EFFECT OF PARTICLE SIZE AND AERATION ON THE BIOLOGICAL DELIGNIFICATION OF CORN STRAW USING *Trametes* sp. 44

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Straw is an agricultural byproduct that can be utilized to obtain bioethanol without affecting animal or human sustinence. This process involves recovering the sugars and reducing the lignin content present through the use of ligninolytic fungi such as the basidiomycete *Trametes* sp. 44. Fermentation was carried out using particle sizes 4 (4.76 mm, No. 4 sieve) and 8 (2.30 mm, No. 8 sieve), and two velocities of airflow (100 and 200 mL/min). Study results showed that particle size affected the production of hydrolytic enzymes, as particle size 8 favored the expression of cellulases and hemicellulases. In addition, both aeration and particle size affected the expression of ligninolytic enzymes, as it was observed that with particle size 8 and airflow of 200 mL/min, the study detected 63 AU/mL of LiP and 11 AU/mL of MnP. In the case of laccase, the enzymatic activity detected reached 220 AU/mL using particle size 8 and an airflow velocity of 200 mL/min. Statistical analysis indicated that the treatment that produced the highest biological delignification occurred when *Trametes* sp. 44 was grown on corn straw at particle size 4 and airflow of 100 mL/min, conditions that yielded 34% delignification at day 12 of fermentation.

Keywords: Particle size; Velocity of aeration; Trametes; Delignification

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INTRODUCTION

Straw (stover) is the main byproduct obtained from cereal cultivation that is commonly used as an alimentary supplement, bedding for cattle, or a substrate in edible mushroom farming. It is estimated that approximately three billion tons of straw from cereals and other fibrous vegetable materials are produced worldwide each year (Mosier et al. 2005), and that an average of approximately 1.5 tons of straw are produced for every ton of cereal harvested. The fact that world cereal production exceeds one billion tons annually means that approximately 1.5 billion tons of straw are available every year (Howard 2003). Today, the enormous amount of straw and fibrous materials available around the world raises the possibility of utilizing these materials in a variety of biotechnological processes designed to obtain products with higher added value, such as pulp for paper production, cattle feed (Ramos 2003), and biofuels (Hoekman 2009), among others.

The basic structure of vegetable material consists of three biopolymers, whose relative proportions depend on the vegetable of origin, such that the concentration of cellulose varies from 30 to 50 %, hemicelluloses from 19 to 45 %, and lignin from 15 to 35 % (Fang et al. 2010). As one of the principal obstacles that complicate the exploitation of straw is the presence of lignin, several processes have been developed for the purpose of eliminating this biopolymer. During the hydrolysis of cellulose and hemicellulose, the presence of lignin generates compounds that inhibit the fermentation process (Wyman et al. 2005); thus, it is important to eliminate this substance from such vegetable residues. Diverse methods of eliminating lignin have been elaborated for the purpose of making the cellulose more easily accessible so that it can then be hydrolyzed through chemical or biological methods for its subsequent fermentation (Mosier et al. 2005). Though each type of vegetable material requires a different strategy, all methods used to pre-treat such materials pursue the following objectives: (1) improve the extraction of fermentable sugars; (2) prevent the breakdown or loss of carbohydrates; (3) avoid the formation of inhibitory byproducts; and, (4) improve the cost-benefit ratio (Sun and Cheng 2002). Among the most commonly used physical-chemical strategies for eliminating lignin are: vapor explosion, exploding the fibers with ammonium, and humid oxidation (Holtzapple et al. 1991; Mosier et al. 2005; Olsson et al. 2005), or a combination of all three with extraction methods using alkalis (Holtzapple et al. 1991; Mosier et al. 2005; Olsson et al. 2005; Martin et al. 2009; Pedersen and Meyer 2009; Saha et al. 2010), or acids (Esteghlalian et al. 1977; Bura et al. 2002). Also, parallel studies have been conducted on biological processes designed to eliminate lignin from various types of cereal straws utilizing filamentous fungi such as *Pleurotus ostreatus* and *Lentinula edodes* for corn straw (Sermanni et al. 1994), *Pleurotus* sp. for wheat straw (Martínez et al. 1994), *Ceriporiopsis subvermispora* and *Cyathus stercoreus* for vegetable residues (Akin et al. 1996), *Pl. ostreatus* for wheat straw (Adamovic et al. 1998), *Panus tigrinus* for sugarcane bagasse (Goncalves et al. 1998), *Phanerochaete chrysosporium*, *Pleurotus eryngii*, *Phlebia radiata*, and *C. subvermispora* for wheat straw (Dorado et al. 1999), *Phanerochaete chrysosporium* for wheat straw (Chen et al. 2002), *Galactomyces geotrichum* and *Myrothecium verrucaria* for rye straw (Varnaite and Raudoniene 2005), *Trametes versicolor, Bjerkandera adusta, Ganoderma applanatum*, and *Phlebia rufa* for wheat straw (Dinis et al. 2009), and *Irpex lacteus*, also for wheat straw (Dias et al. 2010). All of those procedures showed that the lignin present in the different types of straw was affected by the presence of the microorganisms utilized; however, there are few studies of the biological delignification of corn straw and the possible use of this material as a substrate and source of fermentable sugars. Corn is one of the most important cereals in the world, and its cultivation generates enormous amounts of vegetable residues that have little nutritional value. The basidiomycete fungi from white-rot have the capacity to preferentially break down the lignin in wood, while leaving the hemicellulose and cellulose practically intact, a characteristic that makes them of special interest in biotechnology due to their potential utilization in developing delignification processes for agricultural residues (Cardona and Sánchez 2007).

