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ORIGINAL RESEARCH

Effect of particle size, aeration, and substrate height on the production of lignocellulolytic enzymes produced by *Trametes polyzona* HHM001 grown on corn leaf residues.

Efecto del tamaño de partícula, aireación y altura del sustrato sobre la producción de enzimas lignocelulolíticas producidas por *Trametes polyzona* HHM001 crecido sobre residuos de hojas de maíz

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ABSTRACT

The objective of the work was to evaluate the production of lignocllulolytic enzymes produced by *Trametes polyzona* HHM001 during its growth on corn leaf residues. Two particle sizes (PS) (PS8 and PS12), two levels of aeration (1 vvm and 0 vvm), as well as the height of the substrate in the production of lignocellulolytic enzymes were tested. The enzymatic activities of Laccase (Lcc), Lignin peroxidase (LiP), and Manganese peroxidase (MnP) were favored under aerated conditions (1 vvm). The enzymatic activity of Lcc was the most favored, with 80 Activity Units (AU)/mL, compared to the culture without aeration, in which 40 AU/mL was obtained. The production of xylanases (Xyl) and cellulases (Cel) were not influenced by aeration under the tested conditions. The results indicated that the particle size has more effect on enzyme production than the presence or absence of air, with a particle size of 8 where the best levels of

enzymatic activity were observed. It was observed that the height of the substrate in the fermentation column strongly affects the activity of ligninolytic enzymes and not of the hydrolytic; at 5 cm, the highest ligninolytic activity was detected, where aeration favors oxidative conditions.

Keywords: Cellulase, Corn leaf residues, Lignocellulolytic enzymes, *Trametes polyzona*, SSF, Xylanase.

RESUMEN

El objetivo del trabajo fue evaluar la producción de enzimas lignocelulolíticas producidas por *Trametes polyzona* HHM001 durante su crecimiento sobre residuos de hoja de maíz. Se probaron dos tamaños de partículas (PS) (PS8 y PS12), dos niveles de aireación (1 vvm y 0 vvm), así como la altura del sustrato en la producción de las enzimas lignocelulolíticas. Las actividades enzimáticas de Lacasa (Lcc), Lignina peroxidasa (LiP) y Manganeso peroxidasa (MnP) fueron favorecidas en condiciones de aireación (1 vvm). La actividad enzimática de Lcc fue la más favorecida con 80 Unidades de Actividad (AU)/mL en comparación con el cultivo sin aireación en el que se obtuvo 40 AU/mL. La producción de xilanasas (Xyl) y celulasas (Cel) no se vieron influenciadas por la aireación en las condiciones probadas. Los resultados obtenidos indicaron que el tamaño de partícula tiene más efecto en la producción de enzimas que la presencia o ausencia de aire, siendo el tamaño de particula de 8 donde se observaron los mejores niveles de actividad enzimática. Se observó que la altura del sustrato en la columna de fermentación tiene un fuerte efecto sobre la actividad de las enzimas ligninolíticas y no así sobre las hidróliticas; a los 5 cm se detectó la mayor actividad ligninolítica, donde la aireación, favorece las condiciones oxidativas.

Palabras clave: Celulasa, Enzimas lignocelulolíticas, Residuos de hojas de maíz, SSF, *Trametes polyzona*, Xilanasa.

1. INTRODUCTION

Due to their chemical composition, lignocellulosic residues represent the best substrate for obtaining hydrolytic and oxidative enzymes since they function as inducers of the expression and production of these types of enzymes. The production of ligninolytic and hydrolytic enzymes in a solid and liquid medium using white rot basidiomycetes is of great importance since it allows the use of agro-industrial residues. The most studied fungi for the production of lignocellulolytic enzymes are *Pharenochaete chrysosporium* (Martínez *et al*., 2004; Gassara *et al*., 2013; Riley *et al*., 2014), *Trametes versicolor* (Sánchez, 2009; Montoya *et al*., 2021), and some strains of *Pleurotus* (Elisashvili *et al*., 2008b; Hernández-Domínguez *et al*., 2017; Rajavat *et al*., 2020). The enzyme complex responsible for the lignocellulose degradation consists of two groups of enzymes; the group of enzymes responsible for the degradation of lignin constituted of laccase (Lcc), lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP), and the enzymes responsible for degrading cellulose and hemicellulose (Lundell *et al*.,

2010). The first three enzymes belong to the Peroxidases II family and the superfamily of heme peroxidases; these enzymes carry out their catalytic cycle through the use of H_2O_2, which, in turn, acts as a carrier of oxygen and electrons (Hatakka & Hammel, 2011; Chen *et al*., 2012). For the degradation of the polysaccharides present in lignocellulose, the fungi produce hydrolytic enzymes whose catalytic activity is very specific, and their purpose is the complete hydrolysis of cellulose and hemicellulose to their monomers. This hydrolysis is given by the action of endoglucanases, endo-1-4-β-glucanase, cellobiohydrolases, and β-glycosidases enzymes (Sánchez, 2009).

On the other hand, corn is produced in more than 113 countries. Mexico is the main crop, with an annual production of 27.2 million tons occupying the sixth position in production worldwide (SADR, 2020). The production of corn generates large amounts of residues, and a very high proportion is used as fodder; however, its low nutritional content and difficult digestion by livestock make its widespread use unattractive. A viable option is to use lignocellulosic residues as raw material for the synthesis of by-products by applying the concept of biorefineries. Another option is its use as a substrate for solid fermentation processes to produce biomass or other products such as enzymes. In this sense, it has been described that obtaining products of interest from the enzymatic biodegradation of lignocellulosic residues depends on several factors, including the culture conditions (Thomas *et al*., 2013), the ability to produce enzymes by the fungus under study (Fernández-Fueyo *et al*., 2021), water activity (a_w), pH, inoculum type, moisture percentage (Wan & Li, 2012), the chemical composition of the substrate (Elisashviii *et al*., 2008a), temperature, cultivation time, particle size, and aeration supply (Quintanar-Gómez *et al*., 2012). All these variables directly affect growth, enzymatic activity, and product formation (Wan & Li, 2010).

