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Mycelial growth and production of laccase and peroxidases by *Pleurotus ostreatus* and *Agrocybe aegerita* in liquid fermentation

Crecimiento micelial y producción de lacasa y peroxidasas por *Pleurotus ostreatus* y *Agrocybe aegerita* en fermentación líquida

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ABSTRACT

Mycelial growth of *Pleurotus ostreatus* and *Agrocybe aegerita* in malt extract broth was evaluated. Laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and unspecific peroxygenase (UnP) activities were determined in both strains, using biochemical and polyacrylamide gel electrophoresis techniques. No strain showed MnP, LiP, and UnP activities. *P. ostreatus* had higher biomass production and laccase yield values than those shown by *A. aegerita*. *P. ostreatus* produced approximately twice as much biomass (X_{max}) compared to *A. aegerita* (9.12 and 5.27 g/L, respectively). Maximum laccase activity (E_{max}) was nine-fold higher in *P. ostreatus* than in *A. aegerita* (106.5 and 12.23 U/L, respectively). *P. ostreatus* and *A. aegerita* showed 3 bands (estimated to be approximately 42, 55, 85 and 212 kDa) and one band (estimated to be approximately 35.2 kDa) with laccase activity, respectively. These results show that laccases are constitutively produced in both fungal species, whereas MnP, LiP and UnP are inducible enzymes. *P. ostreatus* has a great potential in the biotechnological production of laccases.

Keywords: *Agrocybe aegerita*, laccases, liquid fermentation, peroxidases, *Pleurotus ostreatus*.

RESUMEN

En este trabajo se evaluó el crecimiento micelial de *Pleurotus ostreatus* y *Agrocybe aegerita* crecidos en extracto de malta en fermentación líquida. La producción de lacasa, manganeso peroxidasa (MnP), lignin peroxidasa (LiP) y peroxigenasa inespecífica (UnP) fueron determinadas en ambas cepas, empleando métodos bioquímicos y de electroforesis en gel de poliacrilamida. Ninguna cepa mostró actividad de MnP, LiP, y UnP. *P. ostreatus* produjo mayor cantidad de biomasa y mostró mayor rendimiento enzimático de lacasa en comparación a los valores obtenidos en *A. aegerit*a. *P. ostreatus* produjo aproximadamente dos veces más cantidad de biomasa (X_{max}) en comparación a *A. aegerita* (9.12 y 5.27 g/L, respectivamente). La actividad de lacasa máxima (E_{max}) fue nueve veces mayor en *P. ostreatu*s que en *A. aegeri*ta (106.5 y 12.23 U/L, respectivamente). *P. ostreatus* y *A. aegerita* mostraron 3 bandas (55 kDa, 85 kDa y 212 kDa aproximadamente) y una banda (35.2 kDa aproximadamente) con actividad de lacasa, respectivamente. Estos resultados muestran que las lacasas son enzimas que se producen de manera constitutiva en ambas especies de hongos, mientras que las peroxidasas (MnP, LiP y UnP) son enzimas inducibles. *P. ostreatus* es un organismo con un gran potencial en la producción biotecnológica de lacasas.

Palabras clave: *Agrocybe aegerita*, fermentación líquida, lacasa, peroxidasas, *Pleurotus ostreatus.*

1. Introduction

White-rot fungi are filamentous fungi capable to growth on lignocellulosic substrates due to the enzymatic machinery they possess (Sánchez, 2009; Sánchez *et al*., 2020; Loftus *et al*., 2020). In particular. *Pleurotus ostreatus* and *Agrocybe aegerita* are edible mushrooms belonging to basidiomycetes, which have two phases of growth: the reproductive phase (fruit bodies) and the vegetative phase (mycelia), which is constitute by hyphae. Hyphae are the fungal cells able to penetrate tridimensional substrates, since they secrete extracellular enzymes, which break down complex molecules into smaller organic compound to absorb them back up, releasing CO_2 and H_2O under aerobic growing conditions (Sánchez, 2020; Sánchez, 2021). Mushrooms grown on lignocellulosic substrates produce extracellular lignocellulolytic enzymes, degrading these substrates, in which they are usually cultivated (Sánchez, 2010; Loftus *et al*., 2020). *P. ostreatus* and *A. aegerita* can be cultivated on a large variety of lignocellulosic substrates and other wastes, which are produced primarily through the activities of the agricultural, forest, and food-processing industries (Sánchez, 2010).