The research described here used the basidiomycetic fungus *Trametes* sp. 44, which was collected and isolated in a region known as the *Huasteca Hidalguense* in southeastern Mexico, an area characterized by an average annual temperature of 30ºC and an average

annual relative humidity of 95%. The microorganism studied has been identified as both a white-rot and thermotolerant fungus (Cruz et al. 2010). The objective of this research was to study the process of the breakdown of the lignin present in corn straw due to the action of the basidiomycetic, white-rot fungus *Trametes* sp. 44, under two different conditions of airflow (10 and 200 mL/min) and two distinct particle sizes (sieves No. 4 (4.76 mm) and 8 (2.30 mm)), under conditions of solid-state fermentation, in order to determine the expression of hydrolytic (cellulase and hemicellulase) and ligninolytic (Laccase, LiP and MnP) enzymes, as well as the percentage reduction of residual lignin.

EXPERIMENTAL

Materials and Methods

Conservation of the strain and preparation of the inoculum

This study used the *Trametes* sp. 44 strain, isolated from the *Huasteca Hidalguense* (Mexico) (Cruz et al. 2010). The strain was cultured in potato dextrose agar (PDA) at 37ºC for one week, conserved at 4 ºC, and then re-grown periodically so that it would not lose viability. To prepare the inoculum, ingredients were placed in a petri dish with PDA agar and *Trametes* sp. 40 cultivated for one week in aseptic conditions; slices of agar with fungus of approximately 1 cm^2 were prepared with aid of a sterile blade. The slices of agar with the mycelium were then placed in an Erlenmeyer flask that contained sterile water. The mixture was shaken at 150 rpm at room temperature (28 ºC) during one night (12 h). The suspension with fungus mycelium detached from agar was utilized as inoculum. For inoculation, mycelium suspension was mixed well with the substrate under aseptic conditions. The amount of inoculum was a function of initial substrate moisture (9%) and final moisture content of the fermentation (82%). The inoculum was standardized by taking as a base a petri dish with the fungus cultivated for one week for each 100 mL of sterile water.

Preparation of the corn straw

The corn straw (stems and leaves) was donated for the study by producers belonging to an organization called *Maíz del Valle del Mezquital* (México) and was obtained from the March-April 2010 harvest. They were washed twice in hot water and dried in an oven at 100 ºC for 4 h. The vegetable material was then ground by hand and placed in a shaker with sieves (W.S. TYLER) for 10 minutes (Mani et al. 2004). The particles selected were gathered from the number 4 (4.76 mm) and number 8 (2.30 mm) sieves. Maize straw was sterilized before being inoculated (121 ° C, 1.5 atm, 30 minutes).

Solid-state fermentation

To carry out the delignification process cylindrical glass columns 30 cm long x 3 cm in diameter were utilized, following the procedure described by Roussos and Raimbault (1982). Initial moisture by dry weight was estimated, and the inoculum was added to reach initial moisture of the vegetable material of 82 % at pack densities of 5.6 and 7.83 g/cm^3 for the 4 and 8 particle sizes (PS), respectively. Pack density was defined as the useful space available for growth of the fungus.

Obtaining the enzymatic extract

The enzymatic extract was obtained from a 1 g sample (obtained after homogenization of total material each column analyzed) to which 20 mL of sterile water were added with agitation. Extraction time was set at 30 minutes, after which the sample was centrifuged at 10,000 rpm for 3 minutes. The supernatant thus obtained was utilized to carry out the corresponding analyses.

Analytical Determinations

Enzymatic activity of laccase

Laccase activity was determined spectrophotometrically using 2,2'-azino-bis-ethylbenzthiazoline (ABTS) as the substrate, following the procedure described by Takamiya et al. (2008). One unit of laccase activity (AU/mL) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute per mL under the conditions of the assay.

Enzymatic activity of manganese peroxidase (MnP)

MnP activity was determined using phenol red as the substrate and reading the formation of color at 610 nm utilizing a spectrophotometer (Thermo Scientific), according to the protocol described by Kuwahara et al. (1984). One unit of MnP activity was defined as the amount of enzyme that oxidized 1 μmol of phenol red per minute per mL under the conditions of the assay (AU/mL).

Enzymatic activity of lignin peroxidase (LiP)

LiP activity was assayed using veratryl alcohol as the substrate, following the procedure described by Maganhotto et al. (2005), reading the change in absorbance using a spectrophotometer (Thermo Scientific) at 310 nm. One unit of enzymatic activity of LiP was defined as the amount of enzyme that oxidized the change of 1 μmol of veratryl alcohol into veratraldehyde per minute per mL under the conditions of the assay (AU/mL). The samples were diluted to 10 times to avoid the interference of color in the analysis.

Determination of the activity of cellulases and xylanases

Cellulase and xylanase activity were tested using the DNS method, which determines the liberation of glucose and xylose, respectively (Poutanen and Puls 1988). Extracts were incubated during 10 minutes at 40 ºC in an acetate buffer at pH 5.3, 100 mM. The substrates utilized were carboxymethylcellulose and birch xylan at 0.2%, respectively. One unit of enzymatic activity of cellulase or xylanase was defined as the amount of enzyme that hydrolyzed 1 μmol of either carboxymethylcellulose or birch xylan per minute per mL under the conditions of the assay (AU/mL).

Reducing sugars liberated

The modified method described by Miller (1959) was followed. A reaction mixture that contained 500 μL of enzymatic extract and 750 μL of DNS was heated to a boil for 10 minutes, before being left to cool for 20 minutes and taking the reading in a spectrophotometer (Thermo Scientific) at 640 nm. To estimate the amount of sugars

liberated, a standard curve was prepared using a glucose solution at a concentration of 1 g/L and carrying out the appropriate dilutions.

Quantification of residual lignin

To estimate the amount of residual lignin, the procedure outlined in the TAPPI T-222 OS-74 norm was used, which entails quantifying insoluble and soluble lignin in concentrated and diluted acid mediums, respectively. Quantification of acid insoluble lignin was determined from hydrolysis of 3 mL 24 N sulfuric acid and 0.3 g of lignocellulosic material. The mixture was incubated at 30 ° C for 1 h., added 84 mL of distilled water and filtered using glass fiber pore size M (medium). The acid insoluble lignin was determined by dry weight. Acid soluble lignin was quantified from the waste generated by determining the absorbance at 205 nm. The sum of the two results permits the determination of the amount of lignin present in the samples.

