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Heat stress on *Fomes* culture reduces proteases and improves laccases thermostability

El estrés térmico en el cultivo de *Fomes* reduce las proteasas y mejora la termoestabilidad de las lacasas

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Abstract

The aim of this work was to study the production of laccase and protease activity by *Fomes* sp. EUM1 under heat stress when a temperature shift from 35 to 45°C was applied. The highest values of laccase activity were similar and obtained after the temperature shift, 76 and 81.6 U/ gram of initial dry substrate (gids) at 35 and 45 °C respectively. For protease activity, at 35°C the peak activity (6.4 U/gids) was obtained at day 7 and maintained until the end of the culture and at 45°C the peak activity (4.3 U/gids) was obtained when the temperature shift was realized (day 6) and decreased until the end of the culture. In the zymogram of laccase activity one band with a molecular weight of 45 kDa was observed with extracts from both temperatures evaluated with slight differences in molecular weight and intensity of the bands at the various days assessed. The thermostability of the laccases and proteases produced was evaluated, at the highest temperature assessed (60°C) for laccase activity the inactivation constant was 0.324 h^{-1} and 0.277 h^{-1} from cultures at 35 and 45 °C, respectively. These results showed that a temperature shift from 35 to 45°C in the culture of *Fomes* sp. EUM1 increases laccases thermostability and decreases proteases production and thermostability.

Keywords: *Fomes*, laccase, protease, thermostability.

Resumen

El objetivo de este trabajo fue estudiar la producción de actividad lacasa y proteasa por *Fomes* sp. EUM1 bajo estrés térmico cuando se aplicó un cambio de temperatura de 35 a 45°C. Los valores más altos de actividad lacasa fueron similares y se obtuvieron después del cambio de temperatura, 76 y 81.6 U/gids a 35 y 45 °C respectivamente. Para la actividad proteasa, a 35°C el pico de actividad (6.4 U/gids) se obtuvo el día 7 y se mantuvo hasta el final del cultivo y a 45°C el pico de actividad (4.3 U/gids) se obtuvo cuando se realizó el cambio de temperatura (día 6) y disminuyó hasta el final del cultivo. En el zimograma de actividad lacasa con extractos de ambas temperaturas evaluadas se observó una banda con un peso molecular de 45 kDa, se observaron ligeras diferencias en peso molecular e intensidad de las bandas en los distintos días evaluados. Se evaluó la termoestabilidad de las lacasas y proteasas producidas, a la temperatura más alta evaluada (60°C) la constante de inactivación para actividad lacasa fue de 0.324 h^{-1} y de 0.277 h^{-1} de los cultivos a 35 y 45 °C, respectivamente. Estos resultados mostraron que un cambio de temperatura de 35 a 45°C en el cultivo de *Fomes* sp. EUM1 aumenta la termoestabilidad de las lacasas y disminuye la producción y la termoestabilidad de las proteasas.

Palabras clave: *Fomes*, lacasa, proteasa, termoestabilidad.

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1 Introduction

Laccase (EC 1.10.3.2) is a blue multi-copper oxidase that catalyze the oxidation of phenolic and nonphenolic substrates with the reduction of one molecule of oxygen to two molecules of water (Liu *et al*., 2020; Mayolo-Deloisa *et al*., 2020). Laccases have potential application in food and pharmaceutical industry processes, in water and soil bioremediation, bleaching of paper pulp, decolorization of dyes in textile industry and in lignin degradation for production of biofuels (Arregui *et al*., 2019; Bertrand *et al*., 2013; España-Gamboa *et al*., 2021; Liu *et al*., 2020; Mayolo-Deloisa *et al*., 2020).

It has been reported that thermostable laccases have the greatest potential for industrial application (Liu *et al*., 2020). Atalah *et al*. (2019) mentioned that thermostable laccases can be used in lignin degradation, polymer synthesis and carbon electrodes for biofuel cells. An important source of thermostable laccases is thermotolerant microorganisms (Liu *et al*., 2020).

