Research Paper

Thermotolerant and mesophylic fungi from sugarcane bagasse and their prospection for biomass-degrading enzyme production

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Abstract

Nineteen fungi and seven yeast strains were isolated from sugarcane bagasse piles from an alcohol plant located at Brazilian Cerrado and identified up to species level on the basis of the gene sequencing of 5.8S-ITS and 26S ribosomal DNA regions. Four species were identified: *Kluyveromyces marxianus*, *Aspergillus niger, Aspergillus sydowii* and *Aspergillus fumigatus*, and the isolates were screened for the production of key enzymes in the saccharification of lignocellulosic material. Among them, three strains were selected as good producers of hemicellulolitic enzymes: *A. niger (*SBCM3), *A. sydowii* (SBCM7) and *A. fumigatus (*SBC4). The best β-xylosidase producer was *A. niger* SBCM3 strain. This crude enzyme presented optimal activity at pH 3.5 and 55 °C (141 U/g). For β-glucosidase and xylanase the best producer was *A. fumigatus* SBC4 strain, whose enzymes presented maximum activity at 60 °C and pH 3.5 (54 U/g) and 4.0 (573 U/g), respectively. All these crude enzymes presented stability around pH 3.0-8.0 and up to 60 °C, which can be very useful in industrial processes that work at high temperatures and low pHs. These enzymes also exhibited moderate tolerance to ethanol and the sugars glucose and xylose. These similar characteristics among these fungal crude enzymes suggest that they can be used synergistically in cocktails in future studies of biomass conversion with potential application in several biotechnological sectors.

Key words: β-glucosidase, β-xylosidase, xylanase, *Aspergillus*, sugarcane bagasse.

Introduction

Agro-industrial wastes such as sugarcane bagasse, wheat bran and corn straw have been recognized as important renewable sources of biofuels and other value-added products (Moretti *et al.*, 2012). These residues are widely available in Brazil and are composed of the polymers cellulose, hemicellulose and lignin. In recent years, there has been an increasing trend towards more efficient exploitation of these by-products. Due to their heterogeneity, several microbial enzymes (cellulases and hemicellulases) with specific modes of action are used to catalyze the hydrolysis of these polymers (Matkar *et al.*, 2013).

Cellulose is degraded by the synergistic action of three cellulases: exoglucanases, endoglucanases and cellobiases, which release cello-oligosaccharides and cellobiose, respectively. Exoglucanases or avicelases (EC 3.2.1.91) are responsible for removing the cellobiose from the non-reducing ends and endoglucanases (EC 3.2.1.4) perform the cleavage from the amorphous parts of cellulose in order to reduce the polymerization level. Lastly, β-glucosidases or celobiases (EC 3.2.1.21) catalyze the hydrolysis of cellobiose in free glucose (Martins *et al.*, 2008). Two hemicellulases have a significant role for the depolymerization of hemicellulose: xylanases and the β-xylosidases. The former are applied for xylan hydrolysis, the main polysaccharide of hemicellulose and β-xylosidases act after the hydrolysis of xylan by xylanases, cleaving oligomers of β-D-xylopyranosyl and xylobiose in free xylose (Polizeli *et al.*, 2005). Thus, the cooperative action of cellulases and hemicellulases from microbial origin is needed in order to achieve effective biomass hydrolysis from the residues (Polizeli *et al.*, 2005).

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In industrial processes, thermotolerant hemi-cellulases and cellulases are preferable since high temperatures are required to increase the solubility of substrates, to reduce viscosity and to reduce risk of contamination (Facchini *et al.*, 2011). As a result, the search for thermophilic microorganisms with the potential for the synthesis of such enzymatic complexes has increased considerably in recent times, mainly filamentous fungi, which have been the most employed for it (Moretti *et al.*, 2012).

At present, molecular methods have been largely employed for the identification of microorganisms since they are independent of culture conditions, accurate and rapid (Baffi *et al.*, 2010). Among them, ribosomal DNA sequencing has been one of the main tools for the classification of fungi strains (Baffi *et al.*, 2012). Accordingly, the aim of this study was to identify the fungal microbiota isolated from sugarcane bagasse piles from Brazilian Cerrado by rDNA sequencing. Moreover, we intended to select novel strains able to synthesize thermotolerant key enzymes in the process of degrading lignocellulosic material from agro-industrial residues. The physicochemical properties of the selected enzymes were also investigated.