Quantification of extracellular protein

The colorimetric method described by Bradford (1976) was used, which involves quantifying the comassie G blue-protein complex at 595 nm.

Quantification of CO_2

Quantification of the CO_2 produced by the respiratory metabolism of the fungus was determined through titration. An air intake system was placed in the upper section of the columns, and the trapped air was then bubbled in a titrating solution of NaOH 0.5 M. On a daily basis, 10 mL of this solution was taken and titrated with a titrating solution of HCl 0.5 M with phenolphthalein added as an indicator. The NaOH that failed to react with the CO_2 produced by the fungus that forms Na_2CO_3 estimated the CO_2 produced indirectly. Prior to entering each column, the line of air was bubbled in a solution of NaOH 1M to capture atmospheric CO_2.

Statistical Analysis of the Data

The experimental results were obtained from independent units and in triplicate. To determine whether particle size and airflow have an effect on the biological delignification of corn straw, the study utilized an analysis of variance (one factor ANOVA) with the significance level set at 5 % ($P<0.05$) and using the SPSS statistical program, version 15.

RESULTS AND DISCUSSION

Expression of Cellulases and Hemicellulases

The basidiomycetic fungus *Trametes* sp. 44 was cultured on corn straw using two particle sizes and two airflows, which showed distinct behaviors. A variety of fermentative breakdown processes for the lignin present in diverse types of straw have been developed, including *Pl. ostreatus*, *L. edodes* (Sermanni et al. 1994), *C. subvermispora*, *Cy. stercoreus* (Akin et al. 1996), *I. lacteus* and Euc-1 (Dias et al. 2010), *C. subvermispora, Phlebia brevispora, Phlebia fascicularia, Phlebia floridensis*, and *Ph. radiata* (Arora et al. 2011) and *Pl. ostreatus* (Adamovic et al. 1998). In none of these cases did the fermentation

temperature exceed 30ºC. Because of its origin, *Trametes* sp. 44 presents an optimal velocity of growth at 37ºC and the capacity to grow at 42ºC (Cruz et al. 2010). There are few reports of basidiomycetic fungus with these physiological characteristics. At this temperature, the fungus was able to colonize the entire substrate in 4 days. Reports have described that *Pl. ostreatus* colonizes a variety of vegetable residues and that its speed of colonization depends on the origin of the residue in question; thus, for example, *Pl. ostreatus* colonizes rice husks in 15 days but corn residues in 30 (Obodai et al. 2003). It is probable that the velocity of growth of *Trametes* sp. 44 directly influences the expression of the enzymes examined in this study. From the results, the expression of cellulases and xylanases was most affected by particle size than by airflow. The highest production of xylanases was observed on the third day of fermentation in the medium with particle size 8 and airflows of 100 and 200 mL/min. Under those conditions, 13 and 12 AU/mL of growth, respectively, were detected. In the case of the cellulases, 20 and 21 AU/mL, respectively, were detected for the airflows of 100 and 200 mL/min, with particle size 8. A lag was observed in the production of cellulases when the fungus was cultured in the medium with particle size 8 and airflow of 200 mL/min; under which conditions the maximum activity of cellulase was observed on day 8 of fermentation (Fig. 1).

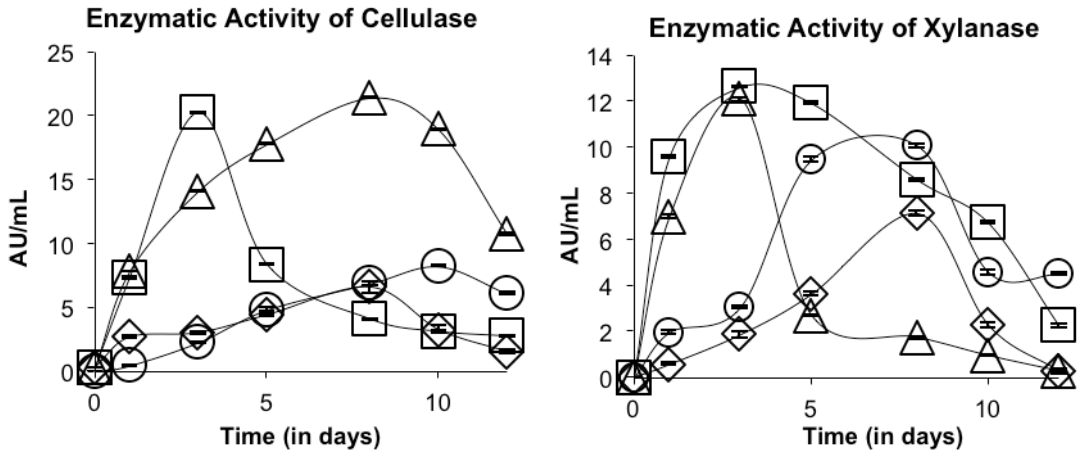

Fig. 1. Production of extracellular hydrolytic enzymes under the conditions assayed. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; □ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