The genus *Fomes* are white-rot basidiomycetes (Papinutti *et al*., 2006) that are widespread in Africa, Asia, Europe, and North America (Wang *et al*., 2021). The genus *Fomes*, being fungi, obtain its nutrients through extracellular digestion of raw materials by the secretion of enzymes (Martínez-Moreno *et al*., 2021). The genus *Fomes* is capable of degrading lignin being laccases one of the enzymes responsible for this process and hence the various reports on the production of this enzyme by various species of this genus (Neifar *et al*., 2010; Papinutti *et al*., 2003; Papinutti *et al*., 2006; Papinutti *et al*., 2008; Větrovský *et al*., 2013). Most fungal species of the genus *Fomes* are mesophilic, with an optimal growth temperature between 20 and 36 °C; although, a thermotolerant strain has been reported (Ordaz-Hernández *et al*., 2016). This strain of *Fomes* sp. EUM1 grows in a temperature range of 20 to 45 °C, with optimal growth at 30 °C (Ordaz *et al*., 2012).

Many organisms change the fatty acid composition of their membrane for the optimal functioning of transporters and enzymes to grow at high temperatures (Ianutsevich *et al*., 2016). Temperature stress causes enzymatic responses, e.g., protease production has been observed in thermophilic and thermotolerant organisms (Ordaz-Hernández *et al*., 2016; Walker and White, 2017). Temperature stress is also related to protein glycosylation that stabilizes protease attacks and protein secretion (Chatterjee *et al*., 2018).

Therefore, in this work, we determined the changes in the production of laccase and protease by *Fomes* sp. EUM1 when the temperature increased from 35 to 45 °C and determined the thermostability of the laccase and protease enzymes produced under these conditions, identifying also possible changes in the molecular mass of the laccases secreted by the fungus that could be identified by zymograms.

2 Materials and methods

2.1 *Fomes sp. EUM1 propagation*

The isolation and identification of the fungus *Fomes* sp. EUM1 was previously described by Ordaz *et al*. (2012). The fungus *Fomes* sp. EUM1 was propagated in a culture medium composed of malt extract (40 g/L) and yeast extract (3 g /L) with a pH of 5.8 ± 0.1. 20 mL of culture medium and boiling beads were added to 50 mL Falcon tubes. The tubes were sterilized in an autoclave for 20 min. at 120 °C and 15 lb/in2. The culture medium was inoculated with 4 six days old mycelium discs with a 1 cm^2 diameter. A Falcon tube with sterile culture medium was used as a control. The propagation culture was cultured at 35 °C for six days. Then the biomass and the boiling beads were separated from the liquid medium by decantation, in this way it was possible to separate the disaggregated biomass from the boiling pearls. The biomass and the boiling beads retained in the Falcon tubes were mixed with 20 mL of Tween 80 (1% v/v) and vortexed for 10 s. to perform mycelium disintegration. The mycelium suspension obtained was used as the inoculum in the agar culture.

2.2 *Agar culture of Fomes sp. EUM1*

The culture medium used for *Fomes* growth consisted of corn stover (40 g/L) and bacterial agar (15 g/L) with a pH of 5.5 ± 0.2. The corn stover used was milled to reduce the particle size between 0.61 and 4.06 mm (Ordaz-Hernandez *et al*., 2016) and sterilized for 25 min. at 120 ºC. Fifty Petri dishes (90 mm × 15 mm) with 30 mL of medium were inoculated with 200 μL of a mycelium suspension from the propagation culture, spreaded over the agar surface with a sterile glass rod and incubated half at 35 °C and the other half at 45 °C for 12 days. The dishes incubated from the beginning at 45 °C showed no appreciable growth and no laccase

activity could be detected due to agar dehydration. Therefore, to investigate the effect of heat stress on *Fomes*, it was determined to incubate a fraction of dishes initially at 35 °C for six days and increase the temperature to 45 °C until the end of the culture. In this way there was growth of the fungus, and the agar dehydration was prevented in the time evaluated.