Trametes polyzona is a white rot basidiomycete fungus that has been used to carry out bleaching processes of textile effluents (Cerrón *et al*., 2015; Chen and Ting, 2015; Pérez-Cadena *et al*., 2020; Uribe-Arizmendi *et al*., 2020), degradation of polycyclic aromatic compounds (Agrawal *et al*., 2018), for the treatment of agricultural effluents (Zeng *et al*., 2017), and removal of compounds in effluents with estrogenic activity (Kasonga *et al*., 2019). This fungus has been described as being capable of producing Lcc (Chairi *et al*., 2013; Rattanarat *et al*., 2016; Zeng *et al*., 2017; Lueangjaroenkit *et al*., 2018) and MnP (Lueangjaroenkit *et al*., 2019) mainly, and has not been reported as a producer of hydrolytic enzymes such as cellulases and hemicellulases. Given its ability to degrade recalcitrant compounds, it is of great interest to study its ability to produce hydrolytic enzymes on lignocellulolytic substrates. The objective of the present work was to evaluate the influence of two particle sizes in combination with two-level of aeration, as well as study the effect of the thickness of the substrate (Corn leaf residuals) in a fermentation column on lignocellulolytic enzyme production of *T. polyzona* HHM001 in conditions of Solid-State Fermentation (SSF).

2. MATERIAL AND METHODS

2.1. Microorganism, strain propagation, conservation, and preinoculum preparation

The basidiomycete *Trametes polyzona* HHM001 was isolated from the Huasteca Hidalguense, México (Cruz-Ramírez *et al*., 2012). For its propagation, the strain under study was plated on potato dextrose agar (PDA) (Dibico®). Plates were supplemented with 0.1 % yeast extract (Sigma-Aldrich®) and grown at 37 °C for 5 days. The plates were maintained at 4° C and periodically reseeded. The preinoculum was prepared according to the method described by Quintanar-Gómez *et al*. (2012). The supernatant containing the mycelium in suspension was added to the previously treated substrate and adjusted to 75 % moisture.

2.2. Molecular Identification

For DNA extraction, AllPrep DNA/RNA/Prep kit (Quiagen) was used. Once the genomic DNA was extracted, a 1 % agarose gel was prepared to verify the DNA integrity.

For PCR amplification, the universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG) and ITS 4 (5'TCCTCCGTATTGATATGC) were used. The total volume of the reaction was 25 µL of the sample, 23 µL of PCR solution (1X PCR, 2.5 Mm $MgCl_2$, 200 µM dNTPs, and 0.1 µg/µL BSA); 1 µL of the primer-Taq-Polymerase mix (0.5 µL of each primer and 0.1 µL of 5 U/µL Taq-Polymerase) 1 µL of DNA. Subsequently, PCR amplification was carried out under the following conditions: 40 cycles at 94 °C starting temperature, 57 °C alignment temperature, and 72 °C final temperature.

The JETquick-PCR purification kit (Thermo Scientific) was used for DNA purification. The DNA concentration was determined in a Nanodrop (Thermo). The sequencing was carried out at the Madrid Science Park (PCM). Finally, the sequence obtained was edited in Genious and submitted to GenBank Blast (NCBI).

2.3. Corn leaf residues pretreatment

The corn leaf residues (CLR) used in this study were collected *in situ* from Omitlán de Juárez, Hidalgo, Mexico (20º10´32.80” N, 98º38´55.70” W), and were obtained between the months of May and August of a hybrid of white corn 9105w (Croplan). The corn leaves were collected and stored at room temperature in dark plastic bags; later, they were ground in a hammer mill and passed through a sieve (WS TILER) to obtain particle sizes (PS) 8 (2.36 mm), and 12 (1.7 mm). The ground substrate was washed with water and dried at 60 °C for 24 h. Finally, the moisture percentage was determined (in triplicate) and sterilized for 30 min (121° C, 15 lb/in^2 pressure).

2.4. Moisture determination

For this analysis, 1 g of CLR was used. The CLR was dry in an oven at 60° C for 48 hours. The samples were taken out of the oven and placed in a desiccator until reached room temperature; subsequently, the samples were weighed until constant weight. The moisture of the CLR was determined by weight difference. All analyzes were performed in triplicate.

2.5. Determination of compounds extractable in solvents, and hot water, lignin, holocellulose, and hemicellulose

The CLR were characterized to know the cellulose, hemicellulose, and lignin contents to estimate their influence on the secretion of lignocellulolytic enzymes. To quantify extractable compounds in solvents, TAPPI Standard T-204 om-84 (1987) was used from 10 g of a crushed sample according to the Standard. The TAPPI Standard T-207 (1993) was used to quantify water-extractable compounds from the residues resulting from the solvent extraction process. For the quantification of lignin, 1 g of sample was used, and the protocol was described in TAPPI Standard T-222 om-88 (1988). To determine holocellulose, 2 g of sample was used, and the method described by Browning (1967) was followed. For the quantification of hemicelluloses (pentosan), the TAPPI 203 cm-09 (2009) standard was used. 1.5 g of the residual resulting from extractable in solvents and hot water was used for this analysis.

