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ZYMOGRAM PATTERNS OF EXTRACELLULAR LACCASES OF *Pleurotus* SPECIES GROWN ON NON-INDUCER AGAR MEDIUM

PATRÓN ZIMOGRÁFICO DE LACASAS EXTRACELULARES DE ESPECIES DE *Pleurotus* CRECIDOS EN UN MEDIO NO-INDUCTOR

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Abstract

Zymogram patterns of extracellular laccases of ten strains of *Pleurotus* grown on agar without addition of inducers, using 2.6-dimethoxyphenol, ρ-anisidine and o-tolidine as substrates, were obtained. Zymogram patterns were only similar for strains within same species, independently of the substrate used, six strains of *P. ostreatus* and one strain of *P. ostreatus* var. florida showed three isoenzymes, two strains of P. pulmonarius showed two isoenzymes and one strain of *P. cornucopiae* also showed two isoenzymes but in a different position in comparison to the other strains. These results showed that *Pleurotus* species produce a basal level of laccase activity and the number of extracellular laccase isoenzymes is species dependent.

Keywords: *Pleurotus*, isoenzyme, enzymatic activity, zymogram, laccases.

Resumen

Se obtuvo el patrón zimográfico de lacasas extracelulares de diez cepas de *Pleurotus* crecido sobre agar sin la adición de inductor, usando 2.6-dimethoxifenol, ρ-anisidina y o-tolidina como sustratos. Los patrones zimográficos fueron similares para las cepas de la misma especie, independientemente del sustrato utilizado, seis cepas de *P. ostreatus* y una cepa de *P. ostreatus* var. florida mostraron tres isoenzimas, dos cepas de P. pulmonarius mostraron dos isoenzimas y una cepa *P. cornucopiae* también mostró dos isoenzimas pero en diferente posición en comparación con las otras cepas. Estos resultados mostraron que las especies de *Pleurotus* producen un nivel basal de actividad de lacasa y que el número de isoenzimas de lacasas extracelulares dependen de la especie.

Palabras clave: *Pleurotus*, isoenzima, actividad enzimática, zimograma, lacasas.

1 Introduction

White rot fungi produce several isoenzymes of extracellular oxidases and peroxidases, which are involved in the degradation of lignin in their natural environments (Palmieri *et al.*, 2000). *Pleurotus* ostreatus belongs to a subclass of lignin-degrading microorganisms that produce laccases, manganese peroxidases and veratryl alcohol oxidases but no lignin peroxidases (Palmieri *et al.*, 1997). Laccases (benzendiol:oxygen oxidoreductases, EC 1.10.3.2) are Cu-containing glycoproteins which require O_2 to oxidize phenols, polyphenols, and aromatic amines as well as non-phenolic organic substrates by one-electron abstractions resulting in the formation of H_2O and reactive radicals undergoing further depolymerization, repolymerization, demethylation, dehalogenation, or quinine formation (Claus, 2004).

Although their specific physiological functions are not completely understood, there are several indications that laccases are involved in the morphogenesis of microorganisms (e.g., fungal spore development, melanization)

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and in the formation and/or degradation of complex organic substances such as lignin or humic matter. Due the catalytic action of laccases, these enzymes can be used for various biotechnological and environmental applications such as textile dye decolouration, delignification, pulp bleaching, effluent detoxification, biosensing, and bioremediation (Thurston, 1994; Hublik and Schinner, 2000; Mayer and Staples, 2002). Solís-Oba *et al.* (2007), produced an active, stable and oxidized form of the chemical mediator ABTS (2,2'-azino-bis-(3- ethylbenzothiazoline-6-sulphonic acid)) and observed that oxidation of some compounds by a mixture of ABTS and laccase can be done in a cyclic manner, such a way the oxidation rate was 94 times higher for indigo, 17 times for brilliant blue G, 34 times for orange 7 and 5 times higher for p-cresol compared with using the mediator or the laccase alone.