Materials and Methods

Isolation of microorganisms

Isolation of fungal strains was performed from piles of sugarcane bagasse obtained from the Alvorada Plant, from the mesoregion of Triângulo Mineiro and Alto Paranaíba (Araporã, west of the state of Minas Gerais, Brazil) which lies at 18°26'13" S and 49°11'13" W, with a tropical climate and is part of Brazilian Cerrado.

Ten collecting points from the internal parts of different piles, where the temperatures are elevated, were randomly chosen to collect samples of thermophilic fungi. For the isolation of mesophilic fungal strains, ten random points were also selected but, from the external parts of the piles. The samples (0.5 g) were harvested and homogenized in a sterile broth containing (g/L): cardboard, 5.0; $(NH_4)2SO_4$, 1.4; K_2HPO_4, 2.0; $CaCl_2$, 0.3; peptone, 2.0; yeast extract, 2.0; glucose, 1.0; urea, 0.3; $MgSO_4 \cdot 7H_2O$, 0.2; and 10.0 mL of nutrient solution which consisted of (mg/L): $FeSO_4 \cdot 7H2O$, 5.0; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4$ $7H_2O$, 1.4; $CoCl_2$, 2.0, at pH 5.0. The inoculated medium was incubated under shaking at 45 °C for the isolation of thermophilic strains and at 30 °C for mesophilic fungi, for 72 hours. Afterwards, aliquots of liquid medium were streaked on Petri dishes containing Agar Sabouraud medium (45.0 g/L), with chloramphenicol and ampicillin (0.2 g/L) to inhibit bacterial contamination, and incubated at 45 or 30 °C (thermophilic and mesophilic strains, respectively), for 24 to 96 hours.

When colonies appeared, they were subcultured onto fresh plates containing the same medium and allowed to grow for several days. This process was repeated until pure cultures were obtained. The stock cultures were maintained, with periodical subcultures, in Agar Sabouraud medium, at room temperature. Each isolate was also conserved in Castellani flasks with MilliQ sterile water for subsequent studies (Castellani, 1939).

All morphologically contrasting colonies were identified by conventional and molecular methods. The molecular identification of the isolated strains was performed firstly by polymerase chain reaction (PCR), followed by gene sequencing of the ITS intergenic region of ribosomal DNA.

DNA extraction

Each strain was grown on YPD medium (1% yeast extract, 2% glucose and 2% peptone) for 48 hours at 30 and 45 °C (mesophylic and thermophylic strains, respectively). Genomic DNA was extracted from mycelium using the DNAeasy Plant Mini Kit (Qiagen, USA). Fungal mycelia were macerated in liquid nitrogen using a mortar and pestle. Powdered samples were transferred to microcentrifuge tubes and the procedure was carried out according to the manufacturer's protocol. Isolated DNA from each strain was examined on 1% agarose gel using 5 μL of DNA, 2 μL of Load Dye and 1.5 μL of SYBR Green I fluorescent DNA binding dye and stored at -20 °C.

PCR amplification

Amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was performed according to Baffi *et al.* (2012). The forward primer was ITS1 (5 TCC GTA GGT GAA CCT GCG G 3) and the reverse primer was ITS4 (5 TCC TCC GCT TAT TGA TAT GC 3). Polymerase chain reaction (PCR) was performed in a final volume of 50 μL, and contained 0.2 mM of each dNTP, 1x PCR buffer with $MgCl_2$, 1.0 U Taq DNA polymerase, 0.5 μM of each primer, and 10-50 ng of total DNA template. PCR amplification was carried out in a thermal cycler (MyCycler TM Thermal Cycler, Bio-Rad, USA) with the following cycling parameters: an initial denaturation at 95 °C for 5 min, followed by 33 cycles of denaturation at 95 °C for 1 min, primers annealing temperature at 52 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. PCR products were checked by electrophoresis on 1.5% agarose gels (5 μL of each sample + 2 μL of Load Dye and 1.5 μL of SYBR Green I fluorescent DNA binding dye). After electrophoresis, the gel was visualized under a UV transilluminator.

