Production of Lignocellulolytic Enzymes with *Pleurotus ostreatus*-IE8 by Solid Fermentation and its Effect on the Chemical Composition of Sugarcane Bagasse

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Abstract: Sugarcane bagasse (SCB) is infrequently used in feeding ruminants because of its poor nutritional quality. The use of diverse physical, chemical and biotechnological methods for pre-treatment aim to improve its nutritional value. Fermenting SCB with *Pleurotus ostreatus* improves nutrient availability by effect of extracellular fibrolytic enzymes. The objective of this study was to produce lignocellulolytic enzymes with *Pleurotus ostreatus*-IE8 by solid fermentation and its effect on the chemical composition of SCB. The experiment consisted of solid fermentation (SF) of SCB, with no previous physical treatment, and inoculated with *P. ostreatus* strain IE8 with incubation at 25 ºC for 0, 3, 5 and 7 d. At the end of each fermentation period, enzyme activity of cellulose, xylanase and laccase, as well as extracellular protein, was quantified. The SCB chemical composition was determined by analyzing neutral detergent fiber (NDF), acid detergent fiber (ADF), total protein (TP), organic matter (OM) and ash. Data analysis consisted of a completely randomized design and comparison of means with the Tukey test (α=0.05). Enzyme production occurred in different incubation times; the highest production of xylanase was on day 7 (11.8 UI SSi^{-1}), while that of laccase (36.6 UI SSi^{-1}) and cellulase (0.6 UI SSi^{-1}) was on day 5. SCB chemical composition exhibited increments of ADF and NDF when exposed to SF with *P. ostreatus*-IE8. There were no differences in TP, with decreased OM during fermentation and, consequently, the ash fraction increased. Correlations between chemical components and enzyme production indicated that cellulases and laccases correlated negatively with ADF ($p \leq 0.05$) because the components of this fraction are cellulose and lignin. The effects on NDF and ADF are attributed to the conditions in which the SF processes were carried out; fermentation conditions determine the results reported in the literature. The crude enzyme extract of *P. ostreatus*–IE8 can decrease the lignin and cellulose content of the cell wall of fibrous forages such as sugarcane bagasse used to feed ruminants.
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1. Introduction

The cellulose, hemicellulose and lignin components of sugarcane bagasse (SCB) have repercussions in ruminal digestion, making it relatively slow and incomplete (Abdel-Aziz *et al*., 2015). The nutritional value of SCB for ruminants is poor and its inclusion as an ingredient in their diets increases production costs. Diverse studies have focused on elevating its digestibility using chemical and physical treatments and pre-treatments with white rot fungi through solid fermentation (SF) (Yang *et al*., 2002; Okano *et al*., 2007). Fibrolytic enzymes produced in SF are used in partial de-polymerization of fibrous agroindustrial residues for animal feed (Graminha *et al*., 2008). In the process of de-polymerization, cellulose and hemicellulose are hydrolyzed to soluble sugars through the action of cellulases and xylanases (Beauchemin *et al*., 2003). Lignin is heteropolymeric aromatic composed of an aromatic ring with hydroxyl and methoxy functional groups and a propanoid chain (Azadfar *et al*., 2015) and is hydrolyzed by laccases (Liu *et al*., 2008). The genus *Pleurotus* is used in SF because it is flexible in terms of its environmental and temperature requirements. The ability of fungi to degrade lignocellulosic materials is due to their highly efficient enzymatic system (Sánchez, 2009; Khattab *et al*., 2013; Kholif *et al*., 2014). Some studies indicate that treatment of lignocellulolytic material with *Pleurotus* causes changes in their chemical composition, reducing the NDF, ADF, cellulose and hemicellulose fractions associated with the production of fibrolytic enzymes (Márquez *et al*., 2007; Khattab *et al*., 2013; Kholif *et al*., 2014). The objective of this study was to produce lignocellulolytic enzymes with *Pleurotus ostreatus*-IE8 by solid fermentation and its effect on the chemical composition of SCB.

2. Materials and methods

2.1. Experiment microorganism

The *P. ostreatus* strain IE8 was donated by the Centro de Investigación en Ciencia Aplicada y

Tecnología Avanzada of Puebla. The strain was reactivated in agar-malt extract and propagated in an initiating substrate using a mixture of sorghum grain and SCB in a ratio of 80:20, to be used later as inoculum.

2.2. Solid fermentation (SF)

In sterile (15 min, 15 PSI and 121 °C), SCB (85 % moisture) was used with no previous physical treatment. Fifty g of SCB and 2.5 g inoculum were placed in a hermetically sealed bag. The bags were incubated at 25 °C and removed from the incubator after 0, 3, 5 or 7 days of fermentation for analysis.