As well as other enzymes, including tannases, pectinases, chitinases, etc., the laccase expression is affected by culture and environmental conditions, mainly the type and concentration of carbon sources (Belmares-Cerda *et al.*, 2003; Díaz-Godínez *et al.*, 2001; Sastoque-Cala *et al.*, 2007). On the other hand, it has been reported that laccases are secreted in multiple isoforms (Mansur *et al.*, 1998; Téllez-Téllez *et al.*, 2005; Téllez-Téllez *et al.*, 2008). Additionally, the expression of different patterns of laccase isoenzymes coded by gene families is differentially regulated, depending on the growth conditions (Bollag and Leonowicz, 1984; Rogalski and Leonowicz, 1992) and physiological states (Rogalski *et al.*, 1993; Mansur *et al.*, 1998).

There are several reports about the effect of inducer or the cultural composition and environmental conditions on the laccase production. In recent years, there has been a renewed interest in solid-state fermentation processes due to its high productivity of bioactive compounds in comparison to submerged fermentation (Ruíz-Leza *et al.*, 2007). Téllez-Téllez *et al.* (2008), reported an atypical behavior of *Pleurotus* ostreatus, they observed that the isoenzymes laccase number and the laccases activity were higher in submerged than in solid-state fermentation. On the other hand, there are few reports about the laccases production without inducer addition. In this research, the extracellular laccase activity on three substrates and the isoenzymes number, of ten strains of *Pleurotus* sp. grown on agar medium without addition of any inducer were evaluated.

2 Methodology

2.1 Strain, media, and growth conditions

Ten strains of *Pleurotus* were studied; *P. ostreatus* 32783 (Po-83), *P. ostreatus* 38537 (Po-37), *P. ostreatus* 58052 (Po-52), *P. ostreatus* 201218 (Po-7) and *P. ostreatus* 201216 (Po-3) from the ATCC collection, *P. ostreatus* 3526 (Po-26) from the NRRL, *P. pulmonarius* Pp-134 and *P. pulmonarius* Pp-127 from the Chinese University of Hong Kong Collection, *P. ostreatus* var. florida (Pfl) and *P. cornucopiae* (Pcc) from the Mushroom Experimental Station, Horst, The Netherland.

Inoculum was taken from the periphery of colonies growing on potato dextrose agar at 28°C for 7 days, by using a sterile cork borer (4 mm diam.). The mycelial plug was placed (mycelium facing-down) on the center of the culture medium. In all experiments, well-developed colonies grown on Petri dishes were used. The composition of culture medium was (in g/L): starch, 10.5; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $Ca(H_2PO_4)_2 \cdot H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.02; $ZnSO_4 \cdot 7\ H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.02 (Sánchez and Viniegra-González, 1996). All the strains were grown at 25°C, except the strains of *P. pulmonarius*, which were grown at 28°C.

2.2 Extracellular extract

The extracellular enzymatic extract (EE) was obtained from the agar with deionized water after removing the mycelium from the surface of each colony for every strain. All the EE were centrifuged at 20 000 X g for 10 min at 2°C.

2.3 Laccase activity assay

In EE, laccases activity was assayed using 2,6-dimethoxyphenol (DMP), ρ-anisidine and o-tolidine. For DMP the assay mixture contained 950 μl of substrate (2 mM of DMP in 0.1M phosphate buffer, pH 6.0) and 50 μl of EE, which were incubated at 39°C for 15 min. Oxidation of DMP was followed by absorbance increase at 468 nm. For those assays with ρ-anisidine, reaction mixture contained 950 μl of substrate (10 mM of ρ-anisidine in 0.1M phosphate buffer, pH 6.0) and 50 μl of EE, which were incubated at 39°C for 15 min. Oxidation of ρ-anisidine was followed by absorbance increase at 460 nm. With o-tolidine as a substrate, the assay mixture contained 950 μl of substrate (2 mM of o-tolidine in 0.1M acetate

buffer, pH 3.7) and 50 μl of EE, which were incubated at 30°C for 15 min. Oxidation of o-tolidine was followed by absorbance increase at 627 nm.