PCR products' purification

The amplified products were purified using a kit for PCR products (GeneJet PCR purification kit, Fermentas). After purification, the quality of the purified PCR products was evaluated through electrophoresis on 1% agarose gels

and the quantity determined using a NanoDrop ND-1000 UV-Vis spectrophotometer.

Sequencing of 5.8S-ITS rRNA region

Sequencing of purified PCR products was performed on an ABI 3730 XL automated sequencer (Applied Biosystems) by a commercial laboratory (Macrogen Sequencing Service, Korea). After sequencing, chromatograms were visually checked and edited using the BioEdit software (DNAstar Lasergene software, Madison, WI, USA; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The obtained sequences of the ITS1-5.8-ITS2 region were compared with sequences available in the GenBank database (National Center for Biotechnology Information - NCBI), using the BLASTn algorithm software (Local Alignment Search tool) (http://blast.ncbi.nlm.nih.gov). Sequences for the 5.8S-ITS rDNA region with 99% nucleotide identity or higher were considered to correspond to the same species.

26S rDNA region sequencing analysis

Total DNA samples from each strain were also submitted to PCR amplification and sequencing for the 26S (D1/D2) rDNA region. Specific amplifications were attempted from pure cultures, using the pair of primers ITS 1 5' (TCC GTA GGT GAA CCT GCG G) 3' and LR 3R 5' (GGT CCG TGT TTC AAG AC) 3'. The PCR conditions for 26S region were the same as described for 5.8S-ITS rRNA region sequencing but the annealing temperature was increased to 54 °C (Baffi *et al.*, 2010). The obtained sequences were deposited in the GenBank data library under accession numbers.

Morphological identification

Strains with less than 97% similarity with published sequences in the GenBank database were submitted to identification by morphological examination on a stereoscope. The macroscopic variables analyzed included: mycelium aspect, colony diameter, spore coloration, obverse and reverse mycelium color, degree of sporulation and other characteristics (Pitt and Hocking, 1997). To examine fungal structures, mycelia were examined in a lactophenol cotton blue solution using a light microscope. These procedures allowed identification up to genus level.

Enzyme production

The biosynthesis of cellulases and hemicellulases was carried out according to Moretti *et al.* (2012), with some modifications. The fungal strains were cultivated by solid-state fermentation (SSF) using a mixture of wheat bran and sugarcane bagasse as carbon sources (1:1 w/w). The wheat bran and sugar cane bagasse were washed and dried (55 °C for 24 hours). The solid substrate (5.0 g) was placed in 250 mL erlenmeyer flasks and sterilized at 121 °C for 20 min. A volume equivalent to 10^7 spores/mL or 5 g

equivalent of dry mycelial biomass per gram of substrate of each culture was used as inoculum and suspended in a nutrient solution composed of (g/L): $(NH_4)_2SO_4$, 3.5; KH_2PO_4, 3.0; $MgSO_4 7H_2O$, 0.5; $CaCl_2$, 0.5 and Tween 80 (1.0% v/v). Additionally, 20 mL of the same nutrient solution were added to each flask (around 60% of humidity).

The fermentation was performed at 45 °C for seven days for the thermophilic strains and at 30 °C for 14 days for mesophilic strains. At intervals of 24 (thermophilic) or 48 hours (mesophilic), two flasks were taken and the fermented material was solubilized in 100 mL of distilled water, stirred for 1 hour, filtered and centrifuged at 10000 x *g*, at 4 °C. The supernatant was used as the crude enzymatic extract. All the experiments were carried out in duplicate.

Enzymatic assays

Five important hydrolytic enzymes were assayed: avicelase, carboxymethylcellulase and β-glucosidase (cellulases) and β-xylosidase and xylanase (hemicellulases).

