glucosa, extracto de levadura, sales minerales y sulfato de cobre como inductor de lacasas. Se observaron diferentes niveles de actividad en función del sistema de crecimiento y en todos los casos los patrones de zimografía fueron similares. Por lo tanto, en esta cepa, el número de isoenzimas fue independiente de las condiciones de cultivo, pero la cantidad cambió, mostrando los valores más altos en el cultivo en matraz agitado, seguido de la fermentación en estado sólido, luego del reactor de tanque agitado, la actividad más baja se observó en el reactor airlift. Se sugiere que la diferencia en actividad de lacasa podría deberse al tipo de crecimiento dado en cada sistema, siendo los pellets por su pequeño tamaño los metabolicamente más activos en comparación con la gran masa producida en el reactor airlift y el tanque agitado aunque mostró pequeños trozos de micelio, no hubo un adecuado crecimiento por el daño mecánico causado a las células. En el caso del cultivo sólido, el hongo se desarrolló en forma de micelio el cual es lo mas parecido a su habitat natural.

Palabras clave: reactor airlift, matraces Erlenmeyer, isoenzimas, lacasas, fermentación en estado sólido, reactor de tanque agitado.

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1 Introduction

Lignin is a complex and heterogeneous aromatic biopolymer which provides strength to the wood structure and protects it against microbial attack. The most effective lignin degraders in nature are the white-rot fungi which belong to the basidiomycetes (Ander and Eriksson, 1978). Enzymes involved in the degradation of lignin oxidize phenolic structures and catalyze the generation of highly reactive radicals that can also degrade non phenolic structures (Boominathan and Reddy, 1992; Thurston, 1994). The three classes of extracellular lignin modifying enzymes generally recognized are lignin peroxidase, Mn dependent peroxidase and laccase. White-rot fungi produce one, two or the three of these phenoloxidase enzymes and this appears to be a feature among this group of fungi (Orth *et al*., 1993). Laccase (ρ-diphenol: oxygen oxidoreductases) was first demonstrated in the exudates of *Rhus vernicifera*, the Japanese lacquer tree (Yoshida, 1883). Later it was demonstrated in fungi (Bertrand, 1896). Laccases, are either mono or multimeric copper-containing oxidases that catalyze the reduction of oxygen to water accompanied by the oxidation of a phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water, as one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen, the reaction mechanism cannot be straight forward. When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical (Thurston, 1994).

Many laccase-producing fungi secrete isoforms of the same enzyme (Leontievsky *et al*., 1997). These isoenzymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald *et al*., 1997). Some fungi produce isoenzymes with similar Km and kcat values. In wood-rotting basidiomycetes that are usually dikaryotic, this fact probably indicates that allelic variability is responsible for the production of isoenzymes rather than the evolution of enzymes adapted to the special needs of the fungus. The number of isoenzymes differs between species and also within species depending on whether they are induced or non-induced (Bertrand *et al*., 2013). Isoenzymes can differ markedly in their stability, optimal pH and temperature, and affinity for different substrates (Babu *et al.*, 2012).

There are multiple factors influencing laccase production. Téllez-Téllez *et al*. (2005) reported the laccase zymogram profiles of *Pleurotus* species using various substrates, indicating that laccase isoforms were specific for each species. The differences observed in number and position of isoforms in the gel, suggest that laccase zymograms might be a way to differentiate species of this genus. In other study, the cultures of *Pleurotus ostreatus* grown in submerged fermentation produced laccase at 13,000 U L^{-1}, with a biomass production of 5.6 g L^{-1} and four laccase isoforms, however, cultures grown in solid-state fermentation had a much lower laccase activity of 2,430 U L^{-1}, biomass production of 4.5 g L^{-1}, and three laccase isoforms (Téllez-Téllez *et al*., 2008). Díaz *et al*. (2013) reported that the initial pH of the growing medium is an important factor for regulating the expression of laccase genes which have an effect on the activity and number of laccase isoenzymes produced by *Pleurotus ostreatus* in submerged fermentation (SmF).