2.3. Extraction of fungal fibrolytic enzymes and determination of extracellular protein

To measure fibrolytic enzymes and determine extracellular protein (5 independent samples), a crude enzyme extract (CEE) was obtained by macerating the solid ferment for 20 min with distilled water in a ratio of 1:1.5. This was filtered and pressed for centrifugation for 20 min at 12,000 rpm at 4 ºC. Xylanase and cellulose activities were determined with the method of Miller (1959) and that of laccase with ABTS (2,2' azino-bis [3-etilbenzotiazolina-6-sulfónico]) as described by Bressler *et al*. (2000). Extracellular protein was determined following the method of Bradford (1976). Enzyme activity was expressed in international units (IU) per gram of initial dry substrate (iDS) in which one IU was defined as the quantity of enzyme released by 1 µmol xylose or glucose per min under the reaction conditions indicated.

2.4. Chemical analysis of SCB

Chemical analysis determined DM, ash, OM, TP (AOAC, 2007), NDF and ADF (ANKOM Technology Method of Van Soest *et al*., 1991).

2.5. Statistical analysis

The experimental was completely randomized design. Data were analyzed with the GLM procedure of SAS (2011), and means were compared with the Tukey test ($p \leq 0.05$). Correlations between chemical components and enzyme production were analyzed with the CORR procedure of SAS (2011).

3. Results

The enzyme activity of *P. ostreatus* –IE8 on SCB (Table 1). Most of the xylanase activity occurred on day 7 of fermentation (11.8 UI iDS^{-1}). The greatest production of cellulases was quantified on day 5 of SF (0.6 UI iDS^{-1}), compared with days 0, 3 and 7 of SF. Laccase activity exhibited differences ($p \leq 0.05$) on day 3 of fermentation. The largest production of extracellular protein was on day 3 (1.1 mg iDS^{-1}).

Table 1. Fibrolytic enzyme and extracellular protein activity of *P. ostreatus*-IE8 on sugarcane bagasse at different solid fermentation times (D-SF, days of solid fermentation; Xy, xilanases UI iDS^{-1}; La, laccases UI iDS^{-1}; Ce, cellulases UI iDS^{-1}; Pr, extracellular protein mg iDS^{-1}; SF, solid fermentation, UI iDS^{-1}, international units per gram of initial dry substrate; mg iDS^{-1}, milligrams per gram of initial dry substrate; SEM, square mean error).

D-SF	Xy	La	Ce	Pr
0	9.4^{b}	0.8^{d}	0.5^{b}	1.0^{ab}
3	10.3^{b}	36.6^{a}	0.5^{b}	1.1^{a}
5	9.7^{b}	28.8^{c}	0.6^{a}	1.0^{ab}
7	11.8^{a}	33.3^{b}	0.5^{b}	0.8^{b}
SEM	0.23	3.75	0.01	0.35
P-value	0.05	0.05	0.05	0.05

Different letters in the columns are significantly different, with $p \leq 0.05$.

Table 2. Chemical composition of sugarcane bagasse at different times of solid fermentation using *Pleurotus ostreatus*-IE8 as inoculum (D-SF, days of solid fermentation; NDF, neutral detergent fiber; ADF, acid detergent fiber; TP, total protein, DM, dry matter; OM, organic matter, SEM, square mean error).

D-SF	NDF	ADF	TP	DM	OM	Ash
0	68.0^{b}	38.9^{a}	4.1^{a}	96.5^{ab}	88.1^{a}	11.9^{b}
3	73.2^{a}	37.0^{ab}	4.3^{a}	96.7^{a}	86.6^{b}	13.4^{a}
5	72.1^{a}	35.4^{b}	4.4^{a}	96.3^{b}	86.5^{b}	13.5^{a}
7	72.0^{a}	37.5^{ab}	4.5^{a}	96.3^{b}	86.4^{b}	13.6^{a}
SEM	0.47	0.37	0.06	0.05	0.22	0.22
P-value	0.05	0.05	0.05	0.05	0.05	0.05

Different letters in the columns are significantly different, with $p \leq 0.05$.

Table 3. Coefficients of linear correlation between chemical components and fungal fibrolytic enzyme activity (Pr, extracellular protein; Ce, cellulases; Xy, xilanases; La, laccases; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; TP, total protein, OM, organic matter).

	Pr	Ce	Xy	La
DM	0.437*	-0.245	-0.180	0.123
p-value	0.05	0.29	0.46	0.64
NDF	0.034	0.324	0.335	0.926*
p-value	0.89	0.18	0.17	<0.00
ADF	-0.217	-0.521*	0.032	-0.496*
p-value	0.37	0.02	0.90	0.05
TP	0.071	0.189	0.203	0.374
p-value	0.76	0.42	0.40	0.14
Ash	0.044	0.303	0.330	0.702*
p-value	0.85	0.19	0.16	0.00
OM	-0.044	-0.303	-0.330	-0.702*
p-value	0.85	0.19	0.18	0.00

* The correlation between analyzed parameters were considered significant when the p-value ≤0.05.

The bromatological composition of SCB on the days of fibrolytic enzyme production (Table 2). Time 0 was used as the reference to determine the effect of the rest of the incubation times. NDF content on day 0 was less (68.0 %) and increased as of day 3 (73.2 %). ADF content was lower ($p \leq 0.05$) on day 5 of fermentation. Ash increased ($p \leq 0.05$) on days 3, 5 and 7 of fermentation.