Endoglucanase (CMCase) and avicelase activities were determined according to Moretti *et al.* (2012), with some modifications. Reaction mixtures containing 10 µL of crude enzyme and 90 µL of sodium acetate buffer 0.1 M, pH 5.0, containing carboxymethylcellulose (1%) or avicel (1%), respectively, were incubated at 60 °C for thermotolerant or at 40 °C for mesophilic strains, for 10 min. The released reducing sugars were quantified by DNS (3.5-dinitrosalicylic acid) method (Miller, 1959), using a glucose standard curve. One unit of enzyme activity (U) was defined as the amount of enzyme required to release one µmol of reducing sugar per minute under assay conditions. The β-glucosidase activity (cellobiase activity) was determined in reaction mixtures composed of 50 µL of crude enzyme extract, 250 µL of sodium acetate buffer (0.1 M; pH 5.0) and 250 µL of 4-nitrophenyl-β-Dglucopyranoside (4 mM), (PNPG, Sigma), with incubation for 10 min at 60 °C for thermophilic strains or at 40 °C for mesophilic strains (Baffi *et al.*, 2011). The reaction was stopped by the addition of 2.0 mL of 2M Na_2CO_3. One unit of enzyme activity (U) was defined as the amount of enzyme required to release one µmol of *p*-nitrophenol per minute in the assay conditions.

Xylanase activity was determined by the same methods described for endoglucanase (CMCase) and avicelase activities, except that xylan (from Beechwood) at 1% was used as substrate. The released reducing sugars were quantified by DNS method, using a xylose standard curve. One unit of enzyme activity (U) was defined as the amount of enzyme required to release one µmol of xylose per minute under assay conditions. The β-xylosidase activity was determined by the same method described for β-glucosidase activity, except that 4-nitrophenyl-β-D-xylopyranoside (4 mM) (PNPX, Sigma) was used as substrate. One unit of

enzyme activity (U) was defined as the amount of enzyme required to release one µmol of *p*-nitrophenol per minute in the assay conditions. Reaction mixtures without adding enzymatic extract were used as negative controls.

Physico-chemical characterization

The optimum pH was measured by incubating reaction mixtures at 60 °C for thermophilic or at 40 °C for mesophilic strains, for 10 min, using buffer solutions containing a pH range from 3.0 to 8.0, ranging from 0.5 to 0.5. The buffers used were: acetate buffer (pH 3.0 to 5.5); citrate / phosphate buffer (pH 5.5 at 7.0), and Tris / HCl (pH 7.0 at 8.5). In order to evaluate the optimum temperature of the enzymes, assays were carried out by incubating the reaction mixture at temperatures ranging from 30 to 80 °C (varying from 5 to 5 °C), at the optimum pH.

To determine the pH stability, the extracts were diluted with acetate buffer at a pH range between 3.0 and 8.0, and incubated at room temperature for 24 hours. After this period, the residual enzymatic activity was quantified in optimum pH and temperature. To evaluate the thermostability, the enzymatic extracts were incubated for 1 hour at temperatures ranging from 30 to 80 °C. After this period, the residual enzymatic activity was measured in optimum pH and temperature.

The effect of ethanol on the activity of the enzymes was also evaluated in the optimal pH and temperature, using increasing concentrations of ethanol in the reaction mixture (0-40%). Likewise, the enzyme activity in the presence of sugars (glucose or xylose) was evaluated.

All assays were performed in duplicate.

Results and Discussion

Isolation and identification

A total of 19 filamentous fungi and seven yeast strains were isolated from piles of sugarcane bagasse from the Cerrado region of Brazil (mesoregion of Triângulo Mineiro and Alto Paraíba, Minas Gerais) under thermophilic and mesophilic conditions. The isolated strains were submitted to amplification of the rDNA ITS/26S regions and a representative amplicon of each strain was selected to for gene sequencing. High-quality sequences were obtained and analyzed using the BLASTn tool. Consensus sequence alignments which exhibited at least 99% similarity with published sequences in GenBank were considered to belong to the same species. Four representative species were identified: *Kluyveromyces marxianus* (thermophilic), *Aspergillus niger* (mesophilic), *Aspergillus sydowii* (mesophilic) and *Aspergillus fumigatus,* which was isolated from both temperature conditions (Table 1). Upon BLASTn analysis, some fungal sequences were found to exhibit relatively low levels of similarity (below 97%) with Genbank published sequences. These strains were identified based on morphological characteristics up to genus

Table 1 - Molecular identification of fungi isolated from sugar cane bagasse piles.