One enzymatic unit (U) of laccases activity was defined as the amount of enzyme, which gave an increase of 1.0 unit of absorbance per min in the reaction mixture. The laccases activity was expressed in U per g of dry biomass (U/g X).

2.4 Laccase zymograms

The extracellular laccases activity was also detected through zymograms (Téllez-Téllez *et al.*, 2005; Téllez-Téllez *et al.*, 2008). 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 (30 μL aprox.) was mixed with sample buffer without a reducing agent the disulfide bonds. Without heating, the samples were placed in gels (thickness 0.75 mm) of Mini-Protean III electrophoresis system (BioRad) and then 150 V was applied for 1-1.25 h. After the electrophoresis, gels were washed with deionized water on an orbital shaker (20-30 rpm) for 1 h, water was changed every 15 min to remove SDS. Finally, the gels were incubated at room temperature in substrate solutions (either 2 mM of DMP, 10 mM of ρ-anisidine or 2 mM of o-tolidine). Laccase activity bands appeared on the gel by the oxidation of the substrate after approx. 1 h.

3 Results and discussion

The extracellular laccase activity of ten strains of *Pleurotus*, were evaluated on different substrates (Table 1). In general, the strains Po-7, Po-26 and Pcc showed the highest laccase activity in the three substrates. Pfl, Po-3 and Po-52 showed the lowest laccase activity in all the substrates. The highest laccase activity was observed with DMP followed by o-tolidine and ρ-anisidine for all the strains. The laccase activity was result of the action of the all isoenzymes present in each EE, and the same isoenzymes oxidized the three substrates used.

The Fig. 1 shows the zymogram patterns of extracellular laccase activity using DMP, o-tolidine and ρ-anisidine as substrates. The patterns of each strain were independent of the substrate used and were similar between species. The strains of *P. ostreatus* showed three isoenzymes. Li and Eger (1979) isolated a strain of *Pleurotus* from Florida U.S.A., that was called P. florida and later classified as *P. ostreatus* "var. florida". The two strains of *P. pulmonarius* and the strain of Pcc showed two isoenzymes to different position on the gel.

It has been reported that the laccases expression exhibit differential regulation, then, laccases activity and number of isoenzymes are influenced by environmental conditions such as pH, temperature, inductors, and culture medium conditions (Giardina *et al.*, 1999; Tellez-Tellez *et al.*, 2008). Tellez-Tellez *et al.* (2005) reported a basal intracellular laccases activity in *Pleurotus* species, with at least two isoenzymes for *P. ostreatus* and *P. pulmonarius* and one isoenzyme for *P. cornucopiae*. Téllez-Téllez *et al.* (2008) reported that Po-83 produced three and four extracellular isoenzymes using Cu as inducer in solid-state fermentation (SSF) and in submerged fermentation (SMF) conditions, respectively. In this study, the basal extracellular laccase activity was higher than that previously reported for the intracellular laccase activity of the same strains grown under the same fermentation conditions (Tellez-Tellez *et al.*, 2005). On the other hand, the enzymatic activity using DMP as substrate was around twice and eight times lower than those observed in other study by the same strain in SSF and SMF, respectively (Téllez-Téllez *et al.*, 2008). It was also found that the presence of Cu.

Table 1. Extracellular laccase activity of strains of *Pleurotus* using different substrates.

Strain	Activity (U/g X)		
	DMP	ρ-anisidine	o-tolidine
Po-83	1562^{b} (37)	167^{c} (7)	1206^{c} (28)
Po-37	552^{e} (12)	64^{e} (6)	$429^{e,f}$ (17)
Po-52	394^{g} (10)	18^{g} (1)	177^{g} (8)
Po-7	1998^{a} (36)	176^{c} (10)	1599^{a} (36)
Po-3	245^{h} (8)	39^{f} (4)	141^{g} (4,2)
Po-26	1936^{a} (11)	192^{b} (9)	1316^{b} (23)
Pfl	449^{f} (13)	75^{e} (6)	458^{e} (17)
Pcc	1448^{b} (50)	251^{a} (13)	1101^{c} (56)
Pp-134	640^{d} (17)	133^{d} (1)	384^{f} (14)
Pp-127	700^{c} (17)	172^{c} (7)	387^{f} (20)

Means with the same letter within a column are not significantly different. Data were evaluated by ANOVA and Tukey test. ($p < 0.01$). Numbers in parenthesis correspond to SD of three separate experiments.