With respect to basidiomycetic fungi such as *I. lacteus* and Euc-1, it has been reported that the expression of cellulases and xylanases during colonization of wheat straw can reach 0.08 AU/mL (Dias et al. 2010). Upon comparing these results, the enzymatic activity manifested by *Trametes* sp. 44 turns out to be 250 times greater, a finding that may be related to its velocity of growth. It has also been reported that *Trametes* presents a high expression of hydrolytic enzymes, such as cellulases and hemicellulases (Valmaceda et al. 1991), and that the hydrolytic activity of this type of enzyme could increase the degradability of the cell walls of straw treated with fungi (Agosin et al. 1986). Moreover, basidiomycetic fungi perform the rupturing of the lignin-carbohydrate complex of vegetable residues (Dias et al. 2010). These characteristics may favor the liberation of sugars into the extracellular medium through the enzymatic activity of the basidiomycetic

fungi, and could be associated with the liberation of sugars into the extracellular medium (Fig. 2). The results obtained indicate that particle size and airflow favor the liberation of the reducing sugars produced by the fungus hydrolytic activity, because on the third day of fermentation in the case of particle size 8 mesh, 13.5, and 18 mg/mL of reducing sugars were detected for the conditions of 100 and 200 mL of air/min, respectively. The higher expression of enzymes and presence of reducing sugars may be attributed to the greater availability of the substrate, that is, the smaller the particle size, the greater the contact area and the higher the accessibility to the substrate. Ghizzi et al. (2010) found that a particle size below 50 μm equally favored the extraction of both carbohydrates and lignin, while Zhu et al. (2009) reported that an appropriate reduction in the particle size of the biomass increases the accessibility of lignin and carbohydrates.

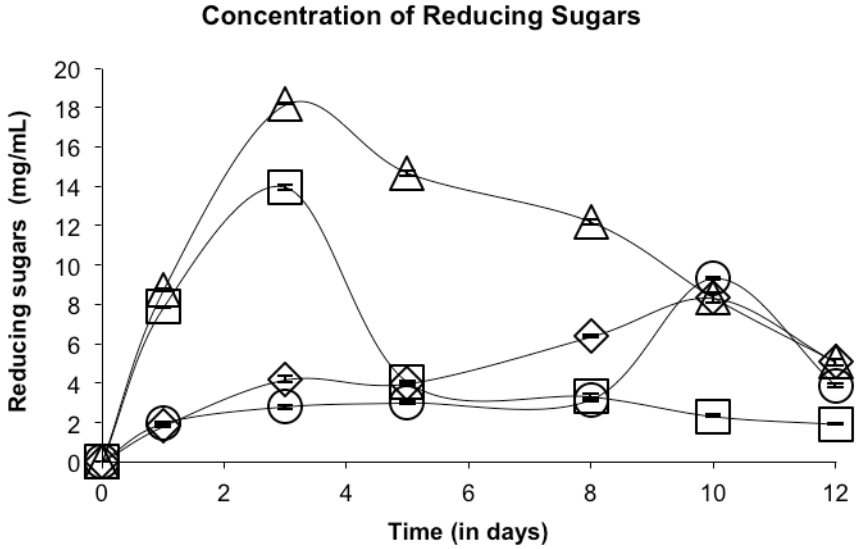

Fig. 2. Total reducing sugars present in the extracts under the different conditions assayed. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; □ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

The pack density in the column is an additional important factor in the fermentation process, as it was seen that particle sizes below 2.3 mm lead to compacting of the corn straw and generate problems of oxygen transference; while, on the other hand, very large particle sizes (>4.76 mm) favor oxygen transference but increase water loss from the solid matrix. Similar results to those obtained in this study have been described: Mani et al. (2004) observed that a smaller particle size increases the pack density and thus affects oxygen transference. In the column with particle size 4, observations detected a lower pack density of 5.6 g/cm^3 that increased airflow through the solid matrix; however, it also produced a greater amount of water loss in that matrix. The low enzymatic activity units detected in both the cellulases and xylanases at particle size 4 are indicative of a low level of hydrolytic activity of *Trametes* sp. 44, which could favor the fungus ligninolytic activity. Reports have described that during processes of solid fermentation moisture plays an extremely important role because an excess of moisture produces anaerobic conditions in the substrate, while low moisture levels reduce the microorganisms' metabolic activity

(Zadrazil and Brunnert 1981). Another factor that may affect water loss could be the water-retaining capacity of the solid matrix. Corn straw shows a low capacity for water absorption that leads to a greater loss of moisture when airflow velocity is increased. The reduced moisture of the substrate can be seen in Fig. 3, which shows that at PS 4 water loss in the substrate is more pronounced than at PS 8, an effect that definitively influences the growth of *Trametes* sp. 44.

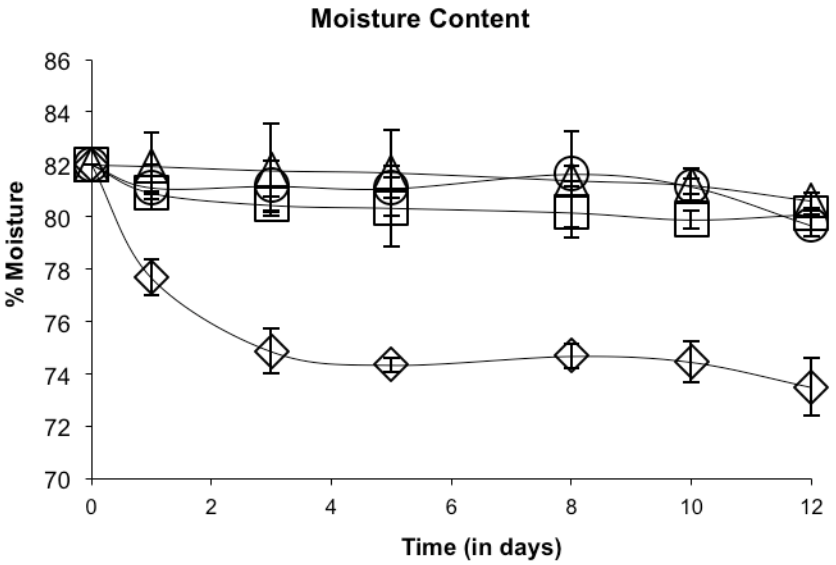

Fig. 3. Moisture present in the samples analyzed. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; □ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

Expression of Ligninolytic Enzymes

An elevated expression of the laccase enzyme was observed at the beginning of fermentation with PS 4 at airflow 200 mL/min, 210 AU/mL were reached. Similarly, but with PS 8 under the same conditions of airflow, it was at 5 days of fermentation that the highest expression of the laccase enzyme was observed: 225 AU/mL (Fig. 4).