Species	Strain	PCR product (bp)	Acession number	Frequency (%)
Aspergillus fumigatus	SCB4	600	KC936269	59
	SBC6	550	KF003422	
	SBC7	600	KF003423	
	SBC10	550	KF003424	
	SBC12	550	KF003425	
	SBC16	600	KF003426	
	L8	600	KF049781	
	SBCM5	600	KF003429	
	SBCM8	600	KF003431	
	SBCM12	600	KF003432	
Kluyveromyces marxianus	L1	800	KF049777	24
	L3	1000	KF049778	
	L4	800	KF049779	
	L6	1000	KF049780	
Aspergillus niger	SBCM3	800	KF003427	12
	SBCM4	800	KF003428	
Aspergillus sydowii	SBCM7	700	KF003430	6

level. Macro- and microscopic analyses allowed the classification of such strains as belonging to the genus *Aspergillus (*SBC2, 5, 9, 13, 14, M1, M2 strains). Some strains (L2, L5 and L7) did not presented satisfactory sequences and identification was not possible.

The low number of species isolated from sugarcane bagasse could be due to the increase in temperature during the decomposition of this waste, which can provide a succession of mesophilic to thermotolerant strains. Moretti *et al.* (2012) reported that few fungi species have been described from environments where high temperatures are shown. Furthermore, the lignocellulosic biomass in this residue can also limit the number of microorganisms, restricting the presence of species which are not able to produce hydrolytic enzymes (Moretti *et al.*, 2012). The results also indicated a predominance of strains from the *Aspergillus* genus in this environment. *Aspergilli* strains with environmental and economical importance have previously been described (Baffi *et al.*, 2012; Souza *et al.*, 2012). Strains from this genus are also known as good producers of extracellular enzymes, giving these microorganisms great potential for growth in wastes (Jaafaru *et al.*, 2007; Gottschalk *et al.*, 2013).

The predominant isolated species was *Aspergillus fumigatus* (59%), an ubiquitous fungus commonly found in agricultural byproducts (Souza *et al.*, 2012). This species is one of a wide range of fibrolytic enzyme-producing organisms, and some strains have been described as having great potential for cellulase and hemicellulase production

(Moretti *et al.*, 2012). The second most frequently isolated species was the yeast *Kluyveromyces marxianus,* with a frequency of 24%. This yeast is known as having a facility to grow in different substrates and in high temperatures (Foukis *et al.*, 2012). Several biotechnological applications have been associated with this species, such as production of enzymes, ethanol, reduction of lactose content in food products, production of bio-ingredients from cheese whey and many others (Fonseca *et al.*, 2008; Arrizon *et al.*, 2012). *Aspergillus niger* strains were isolated at a lower frequency (12%). This species is well known as an enzyme source for several industrial processes (Pawar and Thaker, 2009). Bansal *et al.* (2012) analyzed various agricultural and kitchen wastes to support the production of cellulases by a strain of *A. niger* isolated from decaying residues and obtained appreciable levels of different cellulases. The ethanol production using crude enzymatic complexes produced by *A. niger* and agro-industrial biomass has also been described (Rocha *et al.*, 2013). Lastly, *Aspergillus sydowii* was the least frequent species (6%). This fungus has been isolated in either marine or land environments and enzyme production with biotechnological applications has also been reported (Polizeli *et al.*, 2005). In a recent study, Matkar *et al.* (2013) isolated a terrestrial strain of *A. sydowii* from composting sites which was found to be a potential β-glucosidase producer.
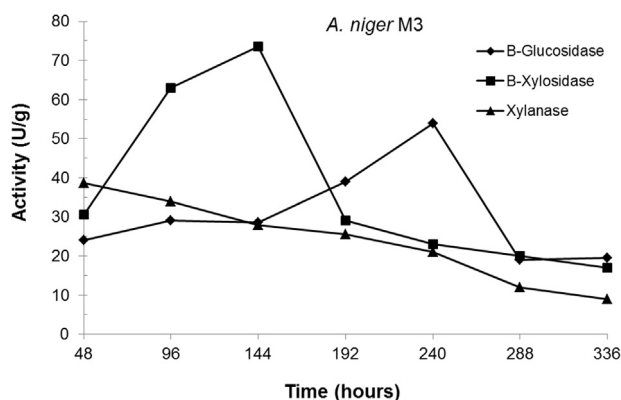
Enzyme production

Amongst the isolates, three strains were selected as good producers of β-glucosidase, β-xylosidase and xylanase after solid state fermentation (SSF): *A. sydowii* SBCM7, *A niger* SBCM3 and *A. fumigatus* SBC4. For other enzymes, significant enzymatic production was not observed under the described conditions.