2.6. Solid State Fermentation (SSF)

For the SSF process, the procedure described by Roussos & Raimbault (1982) was followed. Glass columns of 20 cm long and 4 cm in diameter were used with the conditions described by Quintanar-Gómez *et al*. (2012). The pre-inoculum for fermentation was prepared from a petri dish inoculated with the fungus and incubated for 7 days at 37 °C. The pre-inoculum was standardized using a petri dish with the fungus cut into 0.5 cm^2 squares using a sterile blade and under aseptic conditions and adding it to 100 mL of sterile water contained in a 250 mL Erlenmeyer flask. The flask was kept shaking (200 rpm) overnight to facilitate the detachment of the mycelium. The obtained supernatant was used as inoculum. The packing density was 2.5 mL of inoculum/g of CLR to reach 70-80 % moisture. The incubation temperature was 37 °C. Aeration was 1 vvm. Every 24 h, three columns were taken to carry out the corresponding analyses.

2.7. Obtaining the crude enzymatic extracts

The total content of the columns was weighed and placed in flasks with a capacity of 125 mL. For each gram of culture, 20 mL of buffer solution (10 mM acetate buffer at pH 6) was added and stirred for 30 min at 150 rpm, later was centrifuged at 14,000 rpm for 15 min, and the supernatant was taken to perform the corresponding analyzes (Quintanar *et al*., 2012). To study the effect of column height on enzymatic activities, each of them was packed up to 15 cm high. The column was divided into 3 sections of 5 cm: the bottom of the column (0-5 cm) (BC), the intermediate zone of the column (5-10 cm) (IZ), and the surface of the column (10-15 cm) (SC). The materials of each section were analyzed using their respective extracts obtained as described above.

2.8. Ligninolytic activity

The ligninolytic enzyme activities were measured spectrophotometrically. For Lcc activity, 500 uL of crude extract and 500 μL of a solution of ABTS (500 μM) in 50 mM acetate buffer, pH 5, were taken, monitoring ABTS (Roche®) oxidation at 420 nm for one minute (Eggert *et al*., 1996). To determine the LiP activity, a reaction mixture

compound of 400 μL of 125 mM sodium tartrate buffer, pH3; 200 μL of a 10 mM veratryl alcohol solution; 20 μL of a 2 mM hydrogen peroxide solution, and 200 μL of extract enzymatic crude. The enzymatic activity was obtained by measuring the oxidation of veratryl alcohol to veratraldehyde for one minute at 320 nm. (Tien & Kirk, 1988). To determine MnP activity, 900 μL of reaction mixture composed of 50 mM sodium succinate, pH 4.5; 0.1 mM manganese sulfate; 50 mM hydrogen peroxide, and phenol red (100 μg/mL) (Sigma-Aldrich®) was prepared. To this mixture, 100 μL of crude extract was added. Phenol red oxidation was measured for one minute at 320 nm (Glenn & Gold, 1985). Under the conditions tested, the activity unit (AU/mL) was defined as the amount of enzyme needed to oxidize 1 μmol of ABTS, veratryl alcohol, and phenol red per minute per mL for the activities of Lcc, LiP, and MnP, respectively.

2.9. Cellulolytic and xylanolytic activities

The carboxymethyl cellulose (Sigma-Aldrich®) and birch xylan (Sigma-Aldrich®) substrates dissolved in acetate buffer (0.2 %, 50 mM, pH 6) were used to determine cellulolytic and xylanolytic activity, respectively. To calculate the sugars released by the enzymatic action, a blank (water), sample blank (enzymatic extract), and the test sample (enzymatic extract incubated with the substrate, 15 min at 50 °C) were prepared; later, the DNS technique (Miller *et al*., 1960) was used to estimate the sugars present. A subtraction was made between the sample blank and the test sample to estimate the sugars released exclusively by enzymatic action. The absorbance obtained was compared with the standard curves (glucose and xylose) to determine the amount of sugars released. Under the conditions tested, the AU/mL was defined as the amount of enzyme needed to release 1 μmol of xylose or glucose per minute per mL.

2.10. Analysis of data

The statistical software SPSS (v 20.0) was used to analyze the data obtained. Single-factor ANOVA and means comparison tests (Tukey and Duncan) with a significance level of 0.05 were used. All analyzes were carried out in triplicate.

3. RESULTS

3.1 Molecular identification and CLR composition

Molecular identification using the ITS1-ITS4 region showed 99 % homology with *Trametes polyzona*. The sequence was deposited in GenBank with accession number MW830378. The results of the chemical composition of the CLR indicated 2.73 % moisture, 7.06 % total extractable (in acetone and water), 11.95 % lignin, 41.4 % cellulose, and 24.3 % hemicellulose (pentosans). The quantification of these components is of vital importance since they are the main carbon source present in the substrate and that in the long term, are the components that allow the fungus to survive in natural conditions. The chemical composition of the raw material has a strong

influence on the growth of basidiomycete fungi, hence the importance of its initial characterization.

