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Cellulases production from *Aspergillus niger*-ITV-02 using corn lignocellulosic residues

Producción de celulasas de *Aspergillus niger*-ITV-02 a partir de residuos lignocelulósicos de maíz

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Abstract

Cellulases are enzymes used in the hydrolysis of lignocellulosic residues for second-generation (2G) bioethanol production. However, the low availability in the market, and the high-cost impact in the 40 % of the total cost of 2G bioethanol production. Therefore, it is necessary to look for sustainable alternatives for its production. Cellulases are mainly produced by *Aspergillus niger*, and their activity can be affected by nitrogen concentration, the use of surfactants, and the carbon source. The objective of this work was to identify enzymes with CMCase (Cellulase activity of the crude extracts quantified using as substrate carboxymethyl cellulose: CMC) and β-glucosidase activity of *A. niger* ITV-02 from low-cost lignocellulosic residues such as corn and cob stover in two culture media (M1 and M2). The results showed that using corn stover and medium M2 increased the volumetric activity 1.4 and 1.7 times, respectively compared to corn cob and medium M2. Likewise, enzymes with cellulase activity were identified using carboxymethyl cellulose (CMC) and 4- methyl-lumberyl-β-D-glucopyranoside (MUG). The enzymes identified in MUG correspond to a glucoamylase, two β-glucosidases, and an Exo-β-xylosidase. In CMC the following were identified: Exo-β-1,3-glucanase, Endo-β-1,4 xylanase, Arabinosidase, and Endoglucanase. Corn stover does not showed a difference on CMCase and FPUase activity (cellulase activity of the crude extracts quantified using as substrate filter paper: FP) comparted to corn cob. However, improved β-glucosidase activity, suggesting that corn stover is a good raw material to produce cellulases.

Keywords: Cellulase, *A. niger*, glucoamylase, enzyme activity, fungi.

Resumen

Las celulasas son enzimas que se emplean en la hidrólisis de residuos lignocelulósicos para la producción de bioetanol de segunda generación (2G), sin embargo, su poca disponibilidad en el mercado y su alto costo impactan en un 40 % del costo total de producción de bioetanol 2G, por lo que es necesario buscar alternativas sostenibles para su producción. Las celulasas son producidas principalmente por *Aspergillus niger*, y su actividad puede ser afectada por la concentración de nitrógeno, el uso de surfactantes y la fuente de carbono, entre otros. El objetivo de este trabajo fue identificar enzimas con actividad CMCasa (actividad celulolítica del extracto crudo usando como sustrato carboximetil celulosa (CMC)) y β-glucosidasa de *A. niger* ITV-02 a partir de residuos lignocelulósicos de bajo costo como el rastrojo y olote de maíz en dos medios de cultivo (M1 y M2). Los resultados mostraron que usando rastrojo de maíz y el medio M2 se incrementó la actividad volumétrica 1.4 y 1.7 veces, respectivamente comparándolo con el olote de maíz y el medio M2. Así mismo, se identificaron enzimas con actividad celulasa utilizando como sustratos carboximetil celulosa (CMC) y 4-metil-lumberil-β-D-glucopiranosido (MUG). Las enzimas identificadas en MUG corresponden a una glucoamilasa, dos β-glucosidasas y una exo β-xilosidasa. En CMC se identificaron: Exo-β-1,3-glucanasa, Endo-β-1,4 xilanasa , Arabinosidasa, y una Endoglucanasa. El rastrojo de maíz no mostró diferencia con el olote de maíz comparando la actividad CMCasa y FPUasa (actividad celulolítica del extracto crudo usando como sustrato papel filtro: FP), sin embargo, favoreció la actividad β-glucosidasa, sugiriendo que el rastrojo de maíz es una materia prima alternativa para producir celulasas.

Palabras clave: Celulasas, *A. niger*, glucoamilasa, actividad enzimática, hongos.

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1 Introduction

Lignocellulosic residues are heteropolymers composed of hemicellulose (15-35 %), cellulose (30-50 %), and lignin (10-20 %); these residues are an abundant source of biomass (Michelin *et al*., 2014; Barraza-Gonzales *et al*., 2022). Cellulose is a homopolymer made up of glucose chains linked by β-1,4 bonds, which is why it can release glucose. The lignocellulosic residues are pretreated with chemical hydrolysis and then with an enzymatic, which involves a consortium of cellulases that synergistically hydrolyze the β-1,4 bonds of cellulose (Chuck-Hernández *et al*., 2011). The cellulases involved are: Endoglucanase (Endo-β-1,4-glucanase), Exoglucanase (Exo-β-1,4-glucanase), and β-glucosidase. The endoglucanase acts randomly on the amorphous structure of the cellulose inside the cellulose on the β-1,4 bonds of the cellulose, hydrolyzing them and obtaining a greater number of ends which serve for the Exoglucanases to interact on the reducing ends and non-reducing agents of crystalline cellulose releasing cellobiose, which is hydrolyzed by β-glucosidase to obtain glucose (Olkiewicz *et al*., 2020). Cellulases have a wide range of applications in agriculture, detergents, food, and the textile industry, and recently have been used in the second-generation (2G) biofuel industry to carry out the hydrolysis of lignocellulosic materials such as sugarcane bagasse, sorghum, and corn stover, among others (Kuhad *et al*., 2011; Mohanty and Abdullahi, 2016; Saini *et al*., 2015). Its low availability in the international market and high-cost impact 40 % of the total cost of the 2G bioethanol process (NREL, s/f; Sun and Cheng, 2002; G. Liu *et al*., 2016). Therefore, it is necessary to obtain robust and efficient enzymes that allow reducing operating costs.