The enzymatic crude extract (ECE) was recovered from the agar culture. Two Petri dishes were taken per day of incubation; the culture medium was removed from the Petri dishes by making longitudinal and transversal cuts. The fragmented agar was placed in a 250 mL beaker containing 100 mL of deionized water. The solids were agitated at 30 rpm for 45 min. while keeping the beaker immersed in ice water (10 °C). The extract was filtered with Whatman paper No. 40 to separate the residual solids. The filtrate was used to determine enzymatic activities, as well as the amount of soluble protein and the pH. Throughout the culture, the pH was determined directly from the enzyme extracts using a potentiometer (Conductronic pH120). The amount of soluble protein was determined by the method reported by Bradford (1976). The reaction was carried out in test tubes by adding 800 μL of ECE with 200 μL of Bradford's reagent (Sigma-Aldrich). The reaction was left for 5 min. at room temperature; after this time, the absorbance was recorded at 595 nm. The protein concentration was determined by a standard curve using bovine serum albumin (99%, Sigma Aldrich). The results are reported as milligrams of protein per gram of initial dry substrate (mg/gids).

2.3 *Enzyme activities measurement*

Enzymatic activity of laccases was determined using the substrates syringaldazine (Leonowicz and Grzywnowicz, 1981) and ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Yan *et al*., 2015). Syringaldazine was used to verify that the enzymatic activity detected was specific to laccases, similar profile in vitro with both substrates was obtained (Supplementary information Fig. S1) and is the reason why ABTS was used for further determinations. Both methods are based on the measurement of the oxidation of substrates by laccases by means of the molar extinction coefficient (for syringaldazine, ε at 525 nm is 65,000 M^{-1} cm^{-1}; for ABTS, ε at 420 nm is 36,000 M^{-1} cm^{-1}). A volume of 0.5 mL of a suitable dilution of the ECE was placed in a 1 mL cell with 0.5 mL of 0.5 mM ABTS prepared in citrate buffer (50 mM, pH 5.0) or 0.5 mL of 0.1 mM syringaldazine in acetate buffer (0.1 M, pH 5.0). The syringaldazine was previously dissolved in absolute ethanol. The reaction mixture was incubated at 40 °C, and the enzyme activity was determined by measuring the increase in absorbance every 10 s. for 2 min. at a wavelength of 525 nm corresponding to substrates syringaldazine and 420 nm for ABTS in the spectrum (DU 640 Beckman). A mixture of 0.5 mL of the respective buffer with 0.5 mL of substrate was used as a control. The enzymatic activity was expressed in enzyme units (U) per gram of initial dry substrate (U/gids), where U is defined as the amount of enzyme that oxidises 1 μmol of substrate per minute under the reaction conditions (Junior *et al*., 2020).

The protease activity was measured using a Hammarsten-grade casein solution as a substrate (Lee *et al*., 1997). For preparation of the casein, 1 g/L was weighed and dissolved in 100 mL of phosphate buffer (50 mM, pH 7) and 1 mM $CaCl_2$ in a water bath at 60 °C until completely dissolved (approximately 4 min.). The reaction was started by addition 300 μL of ECE, which was previously centrifuged for 15 min. at 4 °C at 11 200 g. To the centrifuged ECE, 700 μL of casein solution was added. The reaction mixture was incubated for 20 min. at 40 °C. Negative controls were prepared in the same way as the samples, except that no ECE was added to these tubes. For all samples, the enzymatic reaction was stopped by adding 1.5 mL of 10% (w/v) trichloroacetic acid (TCA). Then, 300 μL of ECE was added to the negative control tubes. The negative controls were prepared to quantify the initial amino acids under the same conditions. The samples were gently vortexed for 30 s. and then rested for 15 min. in ice water (10 °C). The precipitate was filtered with Whatman No. 1 paper. The absorbance of the solution was recorded at 280 nm (Beckman DU 640). The amount of aromatic amino acids released in the enzymatic reaction was calculated from a standard tyrosine curve. The protease activity was expressed as the amount of enzyme producing an absorbance change equivalent to 1 μmol of tyrosine per minute under the reaction conditions per gram of initial dry substrate (U/gids).