P. ostreatus has been widely studied due to its ability to secrete extracellular enzymes, such as hydrolases (xylanases, cellulases), oxidases (laccases) and peroxidases (manganese peroxidases, lignin peroxidases) (Bertrand *et al*., 2016; Hernández-Dominguez *et al*., 2017; Bakratsas *et al*., 2023; Krupodorova *et al*., 2024; Hamza *et al*., 2024). Enzymes produced by *P. ostreatus* are also capable of degrading environmental pollutants (Martínez-Berra *et al.*, 2018; Ahuactzin-Pérez *et al*., 2018; Sánchez, 2020; Sánchez, 2021; Khan *et al*., 2023). It has been reported that the edible mushroom *A. aegerita* also grows on lignocellulosic substrates and produces UnP (Ullrich *et al*., 2004;

Tonin *et al*., 2021; Jacob *et al*., 2021; González-Rodríguez *et al*., 2023). UnP is capable of degrading most of the USEPA (US Environmental Protection Agency) priority environmental pollutants, which is due to its ability to catalyze different types of reactions (Karich *et al*., 2017). Several studies on the growth of *P. ostreatus* on different agricultural substrates have been carried out, with the aim of finding optimal cultivation conditions and induction of ligninolytic enzymes (Sánchez, 2010; Muswati *et al*., 2021).
In this context, the selection of the appropriate fermentation system, as well as the use of suitable lignocellulosic materials, are crucial for optimal fungal growth and efficient enzyme production. In this research, the growth and production of laccase, MnP, LiP and UnP of *P. ostreatus* and *A. aegerita* were evaluated in liquid fermentation. pH profile, enzymatic yield parameters of laccases, protein content, and laccase zymographic analysis for both fungi were also carried out.

2. Materials and methods

2.1. Strains and inoculum preparation

P. ostreatus (Po7) and *Agrocybe aegerita* from the culture collection of the Research Centre for Biological Sciences at Universidad Autónoma de Tlaxcala (CICB, Tlaxcala, Mexico) were used. The strains were grown on malt extract agar at 27 °C and kept at 4 °C until used.

2.2. Culture media and culture conditions

Erlenmeyer flask of 125 mL containing 50 mL of culture medium were autoclaved at 120 °C for 15 min, cooled to room temperature, and then inoculated with three mycelial plugs (of 8 mm diameter) taken from the periphery of well-developed colonies of either fungus. Cultures were incubated for 13 d at 27 °C on a rotary shaker operated at 120 rpm. Analyses were carried out on samples taken at 24-h intervals.

2.3. Determination of biomass production

Biomass (X) was harvested from cultures by filtration using filter paper (Whatman no. 4), and the specific growth rate (μ) and yield parameters were calculated by using logistic equation as previously specified (Canavati-Alatorre *et al*., 2016; González-Márquez *et al*., 2019). pH was measured each 24 h using a digital potentiometer (Conductronic, Mexico). Supernatant was used for subsequent analysis (i.e. protein content, enzyme activity and zymography).

2.4. Evaluation of protein content

Water-soluble protein content was measured by Bradford method (Bradford, 1976). For each sample, a reaction mixture was prepared with 200 μL of Bradford reagent (BIORAD), 100 μL of supernatant and 700 μL of sterile distilled water. Samples were incubated for 10 min at room temperature, and absorbance readings at 595 nm were obtained using a spectrophotometer (UV-Vis Jenway 7305, Stone, Staffs, UK) as reported previously (Ríos-González *et al*., 2019; González-Márquez and Sánchez, 2022).