Cellulases are produced by various microorganisms, including *Bacillus subtilis*, *B. licheniformis*, *A. oryzae*, *Trichoderma reseei*, and *A. niger*, being the last mentioned the most important in the production of β-glucosidases at the industrial level. Cellulases are marketed as enzyme cocktails that contain various enzymes that facilitate the degradation of lignocellulosic material and additives to keep them stable during storage and operation (Ravindran and Jaiswal, 2016; Jayasekara and Ratnayake, 2019). Various substrates are used in the production of cellulases, such as carboxymethylcellulose (CMC) and lignocellulosic materials from agro-industrial residues such as coffee pulp, wheat, prickly pear peels, sugarcane bagasse, and corn cob (Peña-Maravilla *et al*., 2017; Vargas-Solano *et al*., 2021). These residues are the most used due to their low cost and high availability. Further, carbon sources with heteropolymers such as delignified sorghum bagasse favor cellulases production compared to CMC sources Infanzón-Rodríguez *et al*., (2020). The production of enzymes using lignocellulosic materials implies the pretreatment of the waste to make the substrate accessible, followed by the addition of the microorganism to produce the enzyme of interest (Islam and Roy, 2018; Ravindran and Jaiswal, 2016; Sánchez, 2009). Cellulase production depends on the carbon source, in this regard, Amore *et al*., (2013) used corn stover, corn cob, and rice straw have been used efficiently to produce cellulases with *A. niger* showing a positive effect on the production of glycosyl hydrolases. Cellulase production is also influenced by the supplementation of urea, peptone, and Tween 80 to the culture medium, suggesting that Tween 80 increases membrane permeability, urea shows a positive effect on cellulase production and peptone can fulfill the function as a carbon or nitrogen source, which can interfere with the expression of cellulases (Antonov *et al*., 2016; García-Reyes *et al*., 2017). In the present study, the production of cellulases from *A. niger* ITV-02 in low-cost lignocellulosic residues was evaluated using two carbon sources, corn stover and corn cob, as well as two culture media enriched with nitrogen sources and Tween 80 to obtain a feasible enzyme extract that could be used in the biofuel industry. Additionally, the enzymes expressed in the enzyme extracts obtained by submerged fermentation were identified.

2 Material and methods

2.1 *Pretreatment of lignocellulosic waste*

The lignocellulosic residues (corn stover and corn cob) were provided by the National Institute for Agricultural and Livestock Forestry Research (INIFAP). A chemical pretreatment was performed to remove lignin from both materials. The corn stover was subjected to alkaline hydrolysis with 6 % H_2O_2 (v/v) in a solid-liquid ratio of 10:1 for 36 h, and the corn cob was delignified with 2 % H_2O_2 (v/v) in a solid-liquid ratio of 13.5:1 for 8 h. All delignified residues and without delignification were subjected

to a particle size reduction to 750 μm, using a model GRT-02A pulverized. Lignocellulosic materials were characterized before and after alkaline treatment through the methodology proposed by Paz *et al*., (2018).

2.2 Microorganism and spread of spores

Aspergillus niger ITV-02 was isolated from wood and belongs to the strain collection of the working group of the Bioengineering Laboratory of the Food Research and Development Unit. The spores of *A. niger* ITV-02 were propagated and stored at 4 °C in Petri dishes with PDA. After, spores were collected in a sterile solution of 6 ml with Tween 80 at 0.1 % w/v.

2.3 Production of enzyme extract

For the enzymatic extract production, two carbon sources were used, corn stover (Cs) and corn cob (Cc) at 10 g/L, which were supplemented with two media M1 and M2. Medium 1 (M1): 2.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 0.9 g/L Urea, 2.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $CaCl_2$, 0.005 g/L $FeSO_4.7H_2O$, 0.0016 g/L $MnSO_4.H_2O$, 0.0014 g/L $ZnSO_4.7H_2O$, 0.002 g/L $CoCl_2$, 1.0 g/L peptone, and 1.5 g/L yeast extract (Infanzón-Rodríguez *et al*., 2020). Medium 2 (M2): 2.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 0.3 g/L Urea, 1.4 g/L $(NH_4)_2SO_4$, 1.0 g/L Tween 80, 0.3 g/L $CaCl_2$, 0.005 g /L $FeSO_4.7H_2O$, 0.0016 g/L $MnSO_4.H_2O$, 0.0014 g/L $ZnSO_4.7H_2O$, 0.002 g/L $CoCl_2$, and 5.0 g/L peptone. The new media named Cc-M1, Cc-M2, Cs-M1, and Cs-M2 were inoculated with 6x106 spores/mL in 500 mL flasks with a culture medium volume of 250 mL at 250 rpm, 30 °C for 120 h in an orbital shaker (SEV-Prendo). Once the fermentation was complete, the enzymatic extract was collected by centrifugation at 1800 xg for 10 minutes at 4 °C in a Centurion K3 series centrifuge. The enzymatic activity of the crude extracts was quantified using the substrates CMC (CMCase activity), filter paper (FPUase), and β-pNPG (β-glucosidase), a statistical analysis was performed by analysis of variance (ANOVA) in Minitab 19.0 software, using the enzymatic activities as the response variable, were compared by Tukey's test, and the level of significance was $p \leq 0.05$.