3.2. Ligninolytic enzymes production

The production kinetics of the main enzymes involved during the CLR degradation process, such as Cel, Lcc, LiP, MnP, and Xyl were carried out for 15 days without aeration and with aeration, and with different PS (8 and 12). Under the analyzed conditions, it was observed that aeration (1 vvm) favors the production of these enzymes presenting greater catalytic activity in the culture with PS 8 (Fig. 1). The Lcc enzyme was the most favored in a PS 8 with 80 AU/mL (Fig. 1A). Regarding the LiP activity, the maximum activity was observed on day 15 with 10 AU/mL in aerated conditions (Fig. 1B) when a PS 12 was used. In contrast, in the absence of aeration, the higher LiP activity was 5 AU/mL with a PS 8 culture. In the case of the MnP activity, the maximum enzyme activity was detected on day 11 of fermentation with 18 AU/mL under aeration conditions and a PS 8 (Fig. 1C).
In general, it was observed that the enzymatic activity of Lcc, LiP, and MnP was greater with a PS 12 with aeration. This result is also repeated with PS8. In the case of laccase, a 50% increase in enzyme activity is observed when fermentation occurs under aerated conditions (PS8). In the case of LiP, no relevant results were obtained regarding enzymatic production with other assay conditions. For MnP production, PS 12 with aeration was where the following production condition of this enzyme was observed with 11 AU/mL at 14 days of growth of the fungus. In PS 12, the production of MnP was 2 and 3 AU/mL for the culture without and with aeration, respectively. The activity was slightly higher, with 3 and 11 AU/mL for PS 8 being significantly different when aeration was applied. It is evident that regardless of the PS, aeration (1 vvm) increases the activity of the ligninolytic complex produced by *T. polyzona* HHM001.

3.3. Cellulase and xylanase production

The enzymatic cocktail responsible for the degradation of lignocellulolytic residues is complemented with the expression and secretion of hydrolytic enzymes such as cellulases and hemicellulases responsible for the hydrolysis of cellulose and hemicelluloses to reach the complete mineralization of this type agro-industrial waste. *T. polyzona* HHM001 showed the same trend in producing these enzymes in the four treatments tested. The highest activity was obtained in PS 8 (Fig. 2A, 2B) under aeration conditions with 3.8 AU/mL and 5 AU/mL for Cel and Xyl activity, respectively, while for PS 12 in the absence of aeration, the lowest activity occurred with 2.5 AU/mL and 2.8 AU/mL for Cel and Xyl activity respectively (Fig. 2C, 2D). The profile of enzyme production shows that Xyl and Cel activity increases with the growth of the fungus under study.

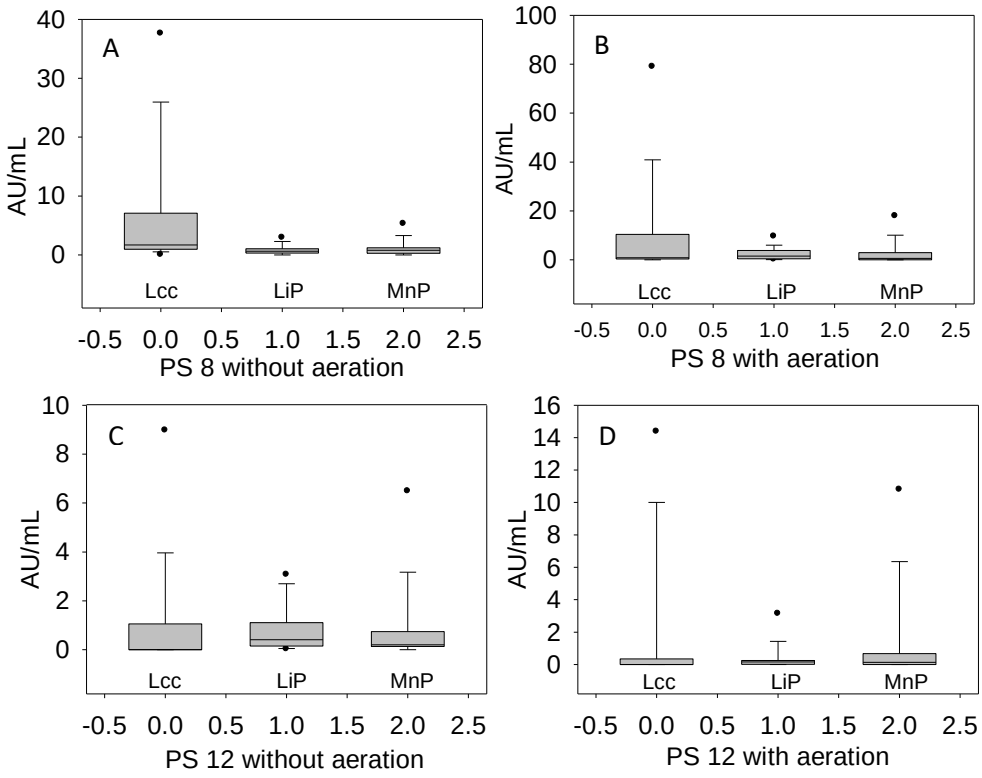

Fig. 1. Effect of particle size (PS 8 and 12) in the production of ligninolytic enzymes by *T. polyzona* HHM001 when CLR is used in SSF, without, and with aeration.

3.3. Effect of substrate height inside the column on enzymatic activity

The lignocellulolytic enzymatic activity did not remain constant along the glass column utilized for the SSF. The greatest enzymatic activities were detected at the bottom and top of the column for enzymes such as Lcc, LiP, and MnP, which are favored by the oxidative conditions of the environment. In these zones, there is probably greater availability of oxygen, which affects the catalytic activity of these enzymes. In the intermediate zone of the column (5-10 cm), air diffusion decreases over time since fungus growth avoids air passage, significantly affecting the enzymatic activity of the fungus. Even when hyphal growth in the entire column was observed, when extracting samples at different heights, the enzymatic activities showed, in some cases, significant differences. The cellulolytic activity was higher at the top zone of the column (10-15 cm) with 34 AU/mL after 8 days of fermentation (Fig. 3A); as regards the xylanolytic activity, the highest activity was observed in the bottom zone of the column (0-5 cm) with 26 AU/mL (Fig. 3B).