Guzmán (2003) considers that *Pycnoporus sanguineus* is a tropical variant of *Pycnoporus cinnabarinus* from the temperate zone, adapted to man disturbed sites, where it is common in fallen logs and fences, always in sunny places. It is closely related species, *Pycnoporus coccineus*, and *Pycnoporus sanguineus*. These fungi are recognized as efficient lignin decomposers, in spite of its relatively simple lignin modifying enzyme system composed of laccases (Eggert *et al*., 1996). These features make *Pycnoporus* species an attractive group of white-rot basidiomycetes for the production and purification of laccases.

2 Methods

2.1 Organism and culture conditions

Pycnoporus cinnabarinus (HEMIM-79) was employed. The fungus was grown by triplicate in four culture systems, solid-state fermentation (SSF) and SmF in shake flasks, airlift reactor and stirred tank reactor. For all culture systems, a liquid medium containing in g L^{-1}: glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$ $7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$ $5H_2O$, 0.25; $FeSO_4$ $7H_2O$; 0.05; $MnSO_4$ H_2O, 0.05; $ZnSO_4$ $7H_2O$, 0.001 was used (Téllez-Téllez *et al*., 2008). The pH was adjusted at 6.5 using NaOH 0.1M.

The SSF was carried out in Erlenmeyer flask (250 mL) containing 1 g of polyurethane foam of low density (PUF; 17 kg m^{-3}) cubes (0.5 height x 0.5 width x 0.5 depth) (Diaz-Godinez *et al*., 2001) as an

inert support impregnated with 30 mL of sterile culture medium. Previously, the cubes were washed twice with boiled distilled water and oven-dried (at 60°C) for 24 h and then autoclaved at 15 psi for 15 min. The SmF was undertaken in Erlenmeyer flasks (125 mL) containing 50 mL of culture medium. All flasks were inoculated with three mycelial plugs (4 mm diam) taken from the periphery of a colony grown on PDA at 25°C for 7 d. The cultures were incubated at 25°C for 25 days on a rotary shaker at 120 rpm. Samples were taken every 24 h after third day of growth. The enzymatic extract (EE), was obtained from the SSF by soft pressing the PUF cubes and from the shake flasks was obtained by biomass retention, the broths were filtrated using Whatman paper No. 4.

Fungus was also grown in SmF using a 3 L stirred tank at propeller speed of 120 rpm and in 5.5 L airlift bioreactor with an air flow of 1 vvm, in both at 75% of their capacity with culture medium, Agitator speed and aeration rates were kept constant over the whole cultivation time. The reactors were inoculated with mycelium of one colony per L of culture medium, obtained from a Petri dish (100 x15 mm) with PDA at 25°C for 7 d. The operation temperature was 25 °C. Five mL of culture medium considered as EE was taken from 72 h after inoculation and then every 24 h. In both reactor cultures, was impossible to directly quantify fungal growth. To indirectly measure fungal growth, glucose disappearance from the culture broth was quantified using a refractometer, and in both cases, fermentation was stopped when the glucose concentration was close to zero.

2.2 *Enzyme assays*

In each EE, the activity of laccases was evaluated by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol (DMP) as substrate. The assay mixture contained 900 μL substrate (2 mM DMP in 0.1 M acetate buffer pH 4.5) and 100 μL EE, which were incubated at 40 ºC for 1 min. One enzymatic unit (U) of laccases activity was defined as the amount of enzyme which gave an increase of 1 unit of absorbance per min in the reaction mixture. The U L^{-1} values were obtained as the mean ± standard deviation of three replicates.

Fig 2 shows the laccase activity zymograms, however, the activity obtained in the airlift reactor was so low that it was not possible to observe the isoenzymes in the gel. In the other cases, apparently the isoenzymes are the same regardless of the production system. It is suggested that the regulation of the production of laccases from *Pycnoporus cinnabarinus* is equal for the two isoenzymes and only the amount produced is modified. It has been reported that the composition of culture media induce the synthesis of isoenzymes which show the same activity but different physicochemical characteristics (Téllez-Téllez *et al*., 2005; Castro *et al*., 2013).