2.5. Enzymatic assays and estimation of enzymatic parameters

Laccase, LiP, MnP, and UnP activities were evaluated as previously reported (Ocaña-Romo *et al*., 2024). Briefly, laccase activity was evaluated using 2,6-dimetoxyphenol (DMP) as a substrate. The reaction mix comprised 900 µL of DMP dissolved in 0.1 M acetate buffer at pH 4.5 and 100 µL of supernatant and incubated at 40 °C for 1 minute. LiP activity was evaluated using veratryl alcohol (VA) as a substrate. The reaction mixture comprised 200 µL of 40 mM VA, 200 µL of 40 mM H_2O_2, 580 µL of 0.1 M tartrate buffer (pH 4.2), and 20 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. The MnP activity was measured using guaiacol as the substrate. The reaction mix comprised 75 µL of 1 mM $MnSO_4$, 790 µL of 0.1 M tartrate buffer (pH 4.2), 50 µL of 40 mM H_2O_2, 75 µL 10 mM guaiacol, and 10 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. The UnP activity was determined using VA as the substrate. The reaction mixture comprised 100 µL 40 mM VA, 50 µL of 40 mM H_2O_2, 840 µL of 0.1 M citrate buffer (pH 4.5), and 10 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. Absorbances were measured at 468 nm (Camacho-Morales *et al*., 2017), 310 nm (Arora and Gill, 2001), 334 nm (Camacho-Morales *et al*., 2017), and 310 nm (González-Rodríguez *et al*., 2023) for laccase, LiP, MnP, and UnP, respectively, using a Jenway 7305 UV-Vis spectrophotometer (Stone, Staffs, UK). One unit (U) of enzyme activity was defined as the amount of enzyme required to obtain 1 µmol of product per minute. The enzymatic parameters, namely, the yield of esterase per unit of biomass ($Y_{E/X}$), specific esterase activity (in U/mg protein), esterase productivity (P), maximum enzymatic activity (E_{max}), and specific rate of enzyme production (q_p), were evaluated as reported previously (Ahuactzin-Pérez *et al*. 2016; Ferrer-Parra *et al*., 2018).

2.6 Zymographic analysis

Zymographic analysis of the samples with laccase activity was carried out using polyacrylamide gels (PAGE). 14% polyacrylamide gel and 4% acrylamide as separation and packaging gels, respectively under non-reducing conditions were prepared (Leammli, 1970). Samples were analyzed using 1 mm gels and a Mini Protean Tetra Cell (Bio-Rad) electrophoresis system at 150 volts for 1.5 h. Gels were incubated at 40 °C for 30 min in DMP as substrate in acetate buffer 0.1 M at pH 4.5. Laccase activity was detected by the appearance of brown-colored bands in the gels. Gels were digitized using a Gel Doc EZ Imager (BIORAD) and lane bands were detected by their density using Image Lab program, Version 6.0.0 (BIORAD).

2.7 Statistical analysis

Data for analysis were obtained from three independent samples. Statistical analysis was performed using one-way ANOVA and Tukey post-test using SAS® OnDemand for Academics (SAS Institute, Inc., Cary, NC).

3. Results

Fig. 1 shows the biomass production by *P. ostreatus* and *A. aegerita* at 312 h of fermentation. It was observed that both strains reached their stationary phase of growth at

216 h approximately. *P. ostreatus* had higher growth and enzymatic yield parameters than *A. aegerita* (Table 1) In particular, *P. ostreatus* produced approximately twice as much biomass (X_{max}) compared to *A. aegerita* (9.12 and 5.27 g/L, respectively). Maximum laccase activity (E_{max}) was nine-fold higher in *P. ostreatus* than in *A. aegerita* (106.5 and 12.23 U/L, respectively) (Table 1).