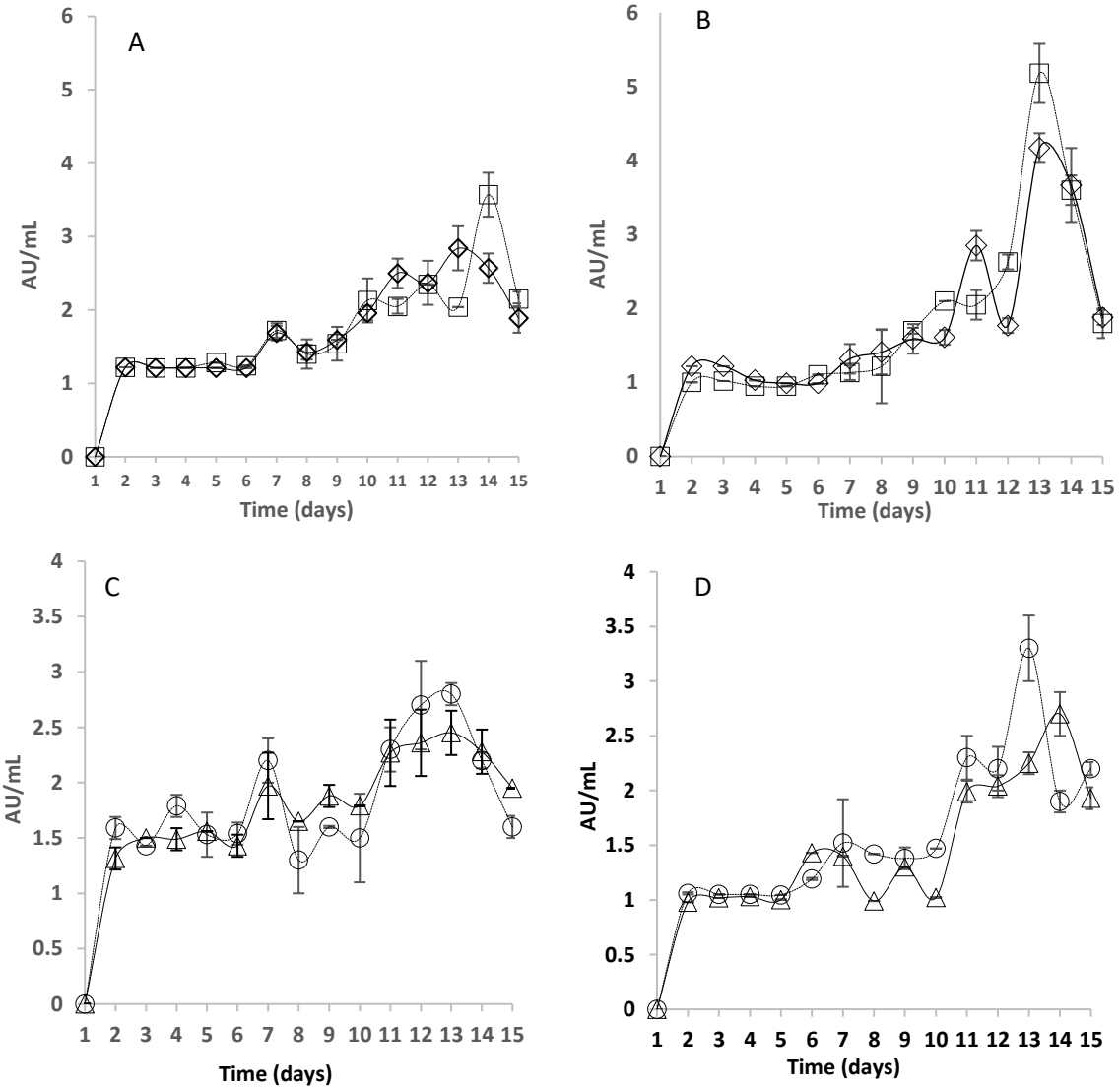

Fig. 2. Effect of PS in the production of cellulases and xylanases of *T. polyzona* HHM001 when CLR is used with a substrate in SSF. A = PS 8 (cellulytic activity); B = PS 8 (xylanolytic activity); C = PS 12 (cellulolytic activity); D = PS 12 (xylanolytic activity); ◊ = Without aeration (PS 8); □ = With aeration (PS 8); Δ = Without aeration (PS 12); ○ = With aeration (PS 12).

Regarding the Lcc activity, the highest activity was observed in the lower zone of the column at 12 days of fermentation with 126 AU/mL. In the upper zone, 113 AU/mL was detected at 10 days of fermentation without significant differences (Fig. 4A). On the other hand, the LiP activity presented its maximum activity values on day 12 of fermentation in the upper zone with 25 AU/mL. In the column lower zone, 23 AU/mL were detected (Fig. 4B).