2.4 Determination of FPUase, CMCase, and β-glucosidase

2.4.1 FPUase

FPUase activity was defined as the enzymatic activity on the filter paper (FP) and taking a glucose calibration curve as a reference. To determine the activity, 50 mg of Whatman filter paper no. 1, 1.0 mL of 50 mM sodium acetate buffer, pH 5.0, and 1 ml of the enzyme extract were added. The reaction was carried out at 50 °C for one hour. In the end, reducing sugars were determined at 540 nm according to the methodology described by Miller (1959) in the Genesys 20 model spectrophotometer (Thermospectronic). One unit of enzymatic activity (U) FPUase was defined as the amount of enzyme capable of releasing 1 μmol of glucose per minute.

2.4.2 CMCase

CMCase activity was defined as the enzymatic activity obtained using CMC as substrate and taking a glucose calibration curve as reference. Sigma brand CMC (C5678) was used at 0.5 % (w/v) final concentration in 50 mM sodium acetate buffer (pH 5.0). One ml of the enzyme extract was added, and the reaction was carried out at 50 °C for 1 min. Reducing sugars were determined at 540 nm according to the methodology described by Miller (1959), in the Genesys 20 model spectrum (Thermospectronic). One unit of CMCase enzymatic activity (U) was defined as the amount of enzyme capable of releasing 1 μmol of glucose per minute.

2.4.3 β-glucosidase

To determine the β-glucosidase activity, a calibration curve of 0.25 mM β-pNPG was taken as reference. Then, 10 mM p-Nitrophenyl-β-D-glucopyranoside (β-pNPG Sigma CASNo. 2492-87-7) were dissolved in 50 mM sodium acetate buffer pH 5.0 and used as substrate. The reaction was performed in a 1:1 ratio of enzyme extract and β-pNPG. The reaction was stopped with 0.2 M Na_2CO_3 in a 1:2 sample and Na_2CO_3 ratio, respectively. The amount of *p*-nitrophenol (pNPG) produced was measured at 400 nm in a Genesys 20 model spectrum of the Thermospectronic brand (Singhania *et al*., 2011). One unit of β-glucosidase enzymatic activity (U) was defined as the amount of enzyme capable of releasing 1 μmol of *p*-nitrophenol (pNP) per minute.

2.5 *Determination of protein*

Protein concentration was measured at 595 nm following the methodology established by the supplier of the Bradford B6916 reagent (Sigma Aldrich, s/f) using bovine albumin serum as standard. All spectrophotometric measurements were performed using a Genesys 20 Thermospectron model spectrum.

2.6 *Determination of enzymatic extracts*

The enzyme extracts were concentrated 10 times with a polyethersulfone (PES) membrane with a cut-off limit of 10 kDa, using an Amicon ultrafiltration system (Millipore Co., Billerida, MA, USA).

2.7 *Identification of enzyme with CMCase and β-glucosidase activity*

2.7.1 *Protein profile and zymogram*

The concentrated extracts were analyzed by SDS-PAGE at 12 % for CMCase activity and 10 % for β-glucosidase, stained with 0.25 % (w/v) Coomassie blue. Zymograms were spiked with CMC at 1 % (w/v) final concentration to reveal CMCase activity and with 1 mM MUG to reveal β-glucosidase activity. In the zymograms, the SDS was removed by submerging the gel with Triton 100-X at 2.5 % (w/v) for 30 min, then three washes of the gel were carried out with 50 mM sodium acetate buffer at pH 5.0. For CMCase activity, the gel was left incubating for 1 h at 4 °C in CMC at 1 % (w/v) final concentration and then incubated for 45 min at 50 °C. The activity in the gel was revealed with a 0.1 % (w/v) Congo red stain and then rinsed with a 1 M NaCl solution until the hydrolysis bands were clear (Cerda-Mejía, 2016).

For β-glucosidase activity, the methodology was adapted from Hernández-Guzmán *et al*. (2016), the gel was prepared with MUG at 1 mM diluted in acetate buffer. The SDS was removed with Triton 100-X at 2.5 % (w/v) for 30 min, then 3 gel washes were performed with 50 mM sodium acetate buffer at pH 5.0. To reveal the activity, the gel was incubated for 1h at 4 °C in 1 mM MUG and then incubated for 1 h at 50 °C. The activity was measured in a 16 mm MiniBis transilluminator.