The expression of this enzyme is subject to catabolic repression in conditions of liquid culture, and in *Trametes pubescens* the expression of the laccase enzyme begins once glucose has been consumed in its totality (Galhuap et al. 2002). In addition, Mikiashvili et al. (2005) described that the laccase of *Trametes versicolor* is expressed with high enzymatic activity in presence of glucose and cellobiose at concentrations of 10 g/L. It has been determined that in conditions of solid-state fermentation, filamentous fungi have the ability to support large concentrations of carbohydrates, thus minimizing the effects of catabolic repression (Aguilar et al. 2001). The high level of cellulase and xylanase activity detected in the experiments conducted likely favored the hydrolysis of the cellulose and hemicellulose present in the corn straw, thus liberating sugars that probably influenced the growth of the fungus and the expression of the laccase enzyme, but did not affect catabolic repression.

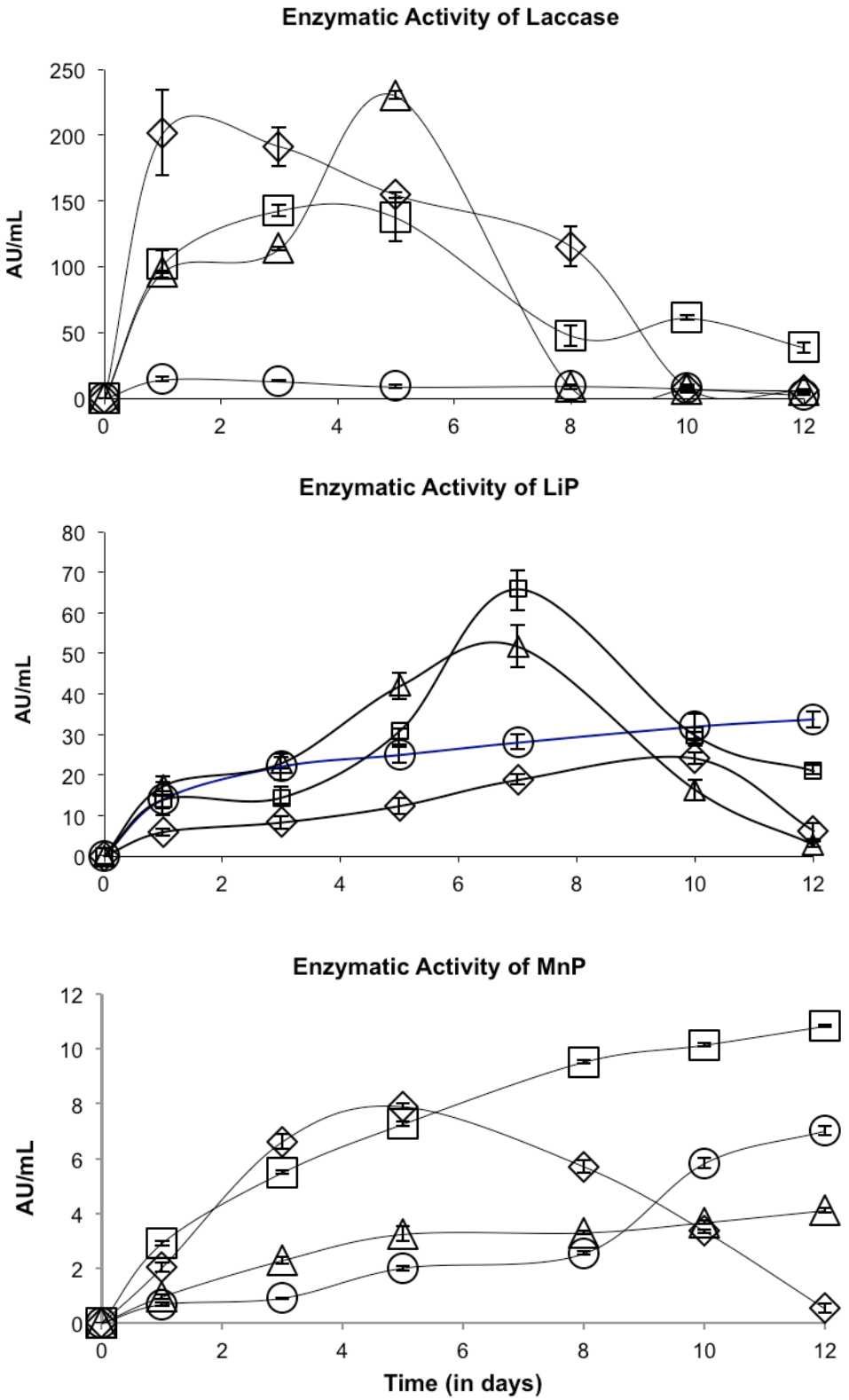

Fig. 4. Production of ligninolytic enzymes under the conditions assayed. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; ☐ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

Similarly, the conditions of aeration favored the activity of this enzyme because, as is well known, O_2 is the final accepter of electrons from laccase and may be an indicator that *Trametes* sp. 44 attacks lignin and the carbohydrates present in corn straw, as described by Zafar et al. (1989). Similar results have been described, but for wheat straw, where it has been demonstrated that *T. versicolor* carries out the breakdown of model phenolic molecules (Kawai et al. 1988) and the partial breakdown of lignin in corn straw (Lapierre and Monties 1989). With respect to the production of LiP (Fig. 4), the particle size of the substrate exercises a marked effect, as there was a higher expression of LiP during the culture of *Trametes* sp. 44 at PS 8 than at PS 4. The production of lignin peroxidase showed well-defined maxima; for PS 8, maximum LiP production occurred on day 7 of fermentation, and it was higher at airflow of 100 mL/min with 62 AU/mL.

