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ORIGINAL PAPER

# Cellulases from *Penicillium funiculosum*: production, properties and application to cellulose hydrolysis

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**Abstract** The objective of this work is to investigate the utilization of two abundant agricultural residues in Brazil for the production and application of cellulolytic enzymes. Different materials obtained after pretreatment of sugarcane bagasse, as well as pure synthetic substrates, were considered for cellulase production by *Penicillium funiculosum*. The best results for FPase (354 U $L^{-1}$) and $\beta$-glucosidase (1,835 U $L^{-1}$) production were observed when sugarcane bagasse partially delignified cellulignin (PDC) was used. The crude extract obtained from PDC fermentation was then partially characterized. Optimal temperatures for cellulase action ranged from 52 to 58°C and pH values of around 4.9 contributed to maximum enzyme activity. At 37°C, the cellulases were highly stable, losing less than 15% of their initial activity after 23 h of incubation. There was no detection of proteases in the *P. funiculosum* extract, but other hydrolases, such as endoxylanases, were identified (147 U $L^{-1}$). Finally, when compared to commercial preparations, the cellulolytic complex from *P. funiculosum* showed more well-balanced amounts of $\beta$-glucosidase, endo- and exoglucanase, resulting in the desired performance in the presence of a lignocellulosic material. Cellulases from this filamentous fungus had a higher glucose production rate (470 mg $L^{-1}$ $h^{-1}$) when incubated with corn cob than with Celluclast®, GC 220® and Spezyme® (312, 454 and 400 mg $L^{-1}$ $h^{-1}$, respectively).

**Keywords** Cellulases · Endoglucanase · $\beta$-Glucosidase · Sugarcane bagasse · *Penicillium funiculosum*

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## Introduction

Brazil has a natural vocation for biotechnology, particularly in the agroindustrial sector, which is evidenced by their high capacity to produce renewable foodstocks. During the processing of this biomass, hundreds of millions of tons of lignocellulosic residues are generated, creating serious environmental problems [1–3]. A classical example of this is sugarcane (*Saccharum* spp.). In 2007, 33% of the entire world's sugarcane production (1.55 billion tons) was harvested in Brazil [4]. The processing of this enormous amount of cane, used for sugar and fuel ethanol production, generates bagasse as the main lignocellulosic residue. About 90% of this residue is used in mills for energy cogeneration [5], but there are enormous surpluses (about 15 million tons in the last harvest season). One alternative for bagasse utilization is to produce cellulases in situ from the cellulosic fraction, which corresponds to 40–50% (w/w) of this residue [6]. These biocatalysts can be further used to hydrolyze more cellulose, with the intention of producing second-generation ethanol through the simultaneous saccharification and fermentation (SSF) process in the same mill, thus increasing the ethanol yield from the feedstock.

Due to its crystallinity, cellulose hydrolysis requires the synergism of different cellulolytic enzymes that together promote higher activity than the sum of the individual enzyme activities [7]. The cellulolytic complex involves

three main groups of enzymes, the first group being the $\beta$-1,4-exoglucanases, which are represented by the cellobiohydrolases (CBH, EC 3.2.1.91) and glucanohydrolases (GH, EC 3.2.1.74). These enzymes catalyze the production of either cellobiose or glucose units from the reducing (CBH I) and nonreducing (CBH II) ends of cellulosic fibrils, and are generally inhibited by their hydrolysis products. The second group involves the $\beta$-1,4-endoglucanases (EG, EC 3.2.1.4), which randomly break internal glycosidic linkages of the amorphous region of cellulose, liberating oligosaccharides of various lengths. Finally, the third group is composed of $\beta$-1,4-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose and soluble oligosaccharides into glucose [8].

Species from the genus *Penicillium* have been reported to possess potential for cellulase production [9–13]; nevertheless, *Penicillium funiculosum* strains have seldom been reported. Van Wyk [14] compared the hydrolytic performances of cellulases from *P. funiculosum* and *T. reesei*, and indicated that, when used separately, the former liberated higher levels of glucose upon incubation with several cellulosic substrates. In addition, a synergy was observed upon mixing the pools from both strains, indicating that the *P. funiculosum* cellulolytic pool efficiently complements the $\beta$-glucosidase-deficient pool from *T. reesei*.

Cellulases can be utilized for applications in the textile and laundry, food and feed, pulp and paper, baking, alcohol from biomass, and waste treatment industries [15–17]. In the textile industry, for example, acid and neutral endoglucanases that are very stable at temperatures above 60°C are used, respectively, in the biopolishing and biostoning techniques that are currently replacing abrasive stonewashing processes [15]. The application of cellulases to the hydrolysis of lignocellulosic materials (biomass) in order to further convert the released fermentable sugars into ethanol has increased due to not its environmental benefits, but also the worldwide demand for renewable fuels.

Therefore, the objective of this work was to evaluate the production of cellulases by a strain of the filamentous fungus *Penicillium funiculosum*, selected previously because it exhibits the three main cellulolytic activities to the same extent, as well as to partially characterize the produced enzymes. Finally, an investigation of their application to the hydrolysis of a lignocellulosic material was also performed.

## Materials and methods

### Cellulase commercial preparations

Celluclast® was kindly provided by Novozymes Latin America (Araucaria, PR, Brazil). Spezyme® CP and GC 220® were purchased from Genencor International Inc. (Rochester, NY, USA).