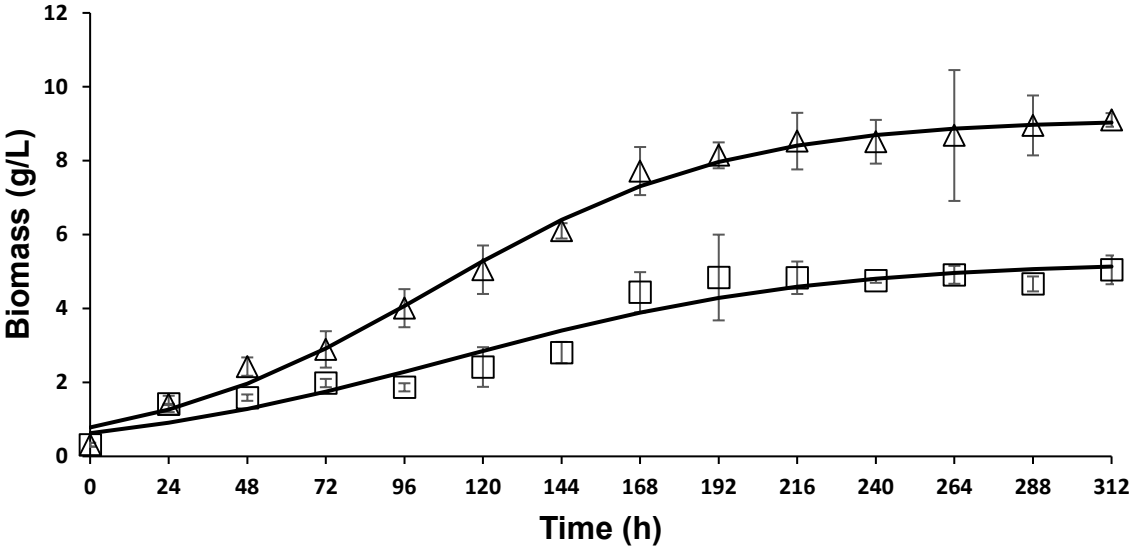

Fig. 1. Biomass production of *P. ostreatus* (triangle) and *A. aegerita* (square) grown in malt extract under liquid fermentation conditions. Biomass curves were fitted (—) using the logistic equation.

Table 1. Growth and enzymatic yield parameters of laccases of *P. ostreatus* and *A. aegerita*.

Parameters	*P. ostreatus*	*A. aegerita*
μ (h^{-1})	0.022^a ± 0.001	0.018^b ± 0.001
X_{max} (g/L)	9.12^a ± 0.03	5.27^b ± 0.02
E_{max} (U/L)	106.5^a ± 1.21	12.23^b ± 0.5
$Y_{E/X}$ (U/gX)	11.6^a ± 0.3	2.32^b ± 0.001
P (U/L/h)	0.34^a ± 0.03	0.07^b ± 0.001
q_p (U/h/gX)	0.25^a ± 0.03	0.04^b ± 0.001

The mean and standard deviation (±) are reported (n=3). Means of the same row with different letters are significantly different (P<0.05). μ, specific growth rate; X_{max}, maximum biomass; $Y_{X/S}$, biomass yield; E_{max}, maximum enzyme activity; $Y_{E/X}$, enzyme yield; P, enzyme productivity; q_p, specific rate of enzyme production.

Fig. 2 shows pH values of *P. ostreatus* and *A. aegerita*. It was observed that the pH value of *A. aegerita* cultures decreased from 6.1 to 5.5 at the end of the fermentation, whereas the pH of *P. ostreatus* cultures decreased during the first 72 h of growth to a minimum of 4.8, then increased and reached 6.7 after 264 h, decreasing to 6.2 by the end of the cultivation (after 312 h).