2.7.2 *Proteins identification*

The protein bands that showed enzymatic activity were selected and subsequently cut from the corresponding Coomassie-stained SDS-PAGE and sent to the Research and Industry Support Services Unit (USAII) of the UNAM chemistry faculty. The analysis was performed by digestion of the proteins with modified porcine trypsin, the peptides from the digestion were analyzed by HPLC and mass spectrometry. The software used for data processing was Protein Lynxs Global Server 2.5.1^{TM} (Waters).

2.7.3 *Bioinformatic analysis*

To identify the expressed enzymes, the obtained peptides were aligned and analyzed using the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore). The proteins were analyzed using other programs and databases such as BRENDA (https://www.brenda-enzymes.org/index.php), UNIPROT (https://www.uniprot.org/), Jalview, Prot pi (https://www.protpi.ch/).

3 Results and discussion

3.1 *Effect of culture medium composition*

The lignocellulosic material was characterized using the methodology of Paz *et al*. (2018) to know its lignocellulosic composition (Table 1).

The structural composition of the crude residues reported in Table 1 is in the range of the reported in the literature for corn cob, cellulose 20-36 %, hemicellulose 33-42 %, and lignin 15-23 % approximately, concerning the corn stover cellulose 24-40 %, hemicellulose 30-45 % and lignin 20-25 % (Hernández *et al*., 2019; Rofiqah *et al*., 2019; Mafa *et al*., 2020; Bohn *et al*., 2021). Regarding delignification, the results obtained in this work are similar to the structural composition for corn cob and corn stover in reported previous research (Chen *et al*., 2013; Rofiqah *et al*., 2019).

Authors such as Amore *et al*., (2013); Gutiérrez-Rojas *et al*., (2015); Nitta *et al*., (2012), reported that the expression of cellulases is regulated by the xylose present in the xylan contained in hemicellulose, while the β-glucosidases are induced by the concentration of cellobiose obtained in the hydrolysis of cellulose. However, lignin can represent a drawback in the hydrolysis of hemicellulose and cellulose since it represents a physical barrier to hydrolysis (Lu *et al*., 2016). The cellulose and hemicellulose ratio accompanied by a low percentage of lignin positively influences the expression of cellulases.

Table 1. Structural composition of corn lignocellulosic residues.

Materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Corn stover	33.93 ± 0.91	31.74 ± 0.05	25.55 ± 0.5
Corn stover delignified	54.8 ± 0.09	29.3 ± 1.53	10.0 ± 2.31
Corn cob	31.95 ± 1.5	37.18 ± 1.00	15.71 ± 0.83
Corn cob delignified	38.0 ± 0.54	43.0 ± 1.22	10.9 ± 0.43

Table 2. Statistical analysis by Tukey of the FPUase, CMCase, and β-glucosidase activities.

Treatment	**Volumetric and specific activities**					
	FPUase (U/mL)	FPUase (U/mg)	CMCase (U/mL)	CMCase (U/mg)	β-glucosidase (U/mL)	β-glucosidase (U/mg)
Cc-M1	0.10^{a}	0.65^{b}	0.92^{cd}	5.98^{e}	2.80^{f}	18.18^{h}
Cs-M1	0.07^{a}	0.49^{b}	0.83^{d}	5.98^{e}	3.56^{f}	25.65^{i}
Cc-M2	0.10^{a}	0.48^{b}	0.86^{d}	4.02^{e}	3.55^{f}	16.35^{h}
Cs-M2	0.10 a	0.43^{b}	1.33^{c}	5.66^{e}	6.34^{g}	27.38^{i}

Different letters indicate a significant difference between activities of each treatment, for $\alpha < 0.05$ and $n = 2$.

Corn stover presented the highest cellulose/hemicellulose ratio (1.87) compared to the corn cob (0.8), which contributes to the expression of enzymes, the percentage of lignin was 10 % for the corn stover and 10.9 % for corn cob, so the lignin concentration of these two residues is relatively similar.

Table 2 shows the results of the activities in the four treatments evaluated (Cc-M1, Cc-M2, Cs-M1, Cs-M2). Considering the volumetric activity and comparing the Cc-M2 treatments with Cs-M2 we can observe a significant difference in the CMCase and β-glucosidase activity. Regarding the specific activity, a significant difference was only observed in the β-glucosidase activity. In these results, the CMCase and FPUase activity are similar even when the carbon source is changed. Some cellulases with activity on CMC and FP are regulated by the concentration of xylose, so their expression may be controlled by this monosaccharide or there may also be a regulatory effect due to the inhibition of products such as cellobiose, which could keep the expression constant and the enzymatic activity CMCase and FPUase (Gutiérrez-Rojas *et al*., 2015; Zou *et al*., 2021). Cellobiose is an inducer of some β-glucosidases, which would explain why the β-glucosidase activity is higher using corn stover as a carbon source. It is suggested that there is a direct relationship between cellulose content and concentration of cellobiose (high cellulose content in the corn stover leads to a high concentration of cellobiose), favoring the expression of β-glucosidases. These results coincide with those reported by Miranda-Sosa *et al*., (2019) where the higher the concentration of cellulose, the greater the β-glucosidase activity. It is worth mentioning that β-glucosidases play an important role in cellulose degradation by removing cellodextrins or endoglucanase-inhibiting cellobiose obtained from cellulose hydrolysis (Guo *et al*., 2015; Salgado *et al*., 2018).