A. sydowii SBCM7 produced good levels of β-glucosidase (60 U/g), but low values of for β-xylosidase (9 U/g) and xylanase (19 U/g). The peaks of production occurred at 240, 144 and 96 hours of SSF, respectively (Figure 1). The production of xylanase by this species was reported (Nair *et al.*, 2010). In a recent study, an *A. sydowii* strain isolated from composting sites was able to produce endoglucanase, exoglucanase and β-glucosidase (Matkar *et al.*, 2013). However, the production of β-glucosidase was lower than in our study.

The *A niger* SBCM3 strain exhibited peaks of maximum production for β-glucosidase (54 U/g), β-xylosidase (73 U/g) and xylanase activities (39 U/g) after 240, 144 and 48 hours of SSF, respectively (Figure 2). Xylanase production was lower than those reported in literature. Higher levels of xylanase activity were obtained (4465 ± 52 IU/gds) after SSF, using wheat bran and soybean cake as substrates (Pal and Khanum, 2010). On the other hand, β-glucosidase production was higher than in other studies (Bansal *et al.*, 2012). In addition, the SBCM3 strain was found to be a po-
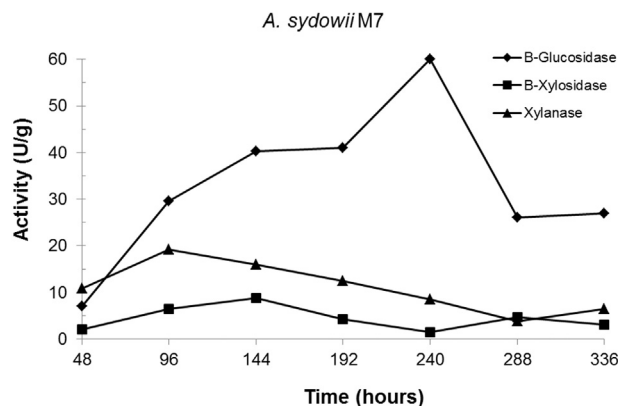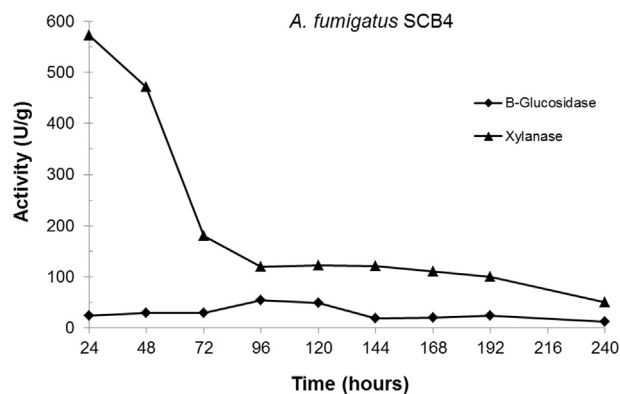

Figure 1 - Kinetics of enzyme production by *A. sydowii* SBCM7 strain. Enzymatic activity was expressed as U/g. Results are mean values from triplicate experiments.

Figure 2 - Kinetics of enzyme production by *A. niger* SBCM3 strain. Enzymatic activity was expressed as U/g. Results are mean values from triplicate experiments.

tential β-xylosidase producer showing high activity (73 U/g). Lower values of β-xylosidase were obtained by other species (Dogaris *et al.*, 2009; Oliveira *et al.*, 2014). In a similar study carried out with an *A. niger* strain isolated from copra paste, the β-xylosidase could not be detected in the crude extract (Díaz-Malváez *et al.*, 2013). These data indicate *A. niger* SBCM3 as a very good β-xylosidase- and β-glucosidase-producing strain.