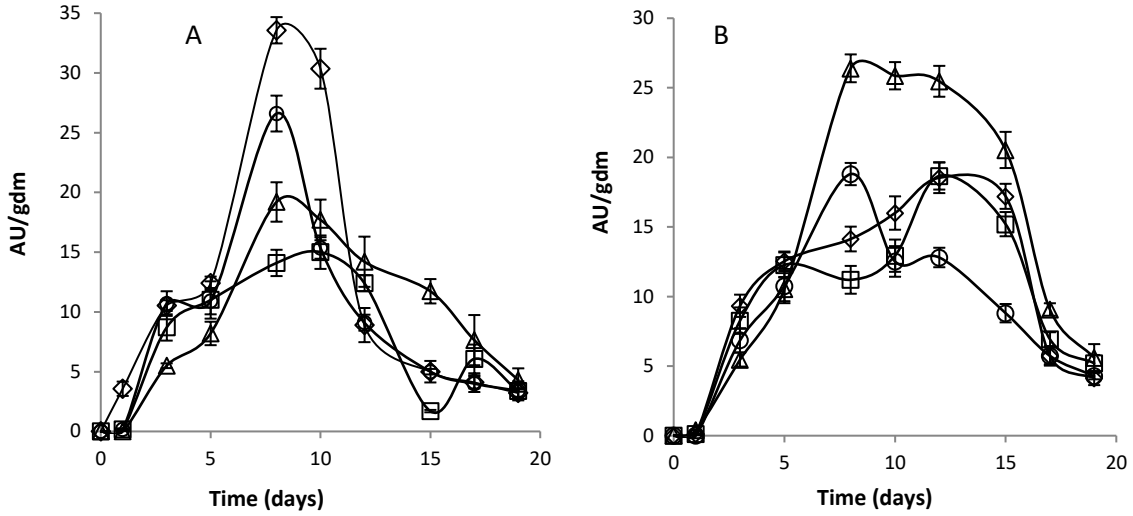

Fig. 3. Cellulolytic (A) and Xylanolytic (B) enzymatic activity in different sections of the fermentation column. ◊ = Column surface (10-15 cm); □ = Intermediate area of the column (5-10 cm); Δ = Column bottom (0-5 cm); ○= homogenized substrate.

Regarding MnP activity, the catalytic activity was slightly higher at the bottom of the column with 26 AU/mL on day 15 of fermentation (Fig. 4C). When comparing all the results, it is evident that in the middle zone of the column, the lowest enzymatic activities prevailed regardless of the type of enzyme.

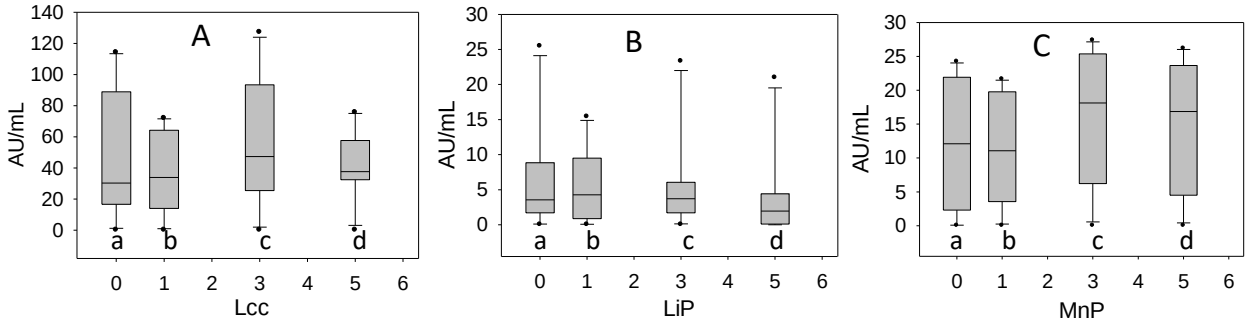

Fig. 4. Production of ligninolytic enzymes of *T. polyzona* HHM001 in solid-state fermentation, using CLR as a substrate in different column sections. A = Laccase; B = LiP; C = MnP; a = Column surface (10-15 cm); b = Intermediate area of the column (5-10 cm); c = Column bottom (0-5 cm); d = homogenized substrate.

4. DISCUSSION

The basidiomycete fungus *T. polyzona* HHM001 is commonly known as a stick ear because of its fruiting body shape. This fungus was previously identified as *Coriolopsis polyzona*; however, a study of the phylogeny of *Trametes* and related genera confirmed by molecular analysis that the genus *Coriolopsis* is part of the *Trametes* species (Justo & Hibbett, 2011; Welti *et al*., 2012). *T. polyzona* is a white-rot fungus that has been used in a wide variety of biotechnological processes (Cerrón *et al*., 2015; Zeng *et al*., 2017; Agrawal *et al*., 2018), and has also been reported to be capable to produce Lcc and MnP (Lueangjaroenkit *et al*., 2018; Lueangjaroenkit *et al*., 2019).

Prior characterization of plant residues is essential since it is necessary information to estimate the potential of the substrates to be used. The values obtained for the three main CLR biopolymers were 41.4 %, 24.3 %, and 11.85 % for cellulose, hemicellulose, and lignin, respectively. These results coincide with those reported for cellulose (30.35-45.5 %), hemicellulose (14.68-31.08 %), and lignin (6.77-16.1 %) in works where corn residues have also been used (Costa *et al*., 2015; Wang *et al*., 2019; Yu *et al*., 2019). The analysis of the chemical composition of the CLR indicates that they can be an ideal substrate to produce lignocellulolytic enzymes used, in this case, *T. polyzona*

It has been described that aeration conditions favor ligninolytic activity; in *P. chrysosporium*, higher production of ligninolytic enzymes was observed at higher aeration flows (Gassara *et al*., 2013), where the oxygen present can increase the growth of filamentous fungi (Durand *et al*., 1993; Quintanar-Gómez *et al*., 2012; Thomas *et al*., 2013). On the other hand, independent of the presence of air or not, the highest activity values were obtained for treatment with PS 8 (Figure 1); this can be associated with several factors, such as the availability of substrate since the larger particle size, the penetration of hyphae in the substrate is more difficult (Wan and Li, 2012), so it is possible that under the conditions tested, *T. polyzona* HHM001 was forced to secrete a greater amount of enzymes to access carbon sources and energy. Membrillo *et al*. (2008) reported similar behavior for two strains of *P. ostreatus* (IE-84 and CP-50) after analyzing three particle sizes (0.92 mm, 1.68 mm, and 2.9 mm), observed that the higher production of ligninolytic enzymes was obtained with the largest particle size.