2.4 *Zymograms*

For the performance of zymograms of laccase activity a modified SDS-PAGE technique (Laemmli, 1970) was used as previously done by Téllez-Téllez *et al*. (2005). Separating and stacking gels were of 12% and 4% polyacrylamide, respectively, and an extract of standardized 119 μg of soluble protein was loaded per lane. To determine the molecular mass of the bands

with laccase activity, Bio-Rad prestained SDS-PAGE standards (cat. 161-0305) were loaded. The proteins included were the following: (Aprotinin 6,500 Da), lysozyme (14,400 Da), soy trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,200 Da), and phosphorylase B (97,400 Da). Separation was carried out at 100 V and 4 °C. After the electrophoresis, the gels were washed once with 0.1% Triton X-100 for 15 min. and 3 times for 15 min. with deionized water. Finally, 50 mL of 50 mM ABTS in 50 mM citrate buffer (pH 5) was added, and gels were incubated at 40 °C for approximately 20 min. to reveal the bands with laccase activity (Ordaz-Hernández *et al*., 2016).

2.5 *Thermostability determination*

To determine the thermostability of the laccases and proteases produced, the enzyme extracts from the day of maximum activity at each temperature (35 °C and 45 °C) were used. 500 μL of ECE and 500 μL of 50 mM citrate buffer (pH 6) were deposited in screw-cap test tubes and incubated in a water bath at different temperatures (45, 50, and 60 °C) for the corresponding time. Samples were taken at different incubation times, removed from the water bath, and cooled to room temperature. Finally, the laccase activity was determined, as mentioned above, and the percentage of active enzyme present in the enzymatic extracts was calculated. The data were adjusted to the following first-order decay model (Eq. 1) with an exponential adjustment. The mean activity time, $t_{1/2}$ (h), and the inactivation constant, k (h^{-1}), were determined.

$$A = A_0 * \exp(-k * t) \tag{1}$$

Where:

$A(t)$ is the enzymatic activity at time t (U/g ids)

A_0 is the initial enzymatic activity at t=0 (U/g ids)

k is the first-order decay coefficient (h^{-1})

t is the residence time in the incubation temperature (h)

2.6 *Statistical analysis*

All measurements and tests were carried out in triplicate in each Petri dish. The statistical analysis was performed with the Sigma Plot package (Systat Software Inc., San Jose, CA). Soluble protein, pH, and enzymatic activity data obtained at both temperatures (35 °C and 45 °C) were compared with a Student's t-test (α=0.05).

3 Results and discussion

3.1 *pH and soluble protein determination*

During fungal growth at both temperatures, the pH gradually increased to a value close to 7 towards the end of the cultivation (Fig. 1). The highest pH was 7.4 on 11 d. at 35 °C. Mani *et al*. (2017) mentioned that the production of filamentous fungi laccase is usually performed at pH intervals of 3-5 and 25-30 °C. Díaz *et al*. (2013) reported that the initial pH of the growing medium can influence the laccase activity and the laccase isoenzymes produced by *Pleurotus ostreatus* in submerged fermentation. However, the pH to produce laccase has been observed to vary from one substrate to another (Novoa *et al*., 2019). The pH detected at the end of this culture could favor the stability of the laccase activity of the extract obtained as it has been reported that fungal laccases are highly stable above pH 7.0 (Torres-Salas *et al*., 2013).