A. fumigatus SBC4 strain was the best producer of β-glucosidase and xylanase (with peaks of activity of 54 U/g and 573 U/g after 96 and 24 hours of SSF, respectively) (Figure 3). Delabona *et al.* (2012) obtained a lower production of β-glucosidase (38 U/g) by an *A. fumigatus* strain (P40M2) after 96 hours under the same conditions. On the other hand, the strain P40M2 exhibited a superior xylanase activity (821 U/g). In a recent study, Moretti *et al.* (2012) also observed a higher xylanase production (1040 U/g), using a mix of sugarcane and wheat bran. Thus, though the xylanase production was smaller than in previ-

Figure 3 - Kinetics of enzyme production by *A. fumigatus* SCB4 strain. Enzymatic activity was expressed as U/g. Results are mean values from triplicate experiments.

ous reports, *A. fumigatus* SBC4 showed a good potential for β-glucosidase production.

Regarding the yeast *K. marxianus*, no enzymatic activity was detected after SSF according to the growth conditions described in the "Material and Methods" section (data not shown). Experiments under submerged fermentation using different combinations of substrates might be conducted in further studies in order to investigate its enzyme production potential.

Physico-chemical characterization of the selected extracts

The physicochemical properties of crude extracts that exhibited the highest enzymatic activities were characterized as follows: β-glucosidase extract from *A. sydowii* SBCM7 (M7-β-glu), enzymatic extract from *A. niger* SBCM3 with high production of β-xylosidase and β-glucosidase (M3-β-xyl and M3-β-glu) and the extract from *A. fumigatus* SBC4 containing both xylanase (AF4-xyl) and β-glucosidase (AF4-β-glu) activities.

The β-glucosidase from *A. sydowii* SBCM7 (M7-β-glu) exhibited maximum activity at pH 4.5 and at 50-55 °C (Table 2). Similar profiles were obtained by Madhu *et al.* (2009), with optimal activity at pH 5.0 and 50 °C. The enzyme was stable at around pH 4.0-6.5 and up to 45 °C, showing a typical profile of enzymes from mesophilic strains. Moreover, it was tolerant up to 10% of ethanol and 100 mM of glucose. The tolerance to glucose is remarkable for biotechnological applications, since the majority of microbial β-glucosidases reported in the literature are inhibited by this sugar (Baffi *et al.*, 2011; 2013).

M3-β-xyl from *A. niger* SBCM3 showed an optimal pH of 3.0 and stability in the range of pH 4.0-7.0 (Table 2). This enzyme exhibited two peaks of optimum temperature at 55 and 70 °C, suggesting the presence of isozymes. β-xylosidases with elevated optimum temperatures were reported for other *A. niger* strains (Díaz-Mal-

Table 2 - Physico chemical characterization of crude enzymes produced by the selected fungal strains.

Enzyme	Optimum pH	Optimum temperature	pH stability	Thermostability	Ethanol tolerance	Glucose tolerance	Xylose tolerance
A. sydowii β-glucosidase (M7-β-glu)	4.5	50-55	4-6.5	Up to 45 °C	Up to 10%	Up to 100 mM	NE
A. niger β-glucosidase (M3-β-glu)	3.0-3.5	50	3-6.5	Up to 40 °C	Up to 15%	Up to 100 mM	NE
A. niger β-xylosidase (M3-β-xyl)	3.0-3.5	55 and 70	4-7	Up to 60 °C	Up to 20%	Up to 600 mM	Up to 50 mM
A. fumigatus β-glucosidase (AF4-β-glu)	3.5	60	3-8	Up to 55 °C	Up to 30%	Up to 50 mM	NE
A. fumigatus xylanase (AF4-xyl)	4	60	3-8	Up to 55 °C	Up to 10%	Up to 20 mM	Up to 20 mM

*NE: not evaluated.