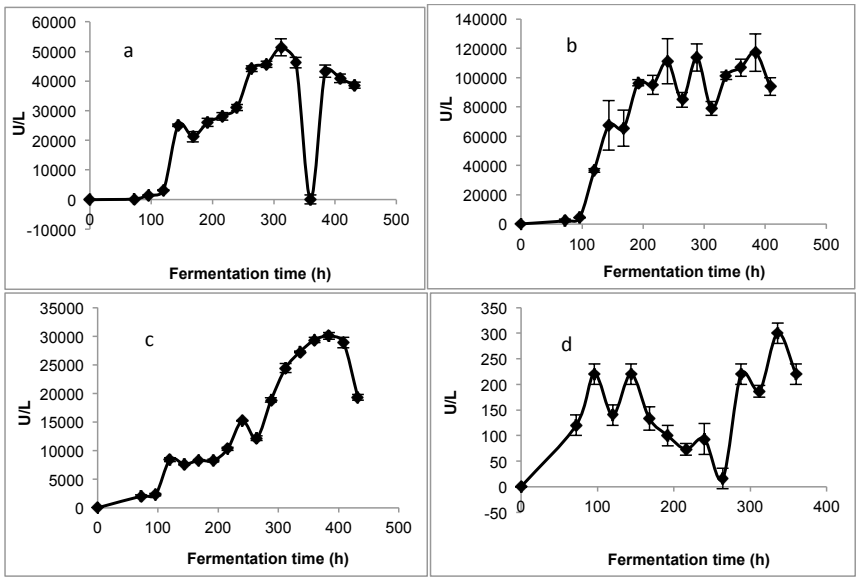

Fig. 1: Laccase activity of *Pycnoporus cinnabarinus* grown in SSF (a), SmF in shake flasks (b), stirred tank reactor (c) and airlift reactor (d).

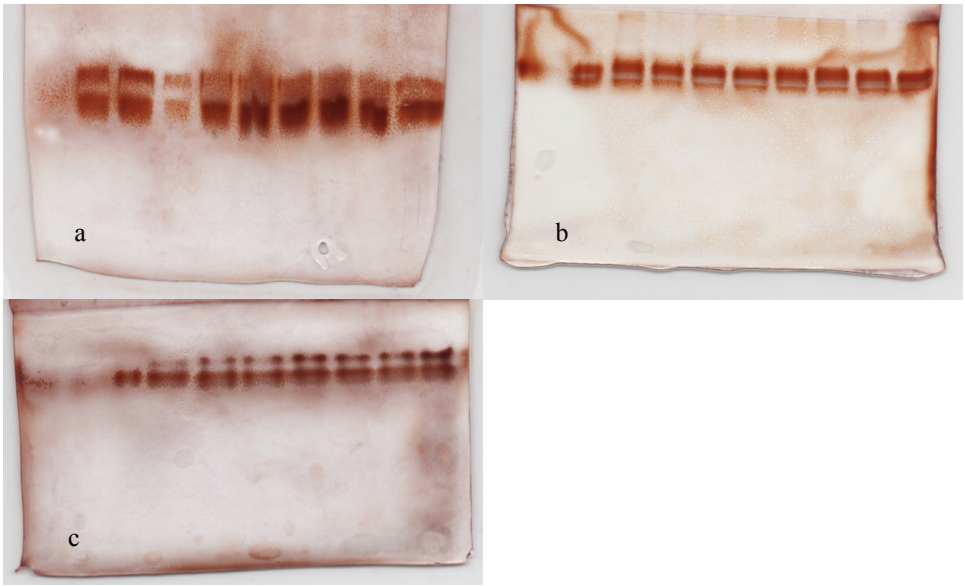

Fig. 2: Zymogram patterns of laccases from *Pycnoporus cinnabarinus* produced in SSF (a), SmF in shake flasks (b) and SmF in stirred tank reactor (c). Samples taken from 144-360 h.