In a similar work with *Aspergillus* spp using corn cob as a carbon source, CMCase activity of 1.42 U/mL was reported, Patel *et al*., (2017), a similar value to those reported in this study. Likewise, *Aspergillus* NRRL 567 with 4 % (w/v) of corn stover produced 0.61 U/mL and 0.67 U/mL of CMCase and β-glucosidase activity, respectively Muhammad ishfaq Ghori, (2011), which compared to those obtained in this work are 2.18 and 6.34 minors, respectively.

It is observed that the specific activity in all the evaluated activities in the media M1 and M2 showed no statistically significant difference using Cs as substrate (Table 2). However, when comparing the volumetric activity in both media, it was observed that the Cs-M2 treatment favored the volumetric activity, with activities of 1.33 U/mL (CMCase) and 6.34 U/mL (β-glucosidase) (Table 2). The main difference between these media was the nitrogen concentration and the presence of Tween 80 where the ratio C:N was

1.7 (M1) and 1.5 (M2), it means M2 contains more nitrogen. The percentage between the organic nitrogen was 43 % (M1) and 75 % (M2) it is indicated M2 have 1.7 times more organic nitrogen.

It is suggested that the reasons why the volumetric activities CMCase and β-glucosidase were significantly favored in the Cs-M2 medium may be due synergy between to the increase in the source of organic nitrogen such as peptone and of the surfactant such as Tween 80. It has been reported that Tween 80 increases protein secretion by increasing the permeability of the cell membrane Pandey *et al*., (2017). Surfactants such as Tween 80 have effects on growth, and metabolite production, and influence mycelial morphology (Liu and Wu, 2012). On the other hand, the free amino acids contained in peptone could facilitate the synthesis of enzymes due to it is not necessary to active amin acid synthesis pathway (Horton *et al*., 2008). Peptone promotes the mycelia growing which is related with the production of metabolites (García-Reyes *et al*., 2017; Yan *et al*., 2012).

In other report with *A. niger* ITV-02, Infanzón-Rodríguez *et al*., (2020) using sorghum bagasse delignified like a carbon source, reports protein concentration of 4.85 mg/L and specific activities of 126.72 U/mg (endoglucanase) and 85 U/mg (β-glucosidase) this result was compared with the results obtained in this work with Cs-M1, the protein concentration reported by Infanzón-Rodríguez *et al*., (2020) is 28.63 times less, whiles the specific activities CMCase and β-glucosidase is 21.19 and 23.87 times highest. It means that other proteins without cellulase activity were produced in this work.

3.2 Identification of β-glucosidase enzyme activity

Figure 1 shows the protein profile and the enzymatic activity, concerning the protein profile, the four treatments presented the same protein profile, although the treatments with the M2 medium presented greater intensity in the protein bands. The main difference between the medium M1 and M2 is the concentration of nitrogen, peptone, and Tween 80. Regarding the zymograms, protein bands with β-glucosidase activity were observed in the four enzyme extracts analyzed, whose total activities were 323.47 U (Cs-M2), 275.85 U (Cs-M1), 156.15 U (Cc-M2), 165.84 U (Cc-M1) where the most intense bands correspond to the treatments with corn stover (Figure 1).

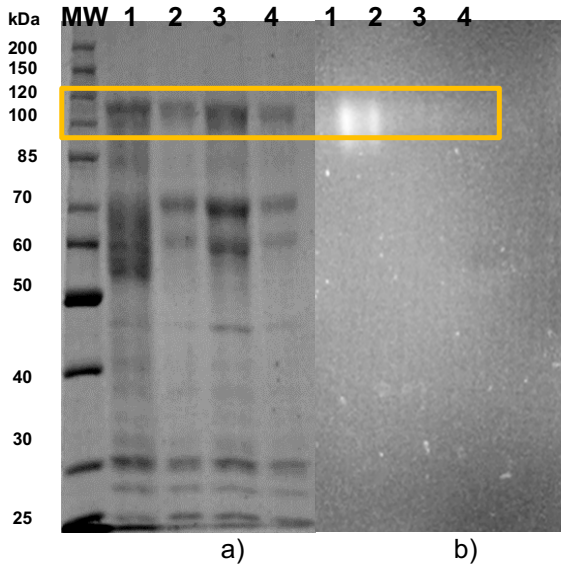

Figure 1. Proteins profile and β-glucosidase activity of the enzymatic extract concentrated of different treatment, a) SDS-PAGE 10 % and b) Zymogram with MUG lane 1. (Cs-M2) 2. (Cs-M1) 3. (Cc-M2) 4. (Cc-M1) and MW (molecular weight).