The soluble protein showed a similar behavior at both temperatures after the increase from 35 to 45°C at day 6 (Fig. 1) but with a higher concentration at 35 °C. As protein secretion has been reported to be associated with growth in fungi (Carrillo-Sancen *et al*., 2016; Da Alves Silva *et al*., 2022), the lower concentration of soluble protein at 45°C by *Fomes* sp. EUM1 can be associated with lower growth of the fungus. Soluble protein value that occurred at time zero could be due to the extracellular material present from the propagation culture of *Fomes* sp. EUM1. Subsequently, there was a decrease in soluble protein until day 3, which could be associated with initial protein consumption by the fungus to start its growth in the culture medium. The increase in soluble protein at the end of the culture at 45°C could indicate stress of the fungus by high temperature. Ado (2019) mentioned that the production of soluble protein is usually induced by stress factors, such as limited carbon and nitrogen levels. The change in soluble protein suggests a possible change in the activity of laccases, proteases and other proteins secreted by the fungus that allows it to adapt to the increase in the incubation temperature of the culture.

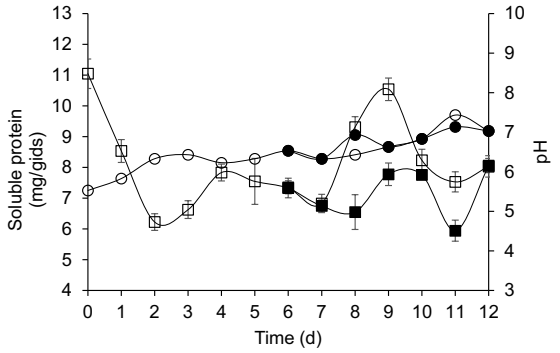

Fig. 1. Measurement of culture pH (circle marker) and soluble protein (square marker). Cultures at 35 °C (unfilled marker) and 45 °C (filled marker). Measurements were carried out in triplicate.

3.2 Kinetics of laccase and protease activities

Laccase and protease activities were analyzed for both culture temperatures, 35 and 45 °C, during the 12 days of culture (Fig. 2a). Laccase activity showed a similar behavior at both temperatures after the temperature shift at day 6 but with different activity values (Fig. 2a). Maximal activities values were not significative different and obtained after the temperature shift, they were 76.04 and 81.55 U/gids, at 35 °C and 45 °C respectively. The time to obtain the maximum values were 8 and 7 days, at 35 °C and 45 °C respectively.

There is a wide variety of works focused on laccase enzyme production in Basidiomycetes. In submerged culture reports, with *Fomes fomentarius* a mean activity of 206.8 U/L extract was obtained after 35 days of culture with cellulose as the source of carbon (Větrovský *et al*., 2013), *Fomes fomentarius* with a mandarin peels-based medium obtained a peak activity of 17 500 U/L extract after 7 days of culture (Elisashvili and Kachlishvili, 2009), *Phellinus robustus* and *Ganoderma adspersum* produced a peak activity of 4 000 U/L extract (14 days) and 34 000 U/L extract (11 days), respectively, growing on mandarin peels medium (Songulashvili *et al*., 2006). In solid state fermentation using wheat bran as substrate, with *Fomes sclerodermeus* a maximum laccase activity of 270 U/g dry wheat bran was obtained after 28 days of incubation (Papinutti *et al*., 2003) and with *Fomes fomentarius* the highest laccase activity obtained was nearly 6 400 U/L extract after 13 days of cultivation (Neifar *et al*., 2010). In the solid state fermentation of *Pleurotus ostreatus*, using polyurethane foam as an inert support and glucose as carbon source, the