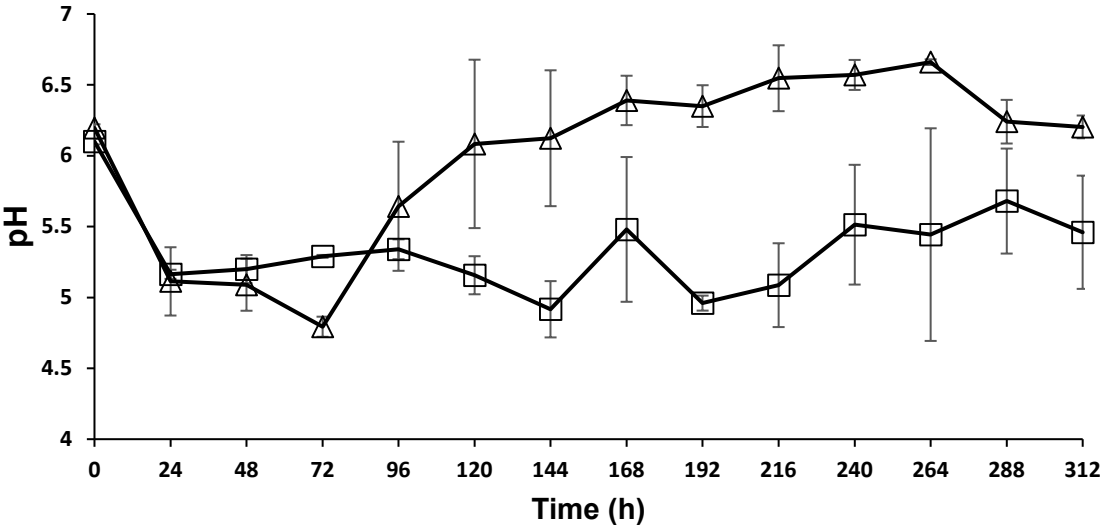

Fig. 2. pH profiles of *P. ostreatus* (triangle) and *A. aegerita* (square) grown in malt extract under liquid fermentation conditions.

Protein content of *P. ostreatus* and *A. aegerita* is shown in Fig. 3. It is observed that *P. ostreatus* had higher protein content than *A. aegerita* during all the fermentation. *P. ostreatus* and *A. aegerita* had the highest protein content at 312 h of growth, however, *P. ostreatus* also showed a high protein content at 168 h.

Laccase activities of *P. ostreatus* and *A. aegerita* are observed in Fig. 4. *P. ostreatus* had greater laccase activity than *A. aegerita* during all the fermentation. The laccase yield parameters were higher in *P. ostreatus* than in *A aegeri*ta. E_{max} was nine-fold higher in *P. ostreatus* than in *A. aegerita* (106.5 and 12.23 U/L, respectively). $Y_{E/X}$ and P were around five-fold higher in *P. ostreatus* (11.6 U/gX and 0.34 U/L/h, respectively) than in *A. aegerita* (2.32 U/gX and 0.07 U/L/h, respectively). q_p was around six-fold higher in *P. ostreatus* (0.25 U/h/gX) than in *A. aegerita* (0.04 U/h/gX) (Table 1).

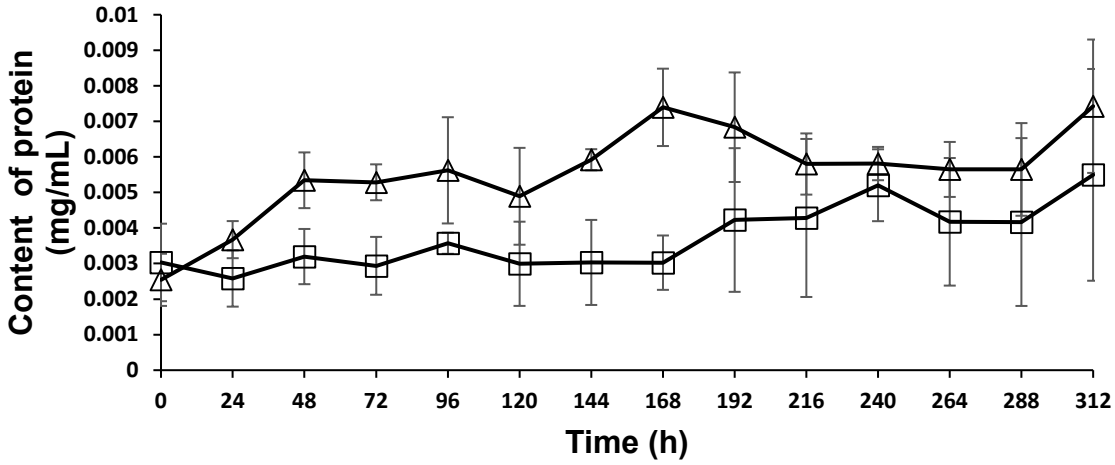

Fig. 3. Protein content of *P. ostreatus* (triangle) and *A. aegerita* (square) grown in malt extract under liquid fermentation conditions.