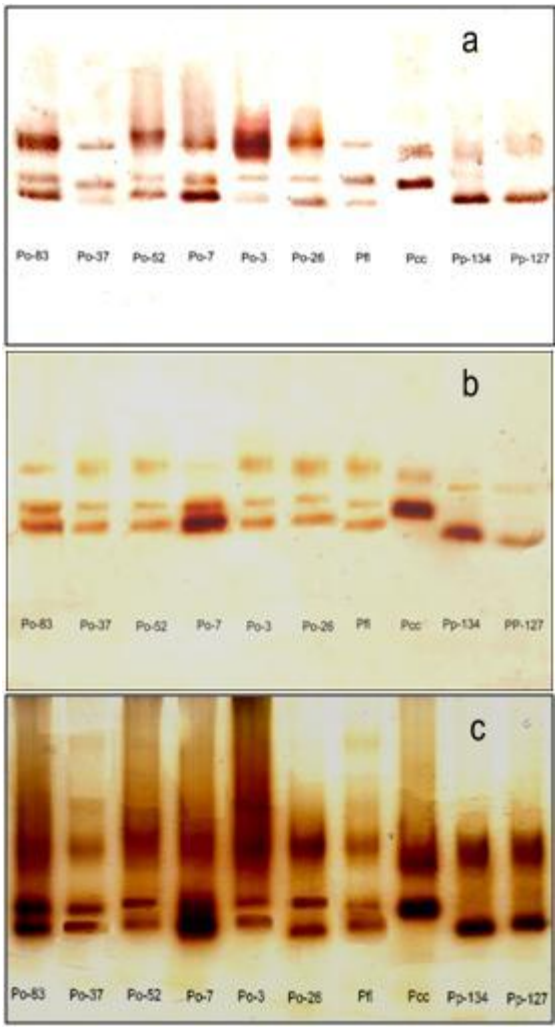

Fig. 1. Zymograms of laccase isoenzymes of ten *Pleurotus* strains using DMP (a), ρ-anisidine (b) and o-tolidine (c) as substrates.

In SMF enhanced the laccase activity around 37, 6, 13, 14 and 60 times which was higher than those previously reported for the strains Po-83, Po-3, Po-7, Po-37 and Po-52, respectively. (Díaz *et al.*, 2011). In addition, the constitutive basal activity enhances when the growing conditions of the fungus are changed. The extracellular laccase activity (1936 U/g X) of the strain Po-26 was approximately 2.4 and 194 times higher than the reported when the same strain was grown on lignocellulosic substrates and in Petri dishes (803 U/g X) respectively (Sainos *et al.*, 2006).

The biological role of the laccases is not fully understood; it appears to vary depending on the type of organism. It has been suggested that fungi have a biodegradation role (degradation of lignin or elimination of those toxic phenols generated by such process), participate in cellular processes, morphogenesis, pigments production, pathogenesis and virulence. However, only few of these functions have been experimentally validated (Eggert *et al.*, 1998). It is known that the extracellular isoenzymes are responsible of the biodegradation role and the intracellular of the rest of the suggested functions, however, some isoenzymes might have both functions (Das *et al.*, 2011).

Conclusion

These results show that the number of extracellular laccase isoenzymes depends on the species, however, the activity is different even between strains from the same species. It could be due to factors related with the habitat conditions of the strain. The zymogram patterns in each strain were similar in all the substrates, showing that all the produced isoenzymes have the ability to catalyze the same substrates. The extracellular laccase activity and the isoenzymes number were higher than those previously reported for the EE intracellular of the same strains.

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