The behavior observed in the expression of LiP is different from that described by Dias et al. (2010) and Hwang and Song (2000), as they observed fluctuations in the expression of LiP over time. Studies have described that LiP production is strongly influenced by the C/N relation (Tien and Kirk 1988); however, it is likely that the airflow accelerated the growth process of the fungus and, therefore, hydrolysis of the constituents of the corn straw, thus allowing the liberation of nitrogenated compounds that favor the expression of LiP. Similar results were observed with the enzyme MnP (Fig. 4). It has been reported that this enzyme has a marked influence on the delignification process, specifically in lignocellulosic residues, where MnP causes the solubilization of lignin (Pérez and Jeffries 1990). The results obtained suggest a gradual increase in the expression of MnP, except in the PS 4 condition at 200 mL of air/min, where it is likely that the drying effect of the substrate is more marked, due to the low pack density. This result coincides with the process of breaking down lignin, as it was the condition in which the lowest delignification was found. The expression of MnP may be associated with the reduction of lignin in corn straw, just as occurs in wheat straw (Dorado et al. 1999), which means that MnP is a predominantly ligninolytic enzyme (Dias et al. 2010; Arora et al. 2011). The tendency observed with respect to MnP production could indicate that *Trametes* sp. 44 continues to utilize the substrate for its growth, result that is reinforced when the amount of CO_2 produced during the fermentation process is quantified, as it is a product of the fungus respiratory process. Figure 5 shows that *Trametes* sp. 44 continues its growth process up to day 12 of fermentation, because CO_2 production manifests a tendency to continue increasing.

Valmaceda et al. (1991) observed that during the growth of *P. ostreatus* and *T. versicolor* on wheat straw, CO_2 production occurred on day 7 of fermentation, followed by a decline, which suggests that during the first days of fermentation, the fungi grow at their maximum velocity in order to rapidly invade the substrate; a similar process can occur with *Trametes* sp. 44, though this fungus continues its growth process up to day 12 of fermentation. Jaszek et al. (2006) reported a gradual reduction in the activity of the MnP produced by *P. chrysosporium* after 10 days of fermentation on wheat straw, and that the delignification process ended a few days later; however, in the case of *Trametes* sp. 44, the fungus continued growing after 12 days of fermentation, so that MnP production continued actively.

The activity of this enzyme is probably associated with the presence of manganese in corn straw. While the amount of ash in corn can vary greatly, it is generally estimated to

be around 6% (Carrasco et al. 2011). The ash in corn straw is made up of micronutrients that are required to activate various enzymes. Among those micronutrients we find manganese and other metals (Xu 2010), so the presence of Mn ions could favor the expression of MnP, as it has been reported that the presence of Mn^{+3} ions oxidized by MnP favors the delignification process by attacking phenolic compounds in the structure of the lignin (Kerem and Hadar 1995).

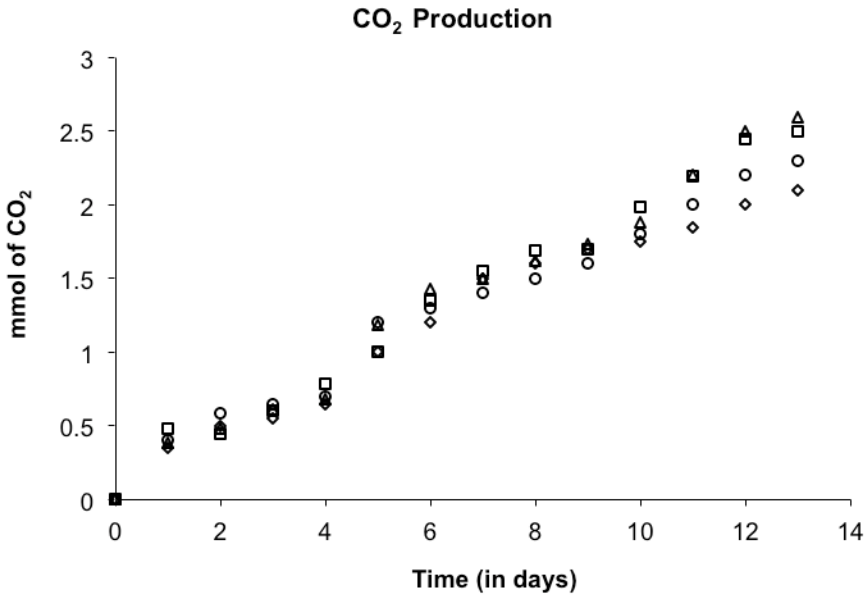

Fig. 5. CO_2 production during growth of *Trametes* sp. 44, a product of the respiratory process. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; □ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

The Delignification Process

The initial content of lignin total raw material did not vary significantly in particle sizes tested, for the particle size 4 total initial lignin was 17.56%, and the particle size 8 total initial lignin was 17.79%. This small difference can be attributed to the increased availability of substrate due to the increased surface area of particles. Table 1 shows the initial composition of the biomass used in this study.

Table 1. Initial Composition of Substrate (maize straw) before being Subjected to Fermentation Process

Particle size	Initial Moisture (%)	Lignin soluble in acid (%)	Lignin insoluble in acid (%)	Lignin Total (%) (g dry matter)
Particle 4	9	0.26	17.30	17.56
Particle 8	9.2	0.32	17.47	17.79