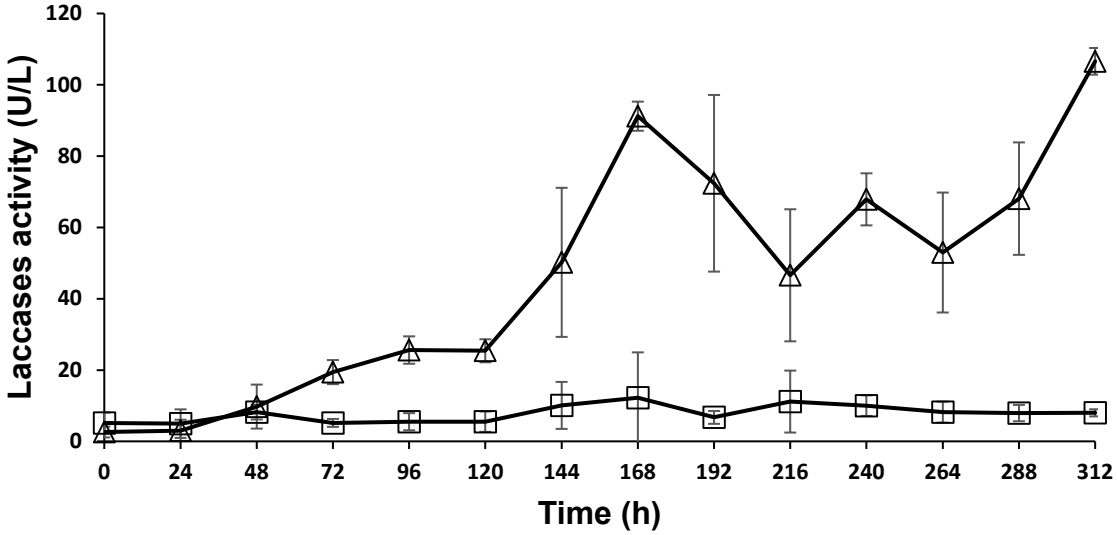

Fig. 4. Laccases activity of *P. ostreatus* (triangle) and *A. aegerita* (square) grown in malt extract under liquid fermentation conditions.

The protein bands presenting laccase activity on gels for samples of *P. ostreatus* and *A. aegerita* cultures are shown in Figs. 5 and 6, respectively. Three bands with laccase activity were observed in the gel for *P. ostreatus* cultures (Fig. 5). A band with molecular weight of 55 KDa approximately was observed in the gel during all the fermentation. A band with a molecular weight of 85 kDa approximately was revealed from 96 h until the end of fermentation. A band with a molecular weight of 212.1 kDa approximately was showed from 120 h until the end of fermentation (Fig. 5). A slightly stained band with a molecular

weight of 35.2 KDa approximately was revealed from 120 h until the end of fermentation in *A. aegerita* cultures (Fig. 6).