The bands with β-glucosidase activity had an approximate molecular weight of 117 kDa, with high intensity for Cs-M2, which coincides with the medium with the highest activity (Table 1). The results are similar to those reported by authors such as Kamaruddin *et al*., (2008) who obtained a β-glucosidase with a molecular weight of 93 kDa from *A. niger* ATCC10574 using crystalline cellulose (Avicel) as a substrate, authors such as Zhao *et al*., (2013) reported a β-glucosidase from *A. niger* NL-1 expressed in Pichia pastoris with an estimated molecular weight of 94.7 kDa. Lima *et al*., (2013) reported a β-glucosidase from a commercial preparation of *A. niger* (Novozymes SP 188; Sigma) with an experimental weight of 116 kDa, while Gong *et al*., (2014) using corn cob and wheat bran as substrate found a β-glucosidase with two subunits of 110 kDa and 120 kDa.

The protein band with β-glucosidase activity was identified by peptide sequencing by mass spectrophotometry as shown in Table 3. An alignment of peptide sequences was performed in the NCBI and MASCOT databases, indicating that the band of 117 kDa contained four different enzymes, one glucoamylase (XP_001390530.1), 2β-glucosidases (XP_001398816.1, XP_001398259.2), and one Exo β-xylosidase (XP_001389416.1).

Table 3. Identified peptides into the 117 kDa protein band with β-glucosidase activity of the Cs-M2 enzyme extract.

Band protein MW	**Identified peptides by mass spectrometry**	**Identified protein by NCBI**
117 kDa	ATLDSWLSNEATVAR FNVDETAYTGSWGRPQR DLTWSYAALLTANNRR ETAYTGSWGRPQR TWSYAALLTANNRR	Glucoamylase (XP_001390530.1)
	GADIQLGPAAGPLGR ITLQPSEETQWSTTLTRR DLANWNVETQDWEITSYPK LYDELIR SPFTWGK IGADSTVLLKNDGALPLTGK DSDYNSAFPAGVNVAATWDKNLAYLR LPLRASLPTVH	β-glucosidase A (XP_001398816.1)
	AIGTTQLFAPLADLAR LSVSFPHYVGDLPIYYDYLNSAR VSDLLSR VSDLLSRMTIEDK SDLLSRMTIEDK DLLSRMTIEDK SDLLSR DLLSR	β-glucosidase (XP_001398259.2)
	ESIAWPGNQLDLIQK NSNNVLPLTEK ELRVPVEVGSFAR YGLDVYAPNINTFRHPVWGR ELRVPVE VGSFAR PGNQLDLIQK	Exo-β-xylosidase (XP_001389416.1)

Comparing the enzymes from the reports of the BRENDA database, 2020 (Table 4) with the enzymes identified in Table 3, they present a molecular weight (117 kDa) different from the observed in Figure 1. However, the four enzymes present glycosylation sites which would increase their molecular weight to the observed molecular weight. Glucoamylase (XP_001390530.1), hydrolyzes the α-1,4 bonds present in starch and is found in the metabolism of carbohydrate transport and degradation. The β-glucosidases (XP_001398816.1 and XP_001398259.2) hydrolyze the β-1,4 bonds of cellulosic materials and are present in the transport and metabolism of carbohydrates. Exo-β-xylosidase (XP_001389416.1), is involved in xylan hydrolysis and glycan degradation, hydrolyzes β-1,4-D-Xylan bonds, is involved in carbohydrate metabolism, and hydrolyzes various substrates such as 4-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside.

No reports have been found of the enzymes previously described and located in the 117 kDa band, (glucoamylase from *Aspergillus* spp.) that hydrolyze the β-1,4 bonds. Therefore, the activity reported in the zymogram is due to the enzymes that hydrolyze the β-1,4 bonds. The β-glucosidases have activity with the MUG reagent. No activity has been reported for xylosidase on MUG, but activity is reported with 4-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, hydrolyzing the bonds β of position 1, so it is suggested that it also performs the hydrolysis of MUG.

Table 4. Theoretical characteristics of enzymes with β-glucosidase activity identified.

Enzyme	Signal peptide	Isoelectric point	Molecular weight (kDa)	Number of potential sites the peptide		Substrates
				glycosylation N	glycosylation O	
Exo-β-xylosidase	1-26	5.5	87.2	15	116	1,4-β-D-xylobiose, 4-nitrophenyl-α-D-glucoside, 4 nitrophenyl-β-D glucopyranose, 4-methylumberyl, -β-D-xylopyranoside
Glucoamylase	1-18	3.5-3.7,4.4	68.3	4	161	starch, 4-nitrophenyl-α-D-glucoside, 4-nitrophenyl-α-D-glucopyranose. 4-nitrophenyl-β-D-glucoside
β-glucosidase A	1-19	4.2	93.2	14	111	4- methylumberyl -β-D-glucopyranose, 4- methylumberyl -β-D-galactopyranoside, 4- methylumberyl -α-D-glucopyranoside, carboxymethyl cellulose (CMC)
β-glucosidase	1-21	5.0	88.0	11	99	β-D- joins

Source: information adapted from BRaunschweig ENzyme Database (BRENDA), Protein tool (Prot pi).