it has also been observed that in smaller PS, there is more nutrient availability because lignocellulose fibers are more exposed, inducing the production of the enzymes involved in their degradation (Yu *et al*., 2019); however, in SSF processes, it is essential to choose an adequate PS to avoid the formation of preferential air flows and an excess of water loss. From the results obtained in this work, it was observed that with PS 12 (1.7 mm), the enzymatic activity was generally lower, probably due to the system heterogeneity, an excessive packing density, and the formation of preferential flows favored by mycelial growth. The PS and the air space between particles possibly impact the production of Lcc, LiP, and MnP. When comparing the PS used (8 and 12), the lower PS (12) has a higher packing density, therefore, greater compaction, which prevents optimal aeration of the substrate. Zadrazil & Puniya (1995) reported for *Pleurotus* sp. P7, *Agrocybe aegarita* A1, *Pleurotus eryngii*, *Pleurotus* sp. P1 and *Kuehneromyces mutabilis*, that the decrease in PS could negatively affect air circulation, negatively affecting ligninolytic enzyme production. Sarikaya & Ladisch (1999) determined that the vegetative growth of *P. ostreatus* is better at a PS of 0.42 mm, while the degradation of lignocellulose is high with a PS of 0.84 mm. In corn wastes, *Cireporiopsis subvermispora* has low lignin degradation at a PS of 15 mm

compared to a PS of 5 to 10 mm (Wan and Li, 2010). Quintanar-Gómez *et al*. (2012) reported that for *Trametes* sp 44, the production of ligninolytic enzymes was less a PS 4 (4.76 mm) and is favored for a PS 8 (2.37 mm); these results are like the ones obtained in this study for the same PS 8 compared to PS 12 which is smaller. Likewise, maintaining constant humidity and temperature levels is among the functions performed by the air supply (Quintanar-Gómez *et al*., 2012; Wan & Li, 2012). It should be mentioned that the colonization of the fungus understudy was more evident from the third day of cultivation in the treatments tested, and this was increasing until 15 days of the process, so the decrease in Lcc activity may be due to the depletion of the substrate or due to space limitations (Quintanar-Gómez *et al*., 2012).

In addition to aeration and PS, another fundamental variable is the substrate type since its complexity and chemical composition influence white-rot fungi metabolic activities. Quintanar-Gómez *et al*. (2012) reported that the quantification of catalytic activity depends on residue type. It should be noted that the lignin and hemicellulose content of the CLR used by these authors (lignin 11.85 %, holocellulose 65.64 %) differs from those reported by this author (lignin 19.52 %, holocellulose 43.05 %) even so, the secretion profile is like that reported by them, observing that the sequence of secretion of the enzymes was Lcc, LiP, and MnP using *Trametes* sp. 44. Vyas *et al*. (1994) found a different enzymatic profile when growing *T. polyzona* on wheat straw where the fungus understudy in the first instance secretes MnP followed by the action of a peroxidase independent of Mn. Lcc and LiP activity was not detected. It should be noted that the PS used by these authors was 20 to 40 mm, and after 25 days of fermentation, the activities reported were 22 AU/mL of MnP and 3 AU/mL of Lcc. These results suggest that the origin of lignocellulosic residues is fundamental for expressing the different lignocellulolytic enzymes.

In contrast, Elisashvili *et al*. (2008a), when using as a substrate maple leaves, wheat straw, tangerine peel, and banana peel with PS from 0.2 mm to 10 mm, for the growth of *Coriolopsis polyzona*, the Lcc activity oscillated between 27 and 290 UA/mL that are similar results to those found in this work. On the other hand, the MnP activity for the same residues ranged between 31 and 308 AU/mL, while in CLR, it was 9 AU/mL. The enzyme profile and the amount of enzyme secreted by *T. polyzona* HHM001 are influenced by aeration conditions, PS, and substrate type. The production of ligninolytic enzymes during the growth of *T. polyzona* HHM001 on CLR does not show a defined pattern, that is, a trend of increase or decrease in their production. This result coincides with what was described by Mendoza (2012), who mentioned that the ligninolytic enzymes produced by basidiomycete fungi occur in pulses and depend on the type of substrate and fungus studied. The enzymes produced by *T. polyzona* HHM001 during the production of Lcc, LiP, and MnP show this type of behavior (Fig. 1), which can be attributed to the type of substrate used and the presence of intermediaries coming from the depolymerization of lignin that potentiates the activity of this type of enzymes (Torres & Sáe, 2003; Elisashvili *et al*., 2008a). It should be mentioned that under the conditions tested, the activities produced by the basidiomycete under study are greater than those reported for *Ganoderma appanatum*, *T. versicolor*, *Phlebia rufa*, and *Bjerkandera adusta*, where the maximum activity Lcc, LiP, and MnP reported for these fungi was: 0.35 AU mL, 0.5 AU/mL and 3 AU/mL, respectively when grown on wheat straw, it is observed a similar behavior using the same type of substrate but for *Irpex*

lacteus and Iuc-1 with ligninolytic activities ranging between 0.12 and 0.9 AU/mL (Dias *et al*., 2010).