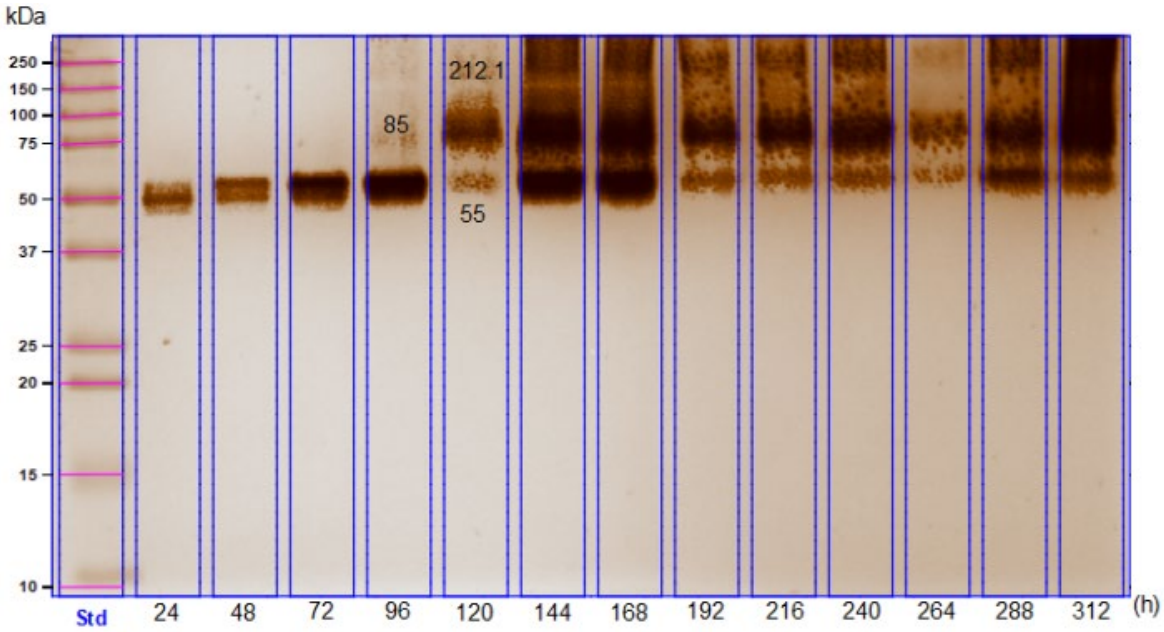

Fig. 5. Zymogram of laccases isoenzymes of *P. ostreatus* grown in malt extract under liquid fermentation conditions.

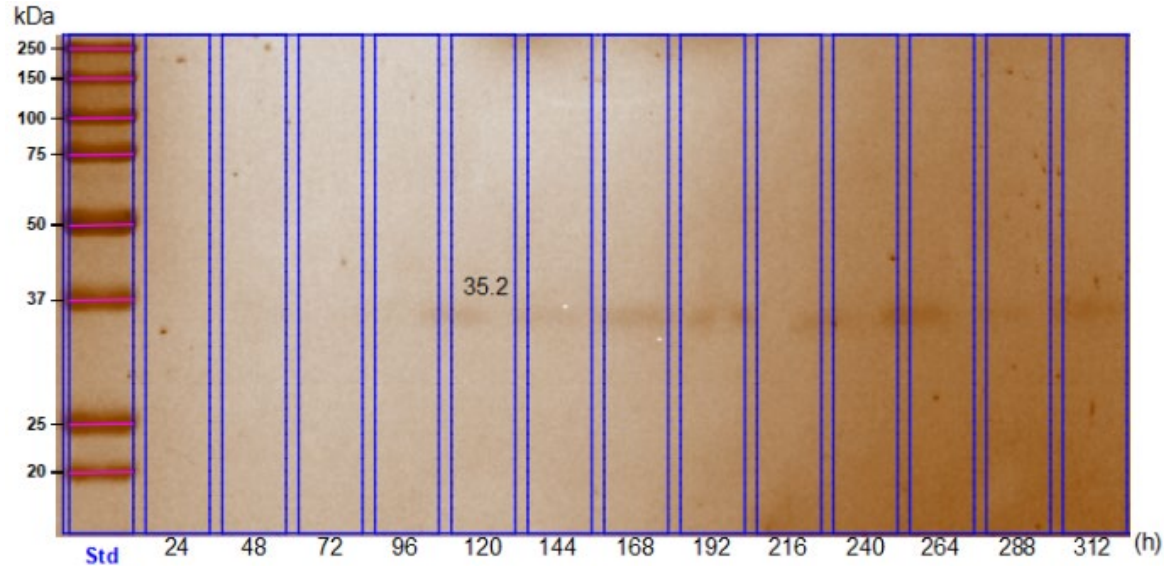

Fig. 6. Zymogram of laccase isoenzymes of *A. aegerita* grown in malt extract under liquid fermentation conditions.