On the other hand, the peptides that correspond to a glucoamylase of *A. niger* were located, this enzyme is inducible by substrates such as starch, maltose, and glucose, among others. Nonetheless, in this work the polymers used were cellulose, xylan, and lignin which are not polymers that induce this enzyme. Some authors reported that xylose affects the expression of the glaA gene that codes for glucoamylase. It is described that in the presence of maltose and xylose the expression of the glaA gene is not induced due to high concentrations of xylose that repress the gene, however, an opposite effect is observed at low concentrations of xylose, because no repressive effect is observed on the gene enzyme expression (Fowler *et al*., 1990; Santerre-Henriksen *et al*., 1999; Ganzlin and Rinas, 2008; Yuan *et al*., 2008). According to the above, it is suggested that glucoamylase was expressed in the presence of glucose as an inducer obtained in the hydrolysis of cellulose, and the concentration of xylose from the hydrolysis of hemicellulose was not sufficient to repress its expression.

3.3 Identification of enzymes with CMCase activity

Figure 2 shows the protein profile (SDS-PAGE) and the enzymatic activity (zymogram). Regarding the protein profile, the four treatments are concentrated by ultrafiltration whose enzymatic activity is 25.02 U (Cs-M1), 27.11 U (Cc-M1), 28.03 U (Cs-M2), 31.15 U (Cc-M2), presented the same profile. For enzymatic activity using CMC as substrate, protein bands were identified whose weights ranged between

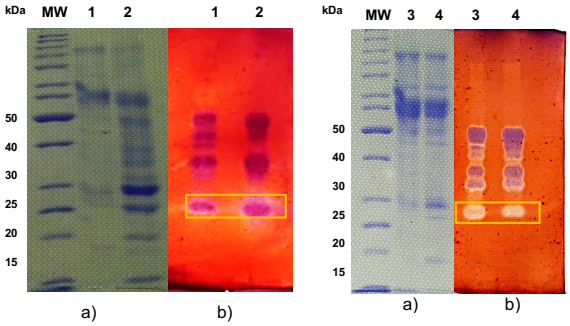

Figure 2. Proteins profile and CMCase activity of the enzymatic extract concentrated of different treatment, a) SDS-PAGE 12 % and b) Zymogram with CMC, lane 1. (Cs-M1) 2. (Cc-M1) 3. (Cs-M2) 4. (Cc-M2) and MW (molecular weight).

approximately 48 and 26 kDa (Figure 2). In both zymograms, the four treatments presented the same profile with CMCase activity. Because in Table 2 no significant difference was observed between the treatments measuring the CMCase activity, the Cs-M2 treatment was selected to identify the proteins expressed in the 48, 33, and 26 kDa bands. These weights were selected because in Figure 2 they presented a greater halo of CMCase activity in the zymogram. The identified peptides are shown in Table 5, finding that the 48 kDa band corresponds to an Exo-β-1,3-glucanase (XP_001389652.2), in the 33 kDa band two proteins were found, one endo-β-1,4 xylanase (XP_001389996.2) and an arabinosidase (XP_001389998.1), finally, in the 26 kDa band endoglucanase (AJ224451.1) was found.

Table 5. Identified peptides into protein bands (48 kDa, 33 kDa, 26 kDa) with CMCase activity of the Cs-M2 enzyme extract.

Bands protein MW	**Identified peptides by mass spectrometry**	**Identified proteins by NCBI**
48 kDa	ASDYWVGTIKR	Exo-β-1,3-glucanase (XP_001389652.2)
	SSSTPLLFDSNYNPKPAYTAIANAL ADFGALTPENSMK KYLGNIGDQYTLTK LYINDYNLDSASYPK	Endo-β-1,4 xylanase (XP_001389996.2)
33 kDa	ANSGATWTDDISHGDLVR DFTDVVSNGK SFTASSLGGEWTAQAASEDQPFAGK SGATWTDDISHGDLVR SIWVLAYQWGSSTFTYR	α-L-arabinofuranosidase axhA (XP_001389998.1)
26 kDa	LVSDVSSIPTSVEWK QIATATVGGK SYSNSGVTFNKK YGNIQPIGKQIATATVGGK	Endoglucanase (AJ224451.1)

Table 6. Theoretical characteristics of enzymes with CMCase activity identified.

Enzyme	Signal peptide	Isoelectric point	Molecular weight (kDa)	Number of potential sites the glycosylation		Substrates
				glycosylation N	glycosylation O	
Exo-β-1,3-glycosylation	1-20	4.5	99.3	13	217	4-nitrophenyl-β-D-cellobioside, cellobiose, cellodextrins, squalene
Xylanase	1-19	5.6	35.4	0	0	4-methyllumberil, β-D-cellobioside, 4-nitrophenyl-α-L-arabinofuranoside, carboxymethylcellulose
Arabinosidase	1-26	4.6	35.8	0	0	4-nitrophenyl-D-cellobioside, carboxymethylcellulose, cellodextrin
Endoglucanase	1-16	4.4	25.8	0	0	Cellobiose, 4-nitrophenyl-D-cellobiose, cellodextrin, carboxymethylcellulose

Source: information adapted from BRaunschweig ENzyme Database (BRENDA), Protein tool (Prot pi)).