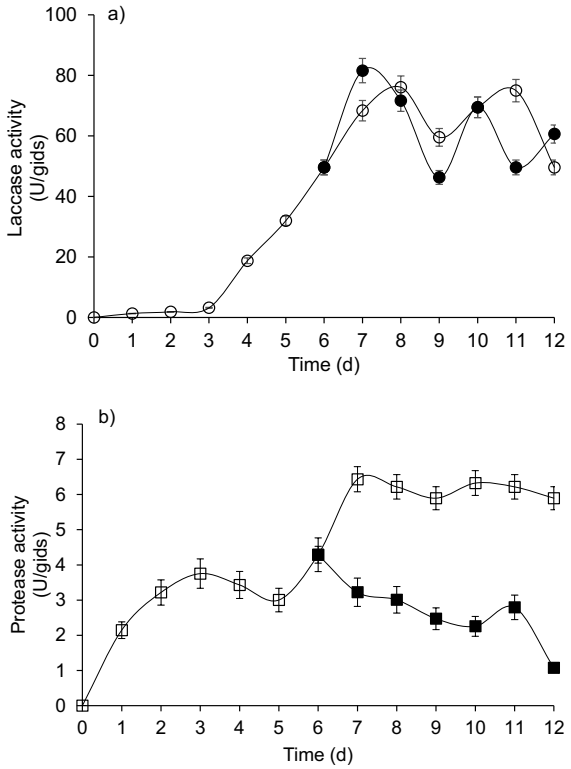

Fig. 2. Activity profiles of laccase (a) and protease (b). Cultures at 35 °C (unfilled marker) and 45 °C (filled marker). Measurements were carried out in triplicate.

highest value of laccase activity obtained was of 20 000 U/L extract after 400 hours of growth of the fungus (Montalvo *et al*., 2020). An advantage of the culture of *Fomes* sp. EUMI is that the culture time to obtain the maximum laccase activity is less than most of the reports mentioned above.

Gokhale *et al*. (2011) reported that *Aspergillus niger* NCIM 1207 produced β-glucosidases and β-xylosidases at 30 °C for the first 5 days, then a change to 36 °C for the 6 following days enhanced the production of both enzymes compared to cultures that remained at 30 °C. The phenomenon of increased enzyme production due to a temperature shift does not agree with the results obtained with *Fomes* sp. EUM1 since the activity values were similar after the temperature change. In some thermophilic microorganisms, modifications in the cytoplasmic membrane alter the transport of solutes as an adaptation to high temperatures (Konings, 2006). Pedneault *et al*. (2007) observed modifications in the composition of fatty acids that compose the cytoplasmic membrane when *Pleurotus ostreatus* and *Pleurotus cornucopiae* were cultivated in a range of

temperatures.

The presence of protease activity was evaluated as a response of the fungus to temperature stress. Protease activity had different behavior after the temperature shift (35 to 45°C), at 35°C the activity peaked at day 7 and was maintained until the end of the culture, at 45°C the activity decreased until the end of the culture (Fig.2b). The phenomenon occurred at 45°C after the temperature shift has not been described before and is possibly due to the denaturation of proteases under these conditions or because the fungus *Fomes* sp. EUM1 reduces the production of these enzymes under thermal stress. Furthermore, proteases had no effect on laccases after the temperature shift since the laccase activity values were similar in the two temperatures evaluated (Fig. 2).

Protease activity has been observed as a response to thermal stress, with an effect on the production of some enzymes (Lim *et al*., 2016; Parsell and Lindquist, 1993). Spiess *et al*. (1999) indicated that in the bacterial periplasm the expression and activity of a protease called DegP are coupled to elevated temperatures. Interestingly, DegP exhibits either chaperone or protease functions, depending on the temperature. It assists in the refolding of proteins if they are not severely damaged and degrades proteins under conditions where refolding is not feasible. In yeast, two vacuolar proteases, the unspecific protease pep4, and ubiquitin are also induced after heat shock, indicating that enhanced proteolysis involving proteasomal degradation is necessary for protein degradation after heat shock. In fact, a small increase in temperature can induce protein unfolding, interlinking, and unspecific aggregation (Richter *et al*., 2010). In contrast to similar reports concerning increased proteolytic activity as a response to heat stress, a higher proteolytic activity when increasing the temperature from 35 °C to 45 °C was not found in this work. Although, the proteases remaining in this culture could still be active, as observed in the ECE thermostability test at 45 °C (described below). Therefore, the activity of the chaperones of the fungus could be the responsible of maintaining the catalytic structure and may not require further production of proteases during cultivation at 45 °C.