4. Discussion

Edible mushroom such as *P. ostreatus* and *A. aegerita* are source of protein from microbial biomass and has a great potential from enzyme production. In this context, these fungi have been studied to find best substrates for mycelial and enzyme production. Bakratsas *et al*. (2023) studied the growth of *P. ostreatus* and found that a mixture of glucose and xylose was ideal for biomass production with high protein content. *P. ostreatus* cultivation in a 4 L stirred-tank bioreactor using aspen hydrolysate has a biomass production of 25 g/L and a μ of 1.8 d^{-1}. In addition, Krupodorova *et al*. (2024) found that *P. ostreatus* was able to produce 36.5 g/L of mycelial biomass in liquid medium using agricultural wastes. Hamza *et al*. (2024) studied the optimization of culture conditions by integrating response surface methodology and artificial neural network to enhance production of mycelium biomass of *P. ostreatus* and found a biomass production of 36.45 g/L. *A. aegerita* produced 6.8 g/L of biomass in a medium added with glucose after 16 d of growth under liquid fermentation conditions (Diamantopoulou *et al*., 2014). Tlecuitl-Beristain *et al*. (2008) reported that *P. ostreatus* showed a μ of 0.02 h^{-1} and had a maximum biomass of 7.8 g/L in glucose-added medium in liquid fermentation. In this study, it was also found that *P. ostreatus* had a maximal laccase activity (12200 U/L) at the beginning of the stationary phase (at 432 h of growth). $Y_{E/X}$, P and q_p were approximately 1564 U/gX, 28 U/L/h and 31 U/h/ gX, respectively (Tlecuitl-Beristain *et al*., 2008). In addition, *P. ostreatus* had a laccase specific activity of 75.48 U/mg protein grown in corn stover medium under submerged fermentation conditions (Elsayed *et al*., 2012). Téllez-Téllez *et al*. (2008) reported that cultures of *P. ostreatus* showed a laccase activity of 13000 U/L, with a biomass production of 5.6 g/L in submerged fermentation. In the present research, higher amount of mycelial biomass was produced by *P. ostreatus*, however, the laccase activity was lower than that found previously (Téllez-Téllez *et al*., 2008).

Isikhuemhen *et al*. (2009) studied *A. aegerita* cultivation and found that laccase and peroxidase activities were higher before fruiting, ranging between 0.1 and 6.2 U/g, and 0.3 and 0.9 U/g, respectively. González-Rodríguez *et al*. (2023) reported that optimized UnP production of 331 U/L was achieved in vinasse, which conditions were scaled-up to a 4 L reactor, achieving a UnP activity of 265 U/L.

Some studies have developed an expression system that aimed to increase the production of peroxygenases from *A. aegerita*. In this context, Jacob *et al*. (2021) studied *Magnaporthe oryzae* as a host for the expression of the unspecific peroxygenase from *A. aegerita to* offer the possibility to establish *M. oryzae* as a broad applicable alternative expression system. Tonin *et al*. (2021) reported that *A. aegerita* secreted peroxygenase into the culture medium to a final concentration of 0.29 g/L in a fed-batch fermentation of the recombinant *Pichia pastoris*.

Tlecuitl-Beristain *et al*. (2008) found that *P. ostreatus* produced four laccase isoforms in a glucose-added medium grown under liquid fermentation conditions. The most abundant laccase isoform had a molecular weight of 43.7 kDa. Mansur *et al*. (2003) found that *P. ostreatus* produced four laccase isoenzymes with molecular masses of 60, 65, 80 and kDa approximately. Furthermore, Patel *et al*. (2014) found that *P. ostreatus* was able to produce an enzyme, which was determined to be monomeric protein with an apparent molecular mass of 68. 42 kDa. In addition, Bertrand *et al*. (2016) found that *P. ostreatus* produced a laccase with a molecular weight of approximately 30 kDa, which was obtained from residual compost, generated from the mushroom production. In the present research, laccase

isoenzymes showed by *P. ostreatus* and *A. aegerita* have similar molecular weights to those previously reported.

5. Conclusion

These results show that laccases are constitutively produced by *P. ostreatus* and *A. aegerita*, while MnP, LiP and UnP are inducible enzymes. *P. ostreatus* grew faster, showed a greater number of laccase isoenzymes, and had greater laccase activity and biomass production, making it an organism with greater potential than *A. aegerita* for the biotechnological production of these enzymes.

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Author contribution

Angel González Márquez performed the experimental work and analyzed data. Carmen Sánchez planned the experiments, conceived the initial idea, supervised the research, analyzed data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

Los autores declaran que no existe conflicto de intereses.

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