Some characteristics of the enzymes were described according to the BRENDA database (Table 6).

All the enzymes identified in the protein bands weighing 33 kDa and 26 kDa in Figure 2 coincide with the molecular weight reported in Table 6. However, the Exo-β-1,3-glucanase identified in Figure 2 with an estimated weight of 48 kDa, has a different weight (approximately half) to that reported in Table 6. Authors such as Montoya-Espinoza *et al*., (2016) report that various fungi produce extracellular β-1,3-glucanases of three or more isoenzymes, some of them constitutive and others induced by substrates. The isoenzymes may be generated by differential maturation which could explain the difference in molecular weight of Exo-β-1,3-glucanase.

It is reported that Exo-β-1,3-glucanase (XP_001389652.2), hydrolyzes the β-1,3 bonds of the non-reducing ends of glucans such as the cell wall of fungi, releasing α-D-glucose, with an estimated weight of 99 kDa. Nevertheless, the Exo β-1,3-glucanase of various microorganisms such as *Saccharomyces cerevisiae*, *Hordeum Vulgare*, and *Helix pomatia*, hydrolyze compounds such as cellobiose, cellodextrins, cellotetraose, and carboxymethylcellulose, which have β-1,4 bonds. The results of Suzuki *et al*., (2001; Schwentke *et al*., (2014); Guo *et al*., (2015), coincide with our results because the Exo β-1,3-glucanase presented CMCase activity.

Endo-β-1,4 xylanase (XP_001389996.2) is involved in xylan hydrolysis, hydrolyzes β-1,4-xylose bonds, and has an estimated molecular weight of

35.4 kDa. It is produced by microorganisms such as *Laetiporus Sulphureus*, *Thermomyces lanuginosus*, *Bacillus*, and *A. niger*. Some authors report that it hydrolyzes CMC Uchino and Nakane, (1981); Ricardo *et al*., (1985); Lee *et al*., (2009), which coincides with the found in this study.

Arabinosidase (XP_001389998.1) is involved in the hydrolysis of the non-reducing ends of the (α-1,5) arabinoxylan present in hemicellulose, releasing L-arabinose. An estimated molecular weight of 35.83 kDa is reported. Some authors such as Gilead and Shoham, (1995), report that this enzyme obtained from *B. stearothermophilus* showed activity on CMC, so the enzyme found in this work could also show activity on CMC, which would explain why it is found in the band with CMCase activity.

Endoglucanase (AJ224451.1) hydrolyzes the β-1,4-D-glucose bonds of various substrates such as CMC, cellobiose, and filter paper, among others, and participates in carbohydrate metabolism and polysaccharide degradation. An estimated weight of 25.86 kDa is reported. Endoglucanases from various microorganisms are reported, such as *A. fumigatus*, *A. oryzae*, *B. subtilis*, and *T. reesei*, which also hydrolyze CMC.

Amore *et al*., (2013) report that the expression of xylanases and arabinofuranosidases is because the production of cellulases and xylanases is positively regulated by cellulose and hemicellulose. The influence of the carbon and nitrogen source on the production of cellulases has been studied, showing that the carbon source has an influence and that the main transcriptional activator XlnR is involved in the regulation of cellulases, hemicellulases, and genes of complementary enzymes for the production of cellulases, cellulose and hemicellulose degradation in *Aspergillus* spp. The transcriptional activator XlnR regulates the transcription of genes such as xlnB, xlnC, and xlnD that code for endoxylanase B, endoxylanase C, and β-xylosidase respectively, this factor is also involved in the transcriptional activation of genes that code for two endoglucanases EglA, EglB, and various enzymes involved in the degradation of hemicellulose and cellulose. The enzymes expressed by the regulation of XlnR with Xylose are agree with the enzymes identified in this study. Interestingly, Exo-β-1,3-glucanase, xylanase, and arabinosidase exhibited enzymatic activity on CMC. Some authors such as Nobeli *et al*., (2009); Tian *et al*., (2016), reported that the enzymatic promiscuity within the enzymes associated with the degradation of lignocellulosic materials may be related to the recognition of the enzymes with the ligand.

Conclusions

All the treatments evaluated presented cellulase activity; however, the corn stover, which contained a high concentration of cellulose, favored the β-glucosidase activity, this indicated the ratio Cellulose/Hemicellulose have more influence than ratio Carbon/Nitrogen with specific activity. The same bands with activity were identified in all treatments. In the identification of proteins with β-glucosidase activity, a band with an estimated weight of 117 kDa was observed, locating four enzymes (glucoamylase, Exo β-xylosidase, 2 β-glucosidases). In the CMCase activity, three bands with a greater halo of activity were identified: 48 kDa (Exo-β-1,3-glucanase), 33 kDa (xylanase and arabinosidase), and 26 kDa (endoglucanase). The enzymes present in the 48 and 33 kDa bands had an affinity to hydrolyze CMC, suggesting enzymatic promiscuity.

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