3.3 Zymograms

In the zymograms shown in Fig. 3 only one band with laccase activity was observed for the times evaluated at the temperatures of 35 °C and 45 °C. The bands with laccase activity have an approximate molecular weight between 45 to 60 kDa (Table S1). Ordaz-Hernández *et al*. (2016) also reported one band of laccase activity (46 kDa) in *Fomes* sp. EUM1 in solid-state culture. Similarly, Neifar *et al*. (2010) reported that a single laccase was found in the solid-state fermentation of *F. fomenatrius* using wheat bran as substrate, with a molecular mass of 51 kDa. Morozova *et al*. (2007) mentioned that laccases from filamentous fungi are proteins with a molecular weight of 60-70 kDa being the bands shown in the zymograms in the present study below these values. However, with the zymograms obtained with *Fomes* sp. EUM1 slight differences in the molecular weight and intensity of some bands were found, which could be due to a different conformation of the protein as an adaptation to temperature. Papinutti *et al*. (2006) using *Fomes sclerodermeus* in a liquid semi-defined media found two laccase isoenzymes with same molecular mass (67 kDa) with small difference in their pI values (3.4 and 3.5). Also, some reports have mentioned that the adaptation mechanism of thermostability is a unique structural-functional property of proteins other than amino acid sequences (Fields *et al*., 2015). Ichikawa *et al*. (2020) analyzed a recombinant tannase in *Aspergillus oryzae* and determined that N-linked oligosaccharides did not affect the enzymatic activity and thermostability. Different from what was obtained in this work, Papinutti *et al*. (2008) reported that with a crude extract obtained from the solid-state fermentation of *Fomes sclerodermeus* using wheat bran as substrate, found three bands of laccase activity (148, 47 and 36.5 kDa) with different thermal inactivation pattern. After 6 hours of incubation at 50°C two bands were practically undetectable and the 47 kDa band remained active.

Further analysis of the enzymes with laccase activity present in the bands of the zymogram of the present work is necessary for a better understanding of possible changes in the structure and/or sequence of these enzymes. In our work, the thermostability of laccases and proteases was analyzed at different temperatures, as shown below.

3.4 Thermostability determination

Thermophilic and mesophilic enzymes share remarkably similar sites of catalytic activity; although, thermostability parameters suggest additional modifications as a function of growth temperature (Ordaz-Hernández *et al*., 2016; Vieille and Zeikus, 2001).

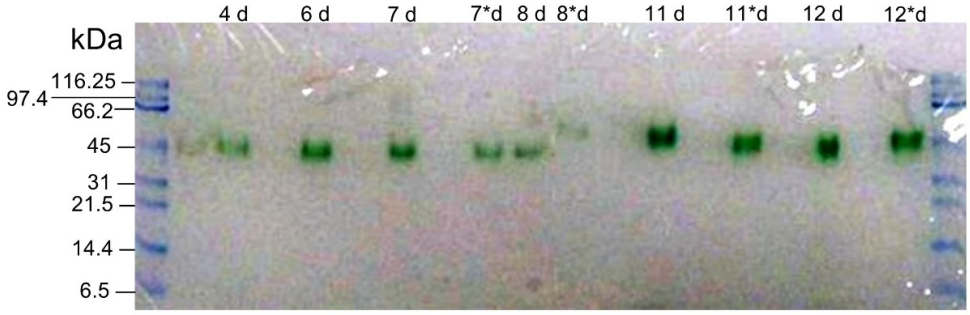

Fig. 3. Zymogram of laccase activity of the extracts produced at 35 °C and 45 °C (numbers with an asterisk) at 4, 6, 7, 8, 10, and 11 days (d). Molecular weights of the protein standards are on the left side of the zymogram images.

The laccase activity of the ECE obtained at 35 °C and 45 °C showed a stability of 100% after 36 hours of testing at 40 °C. In contrast, after 30 min. at 70 °C there was no laccase activity detected (Fig. S2). Therefore, the thermostability tests were carried out at 45, 50, and 60 °C for both laccases and proteases and a first-order decay model were adjusted, the ECE produced in the culture at 35 and 45 °C showed a similar residual activity during thermostability tests, showing slightly lower thermostability for proteases. The experimental data are presented in the Supplementary information (Fig. S2).