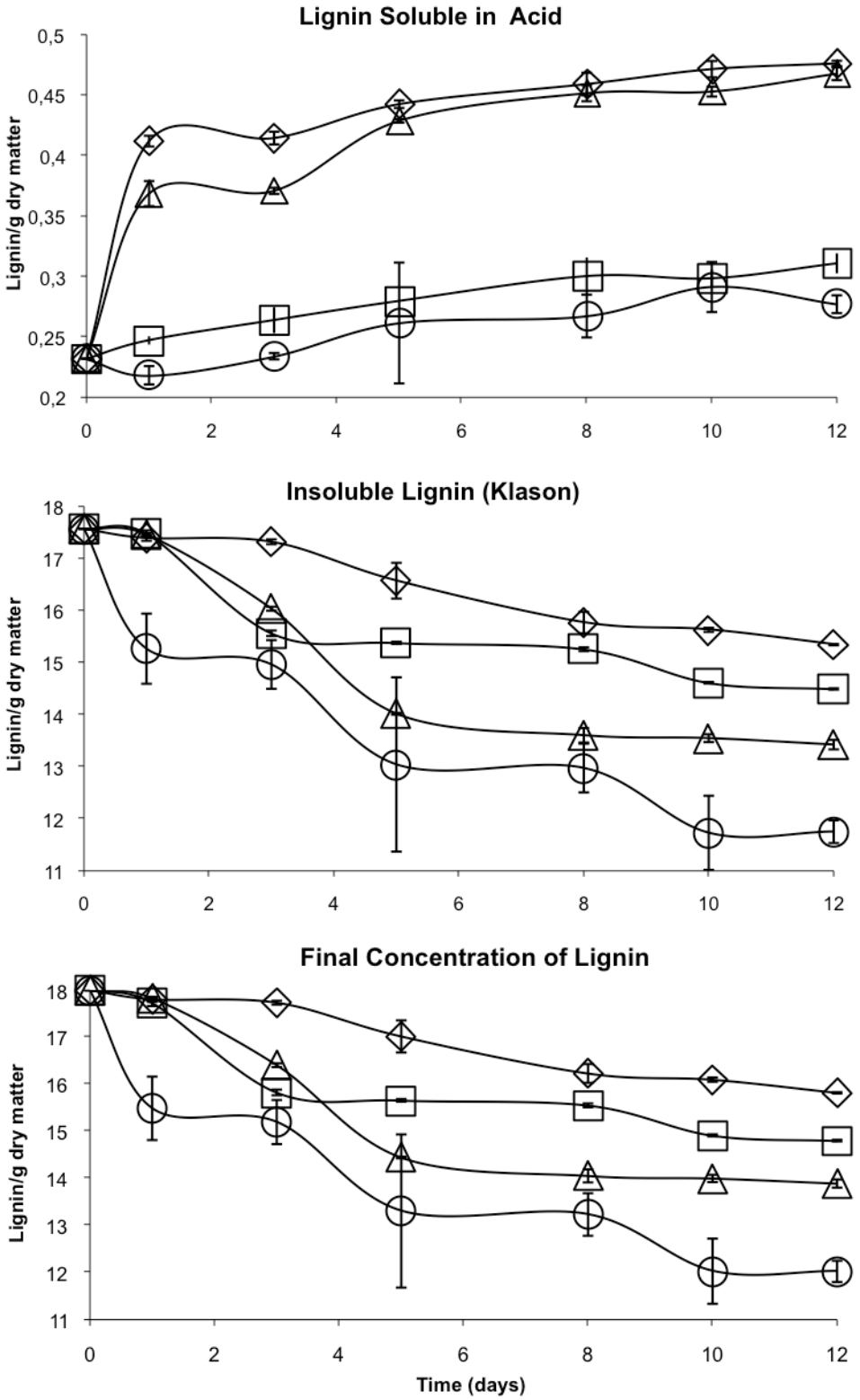

Fig. 6. Behavior of lignin under the different conditions assayed. ○ = Particle size (PS) 4, 100 mL of air/min; ◊ = Particle size (PS) 4, 200 mL of air/min; □ = Particle size (PS) 8, 100 mL of air/min and △ = Particle size (PS) 8, 200 mL of air/min

Figure 6 presents the results obtained during the delignification of corn straw. The results show a marked increase in the breakdown of lignin after the first 24 hours of fermentation due to the presence of soluble lignin. Also apparent is an upwards tendency over time, but only in the condition of the airflow of 200 mL of air/min for both PS 4 and PS 8, illustrating that the aeration conditions may contribute to the process of breaking down the lignin. It has been suggested that an increase in the concentration of soluble lignin is indicative of a rise in the digestibility of the fibers of lignocellulosic materials (Agosin et al. 1985; Valmaceda et al. 1991), in addition to serving as an indicator of the process of delignification. This result is corroborated upon observing that there was a reduction in the amount of Klason lignin and total lignin present in the corn straw. Given that the breakdown of lignin is a strictly oxidative process (Villas-Boas et al. 2002), the presence of oxygen favors its elimination. In addition, atmospheric pressure also plays an important role in breaking down this biopolymer; *i.e.*, conditions in which the partial pressure is equal to the atmospheric pressure favor the breakdown of lignin (Kirk et al. 1978). Because the fermentation process in this study was carried out at atmospheric pressure, conditions favored the breakdown of lignin. Upon comparing the percentage of the breakdown of lignin, it can be seen that after 10 days of fermentation there was a reduction in the amount of lignin from 17.56 % of initial content to 11.73 % in the best-assayed condition (*i*.e., PS 4 at 100 mL of air/min). These results are similar to those obtained in delignification processes involving wheat straw that used *C. subvermispora*, *Pl. eryngii*, and *Ph. radiata*, where a reduction of lignin from 19 % to 12 % was achieved, though in fermentation periods of 15-to-30 days (Dorado et al. 1999). Valmaceda et al. (1991) detected reductions of lignin in wheat straw of 44 % and 31.5 %, respectively, after 60 days of fermentation, when *T. versicolor* and *P. ostreatus* were used. Sermanni et al. (1994) found that *Pl. ostreatus* attacks the lignin present in corn straw after three weeks of fermentation and reduced the lignin present by as much as 30 % after 4 weeks.