The production of hydrolytic enzymes by white-rot fungi has been less studied than lignin-degrading enzymes. According to the results obtained, Cel and Xyl are not influenced by aeration (Fig. 2) since no significant differences are observed in the conditions tested. Elisashvili *et al*. (2008b) observed that as 11 white-rot fungi grew, the hydrolytic enzymatic activity was proportionally increased; therefore, hydrolytic activity is associated with fungus growth. This type of enzyme can be considered a primary metabolite; therefore, the enzyme activity probably increases concerning growth.

This behavior has also been described by Ibarra *et al*. (2010) for *Trichoderma viride* using sugarcane bagasse as substrate; they report that the production of Cel by this fungus has no significant difference concerning the two aeration flows tested (20 and 30 L/min). Besides, compared to the secretion of ligninolytic enzymes for *T. polyzona* HHM001 hydrolytic enzyme production does not show differences for the two-particle sizes used (PS 8 and PS 12) for these enzymes. Similar results were reported by Botella *et al*. (2007) when testing different particle sizes (0.74 mm, 0.063-1 mm, 1-1.6 mm) for *Aspergillus awamori* using grape pomace as substrate, they observed that there is no significant difference in the production of hydrolytic enzymes by this fungus, however, Quintanar-Gómez *et al*. (2012) mention that in Xyl and Cel production by *Trametes* sp. 44, it is affected by aeration.

On the other hand, under the conditions tested, the hydrolytic activity of *T. polyzona* HHM001 showed a minimum variation of 2.8 to 5 AU/mL. When comparing this result with those reported by other authors, results are very similar to those obtained in this work, and it has been described that in the case of *C. polyzona* were observed at 5 AU/mL and 3 AU/mL; 9 AU/mL and 7 AU/mL; 16 AU/mL and 17 AU/mL; 21 AU/mL and 23 AU/mL for Cel and Xyl enzymatic activity grown on wheat straw, tree leaves, apple peel, and banana peels respectively (Vyas *et al*., 1994). It should be noted that these activities were reported for particles with sizes between 0.2 and 10 mm; also, these same authors mention that fruit residues favor the production of xylanase and cellulase enzymes not only for *C. polyzona* but for several white-rot fungi due to high content of free sugars. The lignocellulolytic enzyme production can be induced by the type of substrate used, or by nitrogen sources; for example, for *C. polyzona*, the addition of peptone induces the Cel, and Xyl activity in different lignocellulosic residues (Vyas *et al*., 1994). In this sense, under the conditions tested in this work, no nitrogen source was added to the substrate used in addition to the fact that compared with other residues, the corn leaf contains less sugar; however, it should be noted that the activities quantified are greater than those reported for *Bjerkandera adusta*, *Ganoderma aplanatum*, *Phlebia rufa*, *I. lacteus,* and Euc-1 (Dinis *et al*., 2009; Dias *et al*., 2010) with hydrolytic activities ranging between 0 and 0.25 AU/mL when grown on wheat straw. Enzymatic activities like those produced by *T. polyzona* HHM001 have been reported for *Ganoderma erytropus*, *Pleurotus betulinus*, *T. versicolor* IBB 16, *P. ostreatus* IBB 10, *Fomes fomeius* IBB 38, and *Trametes pubescens* IBB 11 when grown on bran of wheat, grape pomace, maple leaves, and wheat straw respectively where enzymatic activities were reported below 7 AU/mL (Elisashvili *et al*., 2008b; Vetrosvky *et al*., 2012).

Rivela *et al*. (2000) found that the bioreactor configuration is fundamental to obtaining the maximum production of ligninolytic enzymes. In traditional tray fermenters with no possibility of mechanical mixing, the substrate height is essential to obtain the enzyme's

highest production. Brijwani *et al*. (2010) reported that to produce Cel by *Trichoderma reesei* and *Aspergillus oryzae* in co-fermentation using soybeans; the optimal substrate height was 1 cm. A similar case was observed to produce Lcc using orange residues and *Trametes hirsuta* (Rosales *et al*., 2007). It has also been observed that when aeration is lacking, increasing the height of the fermentation bed leads to a decrease in the production of enzymes, Vaseghi *et al*. (2013) observed that increasing the height of the substrate bed of 0.5 to 3 cm resulted in a decrease in lipase production. It is important to note that the decrease in enzymatic activity may also be due to the increase in metabolic heat generated during the fungus growth (Vaseghi *et al*., 2013), so its removal through aeration is essential. Therefore, it is important to increase the production of enzymes and, in turn, increase the amount of substrate; it is necessary to apply airflows that favor the production of enzymes and remove the metabolic heat generated by the fungus simultaneously.

The production of ligninolytic enzymes shows differences due to the absence or presence of oxygen when *T. polyzona* HHM001 is grown in CLR at the same particle size. The Lcc enzyme is the one that shows the greatest effect in terms of the amount of quantified enzyme activity being favored under aeration conditions. Also, the production of these enzymes is sensitive to PS. In contrast, Xyl and Cel production did not show significant differences due to PS or the supply or absence of aeration. Observed a marked effect of the height of the substrate on the production of enzymes. The greatest enzymatic activities were observed on the zones of 0 to 5 cm, and 10 to 15 cm were observed being the intermediate zone (5-10 cm) that presented the lower enzymatic activity; hence fermentative processes in SSF without mixing, a maximum height of 10 cm with aeration is recommended for maximum production of lignocellulolytic enzymes. Finally, *T. polyzona* HHM001 showed the capacity to produce lignocellulolytic enzymes, especially in glass columns with the highest cellulolytic and xylanolytic activities. These results indicate that *T. polyzona* HHM001 can be an excellent source of this type of enzyme.

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Conflict of interest

The authors declare no conflict of interest

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