váez *et al.*, 2013). At optimal conditions (55 °C and pH 3.0), a high level of β-xylosidase activity was obtained (141 U/g of dry substrate). This value of activity was higher than in other studies (Díaz-Malváez *et al.*, 2013). Furthermore, M3-β-xyl maintained 85% of residual activity after incubation at 60 °C for 1 hour (Table 2), showing higher thermostability than that previously reported (Benassi *et al.*, 2013). This thermostability could be of special interest for application in large-scale industrial processes that may be subject to temperature variations (Moretti *et al.*, 2012). M3-β-xyl was tolerant to xylose up to 50 mM (Table 2). These results differ to those reported by Benassi *et al.* (2013), which described a β-xylosidase with atolerance of up to 100 mM of xylose. With regard to glucose, M3-β-xyl was not inhibited, preserving about 78% of residual activity at 600 mM of glucose (Table 2). In addition, at 100 mM, this sugar stimulated the β-xylosidase activity. Higher values of inhibition by glucose have been reported (Benassi *et al.*, 2013). In addition, the enzyme was very tolerant up to 20% of ethanol, reinforcing the potential application of this *A. niger* β-xylosidase in hydrolysis processes.

M3-β-glu exhibited some similar properties with M3-β-xyl (optimal activity at pH 3.0-3.5 and at 50 °C, high stability between pH 3.0-6.5 and up to 15% of ethanol). However, it was stable up to 40 °C and 100 mM of glucose (Table 2). These related characteristics of both enzymes emphasize the simultaneous use of a single crude enzymatic extract from *A. niger* SBCM3 containing both activities, which could be fairly appreciable for a better efficiency in biomass degradation.

Maximum activities were obtained at pH 4.0 and 3.5 for xylanase (AF4-xyl) and β-glucosidase (AF4-β-glu) from the *A. fumigatus* SBC4 strain, respectively (Table 2), revealing typical profiles of acidophilic enzymes. Studies showing acidic xylanases have been reported (Souza *et al.*, 2012). Moretti *et al.* (2012) observed an optimum pH of 4.5-5.0 for a xylanase from *A. fumigatus* isolated from sugarcane bagasse. Previous works also suggested acid optimum pHs for β-glucosidases (Delabona *et al.*, 2012; Baffi *et al.*, 2013). Both enzymes were stable throughout the pH range of 3.0-8.0 (Table 2), with higher pH stability than in other studies (Moretti *et al.*, 2012; Baffi *et al.*, 2013). The enzymes AF4-xyl and AF4-β-glu showed an optimum temperature of 60 °C and high stability up to 55 °C (Table 2). These results strongly suggest that these AF4-xyl and AF4-β-glu enzymes seem to be thermophilic, which could be useful for many biotechnological processes that work in high temperatures. In addition, these similar profiles indicate that a single crude extract containing both xylanase and β-glucosidase activities could be applied during the saccharification processes, functioning properly in acid conditions and elevated temperatures. Consequently, the characteristics of the culture medium could be maintained more easily to achieve high sugar yields, avoiding additional procedures.

AF4-β-glu was active up to 30% of ethanol, demonstrating good tolerance to this compound. However, AF4xyl was stable up to 10% of ethanol (Table 2). Both crude enzymes were inhibited by glucose, with tolerance up to 20 (AF4-xyl) and 50 mM (AF4-β-glu) of this sugar, respectively (Table 2). AF4-xyl was also inhibited at low concentrations of xylose (Table 2).

Conclusions

This work reported the molecular identification of the fungal microbiota isolated from sugarcane bagasse piles from the Brazilian Cerrado and their potential as sources of lignocellulose-degrading enzymes. Three *Aspergilli* strains were selected as good producers of β-glucosidase, β-xylosidase and xylanase. Among them, *A. niger* SBCM3 strain was the best producer of β-xylosidase, with suitable characteristics for industrial applications, such as acid optimum pH, thermostability and tolerance to glucose. This strain was also able to produce high β-glucosidase activity with some similar properties to those detected for β-xylosidase. *A. fumigatus* SBC4 strain was selected as the best β-glucosidase and xylanase producer. Both enzymes exhibited appropriate features for application in procedures of plant biomass reuse, such as optimal activity at acid pH and high temperatures, thermostability, pH stability and tolerance to ethanol. These equivalent properties of the selected enzymatic preparations suggest their promising synergistic use in cocktails in future research on the saccharification of vegetal residues, involving the fabrication of bioproducts with aggregate value, such as ethanol production. Further studies will investigate the combined action of these extracts in the hydrolysis of lignocellulosic biomass from residues.

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