The correlation between enzyme activity and SCB chemical composition (Table 3). The celluloses tended to correlate negatively with ADF. Laccases correlated ($p \leq 0.05$) negatively with ADF and OM. In contrast, they correlated positively with ash and NDF. It should be pointed out that xylanases did not correlate with the variables of SCB chemical composition.

4. Discussion

This study highlighted the particle heterogeneity of sugarcane bagasse collected directly from the sugar factory. This heterogeneity favored the production of laccases and xylanases by the strain. The inoculum *P. ostreatus*-IE8 was activated in a sorghum-sugarcane bagasse substrate, which stimulated the production of enzymes, relative to the activation of the same inoculum grown in potato dextrose culture medium as a substrate. The variability of the results in this type of study is due to the conditions of the experiment. Márquez *et al*. (2007), for example, used SCB (0.5-1 cm diameter, 80% moisture) substrate, *P. ostreatus* as the inoculum, 30 °C incubation temperature for 14 d and reported more xylanase (27.4 UI iDS^{-1}) and cellulase (1.0 UI iDS^{-1}) activity but less laccase activity (15.5 UI iDS^{-1}). Also, Verma and Madamwar (2002) reported greater xylanase activity but less laccase activity; with the same substrate and species; they obtained 112 U g^{-1} laccases. Moreover, Roldán-Carrillo *et al*. (2003) obtained 0.1 U mL^{-1} cellulases with SCB, using *Phanerochaete chysosporium* as the inoculum and 3 d of fermentation at 39°C.

P. ostreatus is recognized as a producer of laccases before cellulases (Márquez *et al*., 2007), coinciding with this study. However, Robles *et al*. (2009) obtained more xylanase activity using the same strain and 7 d of SF in agave bagasse (15.2 IU iDS^{-1}) or bean straw (43.4 IU iDS^{-1}), but less laccase activity in both wheat straw (5.5 IU iDS^{-1}) and agave bagasse (0.2 IU iDS^{-1}). The variation in the results is due to the composition of the cell wall of each substrate since straws have lower lignin content than SCB; variations occur even among different bagasses. The type of cell wall component stimulates the production of certain enzymes during SF. The amount and type of enzymes produced during SF depends on particle size, type of substrate, the microorganism, the nature of the inoculum, temperature and duration of SF (Nsereko *et al*., 2000; Park *et al*., 2002; Kumar *et al*., 2008).

Surface area available for *P. ostreatus* colonization and the chemical composition of the available surface are determining factors in cell wall degradation (Din *et al*., 1991). Studies have analyzed chemical changes during the SF process given the production of lignocellulolytic enzymes by white rot fungi (Okano *et al*., 2007; Bastida, 2007; Peláez *et al*. 2008). A common characteristic of these studies was fermentation for more than 30 d, causing a significant reduction in NDF and lignin; this did not occurred in our study because of the short fermentation period (7 d) to which SCB was subjected. Akhtar *et al*. (1997) mentioned that easily assimilated nutrients are first consumed; then cell wall degradation begins by localized erosion of the cell wall layers. Degradation is progressive through the secondary wall layers and middle lamina. The fungus hyphae, through release of laccases, progressively degrade lignin of the secondary wall toward the middle lamina.

Quantification of NDF and ADF of SCB fermented by *P. ostreatus*-IE8 showed increments ($p \leq 0.5$) after the different periods of incubation because the strain initially used soluble carbohydrates and hemicellulose as energy sources before using cellulose and lignin in the mycelial growth phase (Escalona *et al*., 2001; Okano *et al*., 2007). This phenomenon was confirmed by the increase in percentage of ash ($p \leq 0.05$) and, in contrast, by the decrease in OM ($p \leq 0.05$) when quantified by fermentation time, relative to day 0 of SF. TP did not change as SF progressed ($p > 0.05$) since enzyme production was imperceptible in the quantification and, because of fermentation time, neither fungus fructification nor incorporation of protein into SCB occurred.

The effect of the enzymes produced by fungi basidiomycete is directly related to the degradation of the cell wall for energy production (Pinos-Rodríguez *et al*., 2002; Beauchemin, 2003). Therefore, the obtained linear correlation between the production of enzymes (Table 1) and chemical components (Table 2) was analyzed to determine the coefficients of correlation (Table 3). The cellulases and laccases correlated negatively with ADF ($p \leq 0.05$) indicating that the larger the quantity of enzymes, the smaller the ADF fraction due to the type of components that make up ADF (cellulose and lignin).

Under the conditions of this study, it is inferred that solid fermentation of sugarcane bagasse, without physical or chemical manipulation previous to fermentation with P. ostreatus-IE8, favors the activity of laccases, but is unfavorable for the production of cellulases. The crude enzyme extract of *P. ostreatus*–IE8 can decrease the lignin and cellulose content of

the cell wall of fibrous forages such as sugarcane bagasse used to feed ruminants.

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