There are reports on the existence of different laccase isoenzymes and multiple genes that encode them in various fungi (Yaver and Golightly, 1996; Mansur *et al*., 1997; Smith *et al*., 1998; Giardina *et al*., 1999). García *et al*. (2006) reported two laccase isoforms of *Pycnoporus sanguineus* produced in submerged culture, their molecular masses were 80 kDa (Lac I) and 68 kDa (Lac II) after a partial purification by phenyl-Sepharose chromatography. On the other hand, laccases are regulated by several factors such as pH, temperature, ions, presence of inducers, etc. (Collins and Dobson, 1997; Muñoz *et al*., 1997; Yaver *et al*., 1999). Copper has proven to be an excellent inducer, increasing the transcription of laccase genes (Collins and Dobson, 1997; Karahanian *et al*., 1998; Palmieri *et al*., 2000; Soden and Dobson, 2001; Galhaup *et al*., 2002). In this study, the same culture medium added of cupper was used in all production systems. It has been reported on changes in the composition of the culture medium favoring increased production of laccases of fungi of the *Pycnoporus* genus. Eugenio *et al*. (2009), reported the effect of carbon and nitrogen sources on the activity of laccase from *Pycnoporus sanguineus*. All carbon and nitrogen sources showed an important influence on laccase activity, where a sucrose-asparagine medium reported 320 mU mL^{-1}, but the laccase activity was increased to 820 mU mL^{-1} using 5 times higher of asparagine concentration. In other study, the presence of 20 μM xylidine and low nitrogen amount increased 50 fold the laccases activity (1,368 U L^{-1}) from *Pycnoporus sanguineus* grown in submerged liquid culture (Pointing *et al*., 2000).

A strain of *Pycnoporus cinnabarinus* produced laccase up to 29,000 U L^{-1} in the presence of ferulic acid as aromatic inducer. Two laccase isoenzymes (LAC I and LAC II) encoded by two genes were reported (Figueroa-Espinoza and Rouau, 1998). In other study, ethanol increased the laccase activity nine times than those of ferulic acid-induced cultures, and 155 and 65 times than those of control cultures (Herpoël *et al*., 2002). Previous reports have demonstrated the production of two laccase isoforms (LacI and LacII) by *Pycnoporus sanguineus* under different conditions (Garcia *et al*., 2006; Dantán-González *et al*., 2008; Lu *et al*., 2008; Vite-Vallejo *et al*., 2009). Ramírez-Cavazos *et al*. (2014a) purified two laccases by ultrafiltration, ion exchange and hydrophobic interaction chromatography. The molecular weights of LacI and LacII, determined by SDS-electrophoresis, were 68 and 66 kDa, respectively. Ramírez-Cavazos *et al*. (2014b) reported the production of thermostable laccases from a native strain of the white-rot fungus *Pycnoporus sanguineus* isolated in Mexico, the activity was enhanced by testing different media and a combination of inducers including copper sulfate ($CuSO_4$). The best conditions obtained from screening experiments in shake flasks using tomato juice, $CuSO_4$, and soybean oil were integrated in an experimental design. Enhanced levels

of tomato juice as the medium, $CuSO_4$ and soybean oil as inducers (36.8% v/v, 3 mmol L^{-1}, and 1% v/v, respectively) were determined for 10 L stirred tank bioreactor runs. This combination resulted in laccase titer of 143 000 U L^{-1} with ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) as substrate at pH 3.0.

In this work laccase activity was determined with 2,6 DMP a phenolic compound. Some laccases activity give higher results when evaluated with nonphenolic substrates such as ABTS (More *et al*., 2011). On the other hand, thirty strains of *Pycnoporus coccineus* and *Pycnoporus sanguineus* from subtropical and tropical environments, mainly isolated from fresh specimens collected *in situ*, were screened for laccase activity. On the basis of levels of enzyme activity and percentage of similarity between protein sequences, the laccases from three strains (BRFM 938, BRFM 66 and BRFM 902) were selected for purification and characterization. Each laccase gene of those strains encoded a predicted protein of 518 amino acids; the three deduced proteins showed 68-97% similarity with other Polyporale laccases. The laccases showed a molecular weigth of 59-62 kDa with 7-10% carbohydrate content, remained highly stable up to 75-78°C and at pH 5-7 mixtures, and were resistant to methyl and ethyl alcohols, acetonitrile and dimethylsulfoxide at concentrations as high as 50% (v/v). The best laccase-1- hydroxybenzotriazole systems permitted almost 100% of various polyphenolic dye decolourization and oxidation of adlerol and veratryl alcohol (Uzan *et al*., 2010).