That same study showed that *L. edodes* initiates the delignification process after 7 days, reducing the lignin content by up to 20% at 11 days of fermentation, while after 7 weeks the percentage of breakdown rose to 43% of the lignin present in the corn straw. *Trametes* sp. 44 showed the ability to attack the lignin in corn straw from the beginning of fermentation, achieved a breakdown rate of 34% at 12 days of fermentation and showed a tendency to continue breaking down the lignin. The results show a decrease of 33% lignin, but also a tendency for the fungus to the degradation continues. Likely to increase the percentage of delignification need more fermentation time or smaller particle and yet the amount of lignin is high and possibly interfere with fermentation processes.

In the treatment of maize straw on condition of 100 mL of air/min best results were observed for lignin degradation (Fig. 7). By comparing the decrease in total lignin and ligninolytic enzyme expression shows that the enzyme laccase is the enzyme that is expressed in the first place. These results suggest that this enzyme that under these conditions, starts the attack of lignin. Subsequently, other enzymes are expressed ligninolytic completing the process of delignification. In the other treatments tested, the expression of ligninolytic enzymes changes and is subject to both the aeration and particle size.

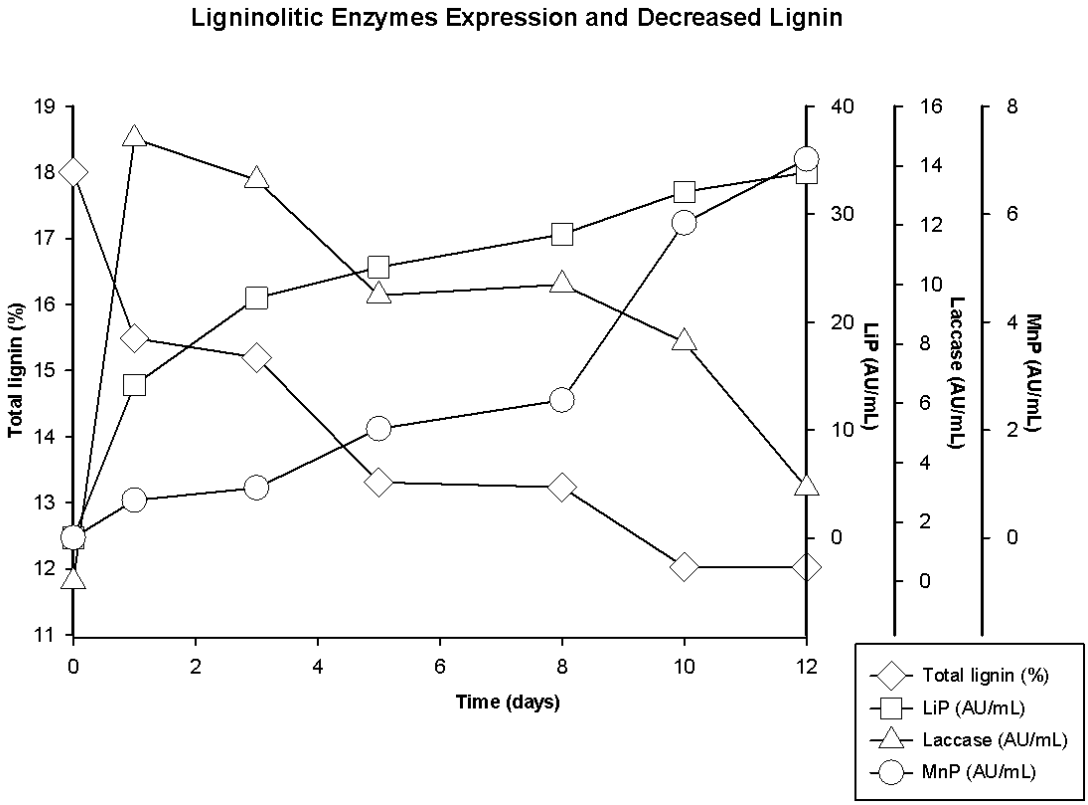

Fig 7. Expression of ligninolytic enzymes (laccase, Lip and MnP) for biological delignification of maize straw in the best condition observed (100 mL air/min and particle size 4).

The data on delignification were subjected to statistical analysis to determine whether statistically significant differences existed among the four treatment regimes used in this experiment; the tests used included Duncan, Dunnet, and Tukey tests, and homogeneity of variances. The results obtained indicated that the best treatment was the condition that used particle size 4 at 100 mL of air/min, with $P \leq 0.05$. In general, all 4 treatments were statistically different, but the results of the treatments at PS 8 were very close to each other. The treatment that showed the greatest statistical difference was PS 4 at airflow 200 mL/min, probably, as mentioned above, because the effect of the drying of the solid matrix was more evident in this treatment and it was the one that obtained the lowest rate of delignification. Statistical analysis showed that the best treatment conditions were PS 4 at 100 mL of air/min, a result that indicates the importance of both particle size and the amount of air present during the fermentation process. When the pack density exceeds 5.6 g/cm^3, problems of oxygen transference arise, while below that level of pack density complications arise due to the loss of water from the solid matrix. The delignification carried out by *Trametes* sp. 44 may involve a synergetic process between, primarily, the LiP and MnP enzymes, due to the fact that in the best delignification condition a progressive increase in the expression of these enzymes was observed over time, and this might favor lignin breakdown. On the other hand, the behavior observed in the delignification process is very similar to results described by other authors, in that a rapid decrease in the amount of lignin present is found early on, but over time the rate of lignin breakdown tends to decrease.

CONCLUSIONS

The breakdown of lignin is an extremely complex process that probably includes synergies among the enzymes involved. The conclusions of this work are as follows:

1. This study showed that *Trametes* sp. 44 is a predominantly ligninolytic fungus with a high capability of producing the laccase enzyme.
2. Also observed was a parallel production of hydrolytic enzymes that under experimental conditions likely favored the growth of the fungus and accelerated the delignification of the substrate. Finally,
3. It has been shown that particle size and airflow favor the expression of hydrolytic (cellulases and hemicellulases, PS 8) and ligninolytic enzymes (laccase, LiP and MnP, 200 mL of air/min), respectively.

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