Table 1 shows the stability parameters of laccase and protease activity of the ECE produced at 35 °C and 45 °C. By testing at the highest temperature (60 °C), the inactivation constant determined for the laccases was 0.324 ± 0.03 h^{-1} and 0.277 ± 0.03 h^{-1} for the ECE harvested from cultures at 35 °C and 45 °C, respectively. The inactivation constants were higher for proteases at 60 °C, with values of 0.414 ± 0.05 h^{-1} and 0.354 ± 0.06 h^{-1}, respectively.

In terms of thermostability, for laccases produced by the genus *Fomes* and other microorganisms, the following reports are available. When performing a thermostability analysis at 50 °C with ECEs obtained from *Fomes sclerodermeus* in solid state fermentation using wheat bran as a substrate, Papinutti *et al*. (2008) observed a loss of laccase activity after 6 hours. Neifar *et al*. (2010) measured the thermostability of laccases produced in solid state fermentation with wheat bran as substrate at 60 °C by *Fomes fomentarius*, obtaining a half-life of 66 min. If we compare with laccases of other fungus, Zheng *et al*. (2017) indicated that a laccase from *Trametes orientalis* at 50 °C showed an 80 % of residual activity after 2 h. Ding *et al*. (2012) assayed a laccase of Ganoderma lucidum and reported a 46 % of residual activity after 80 min at 60°C. The thermostability parameters found in our work shows a better thermostability than those previously reported for the genus *Fomes*, and the other fungus mentioned before. Nonetheless, concerning the mesophilic genus *Trametes*, Haibo *et al*. (2009) obtained a half-life of 5 hours at 60 °C, with laccases produced by *Trametes hirsuta* in solid culture with wheat bran as a substrate. Yang *et al*. (2020) reported a half-life of more than 6 hours at 60 °C for a laccase from *Trametes trogii*. With respect to a bacterial laccase Rai *et al*. (2019) mentioned that a laccase from *Geobacillus* sp had a half-life of 120 h at 50 °C.

Table 1 Thermal stability parameters of laccase and protease activities of the ECEs produced at 35 °C and 45 °C. All measurements were carried out in triplicate.

ECE	T(ºC)	$k(h^{-1})$	$t_{1/2}$ (h)
Lacasse activity			
35 °C	45	0.007±0.01	97.8±1.7
	50	0.058±0.0.7	12.0±3.5
	60	0.324±0.034	2.1±0.3
45 °C	45	0.017±0.002	42.0±2.3
	50	0.045±0.06	15.4±1.7
	60	0.277±0.03	2.5±0.4
Protease activity			
35 °C	45	0.009±0.001	76.5±2.1
	50	0.074±0.008	9.4±0.8
	60	0.414±0.05	1.7±0.4
45 °C	45	0.021±0.009	32.9±1.9
	50	0.058±0.003	12.1±0.3
	60	0.354±0.06	2.0±0.7

Although there is the presence of protease activity in the ECE obtained with *Fomes* sp. EUM1, the protease activity and thermostability decresased with the temperature shift to 45°C. The extracts with laccase activity and with stability up to 60 °C obtained in this report has industrial interest in the production of laundry detergents and animal feeds (Pelaez *et al*., 2022) as well as the other applications mentioned above for thermostable laccases.

Conclusion

The thermostability parameters of laccases analyzed were higher than those previously reported for the genus *Fomes*. The temperature change decreased the protease production and reduced thermostability. Moreover, laccases produced under these conditions were stable, even in the presence of proteases, which is beneficial since some industrial processes involve harsh conditions, such as high temperatures. Additional studies on protein structure and folding will help in understanding the thermostability of enzymes produced by the thermotolerant strain *Fomes* sp. EUM1.

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