Recently was reported the secuence of a laccase gene of *Pleurotus ostreatus* called LacP83 and its promoter region (466 bp upstream of ATG) contains putative binding transcription factors such as metal response element, xenobiotic response element, a defense response element, and a stress response element (Téllez-Téllez *et al*., 2012a). Then, it is possible that differences in laccases activity observed between the production systems is due to stress that the cells presented in each culture system. There is a difference in the amount of dissolved oxygen between the SSF and all SmF´s, also in this last, exist mechanical stress caused by agitation and/or aeration which produced different types of fungus morphology. In the SSF, the PUF cubes used, were of low density, with high water retention (30 mL g^{-1} PUF; Díaz-Godínez *et al*., 2001), which allowed the mycelial growth of the fungus forming networks of very small thickness (less than 1 mm), on the other hand, it is known that the solubility of oxygen in air is about 298 mg L^{-1} and it has been reported that the K_{La} value in similar SSF was of approximately 0.344 s^{-1} (Thibault *et al*., 2000), whereas the solubility of oxygen in water is very low (Estela-Escalante *et al*., 2012), about 30 times less than in air (8.11 mg L^{-1} at room temperature), and K_{La} values are reported up to 0.04 s^{-1} for shake flask (Maier *et al*., 2004), up to 0.158 s^{-1} in stirred tank reactor (Flores *et al*., 1997) and up to 0.04 s^{-1} in airlift reactor (Merchuk and Siegel, 1988); in shake flasks, the biomass was produced in pellets form that can measure up to 2 cm in diameter, on the other hand, in the stirred tank a very large mechanical stress caused the disintegration of mycelium during the fungus growth with size particle of about 0.5 cm and in the airlift reactor a big static mass fixed to along the concentric tube on the outside was observed. Based on the above may suggest that the SSF is a system with greater availability of oxygen that allows fungus growth similarly to its natural environment, in this study was reported a X of 5.66±0.3 g L^{-1} at 15 days of culture and the laccases yield according to the produced biomass (Y) was of 9,081 U g^{-1}X, while in the shake flask was reached a X of 5.60±0.2 g L^{-1} at 12 days of culture, the stirred tank produced an X of 1.60±0.2 g L^{-1} and the airlift reactor showed an X of 3.50±0.4 g L^{-1}, both was observed at 15 days of culture and the Y values in shake flask, stirred tank and airlift reactor were 19,821, 18,750 and 85 U g^{-1}X, respectively. In this case it is suggested that the small pellets with large surface area are more productive than the great mass of mycelium with little surface area obtained in the airlift reactor, where the anoxic zone formed is related to the size of the biomass agregation (Ortega-Sánchez *et al*., 2012), since there is higher proportion of metabolically active cells in the pellets than in the big biomass. Although the amount of laccase produced in stirred tank was approximately one third of that observed in shake flasks, the Y values were similar, indicating that in the stirred tank occurs greater mechanical damage to cells impeding proper growth and the amount laccase is related to the X produced.

Conclusion

The growth system determines laccase activity levels of *Pycnoporus cinnabarinus*, being the liquid culture in shake flasks the best, two isoenzymes of laccase were produced by whatever growth system of the fungus. It is recommended, to make studies on the

stirring rate in the flasks and reactor as well as the air flow in the airlift reactor, to increase the activity, considering the conditions of the culture medium such as the C/N ratio, the pH, working volume, agitation speed and the incubation temperature.

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