### Raw materials and pretreatments

Sugarcane bagasse was provided by Costa Pinto Mill (Piracibaca, SP, Brazil). This biomass was subjected to several pretreatments to generate different carbon sources (Fig. 1). Acid and alkali pretreatments were carried out to increase the cellulose content in the materials by removing the hemicellulose fraction and partially removing the lignin fraction, respectively. The acid pretreatment consisted of incubating the solid material with a 3% (v/v) sulfuric acid solution (solid:liquid ratio of 1:4), while the alkali pretreatment was performed by incubating the material with a NaOH 4% (w/v) solution (solid:liquid ratio of 1:20). Both pretreatments were carried out at 121°C (1 atm) for 20 min. Granulometric determinations were done using a vibratory shaker (model 76773, Viatest, Kuhardt, Germany) coupled with sieves (Tyler 5–100 mesh). The compositions of the obtained materials were determined according to the Klason method [18], and sugars were detected using a Waters® Corporation (Milford, MA, USA) high-performance liquid chromatograph (HPLC) with Milli-Q water used as the mobile phase at a rate of 0.6 mL $min^{-1}$, and an Aminex® HPX-87P column (Bio-Rad Laboratories, Hercules, CA, USA). Oven and detector temperatures were set to 75 and 40°C, respectively.

Another lignocellulosic residue, corn cob, was harvested from a farm (Pinheiral, RJ, Brazil). It was milled, sieved, and the particles corresponding to Tyler meshes of between 28 and 42 were stored for subsequent investigations. The cellulases produced from pretreated sugarcane bagasse were evaluated for their corn cob hydrolysis efficiencies (Fig. 1).

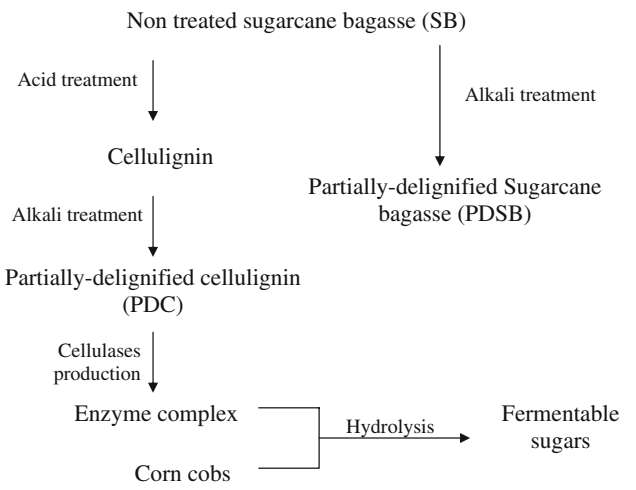


**Fig. 1** Raw materials obtained from sugarcane bagasse pretreatments

### Microorganism and growth conditions

*Penicillium funiculosum* (ATCC 11797) was maintained in PDA plates (DIFCO, Franklin Lakes, NJ, USA) at 30°C for 9–10 days before inoculation.

Resuspended spores of *P. funiculosum* ($5.33 \times 10^7$) were inoculated in 100 mL of modified Mandels & Weber medium [19] in 500 mL conic flasks, and incubated at 200 rpm. After 3 days, 10 mL of the medium containing growing cells (3.83 g $L^{-1}$) were transferred to 1 L conic flasks containing 200 mL of modified Mandels–Weber medium with 7.5 g $L^{-1}$ of pretreated sugar cane bagasse instead of glucose, and incubated at 200 rpm for 15 days. At regular intervals, samples were taken and sonicated for 1 min for enzyme desorption, and then centrifuged at 20,000*g* for 5 min for cell and residual substrate harvesting. Supernatants were frozen and stored for the assays.

### Assays

FPase, endoglucanase and $\beta$-glucosidase activities were determined using Whatman no. 1 filter paper, carboxymethylcellulose (CMC) (Sigma, St. Louis, MO, USA) and cellobiose (Sigma) as substrates, according to previously described standard conditions [20]. These protocols were used as standards for the subsequent analysis. Exoglucanase activity was determined under the same conditions used for the filter paper, with the exception that the $1 \times 6$ cm filter paper strip was replaced with 50 mg of Avicel. The total reducing sugars (TRS) liberated during the enzymatic assays and corn cob hydrolyses were quantified by the DNS method [21], using glucose as a standard, and the glucose liberated during the $\beta$-glucosidase reaction was quantified using an analytical kit based on the enzymes glucose oxidase and peroxidase (Laborlab, São Paulo, SP, Brazil). Endoxylanase and protease activities were determined according to the methods of Bailey et al. [22] and Charney and Tomarelli [23], respectively. For all activities, except that of protease, one enzyme unit (U) was defined as the amount of biocatalyst that liberated 1 micromole of the corresponding monosaccharide (xylose for xylanase and glucose for the other groups of enzymes) per minute, under the assay conditions used. For protease, 1 U was defined as the enzyme quantity that produced an increase of 1 absorbance unit per minute, under the assay conditions used. Total extracellular protein content was measured using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA, USA), according to the Bradford method [24], and BSA (Sigma) was used as the standard. All analyses were done in triplicate in a temperature-controlled incubator (Dubnoff, Nova Técnica, São Paulo, SP, Brazil). Unless otherwise stated, data are presented as mean $\pm$ 1 SD.

### Characterization of crude extracts

To investigate the dual influence of pH and temperature on the catalytic power of each cellulase, these factors were manipulated simultaneously. The evaluated temperatures ranged from 30 to 80°C and the pH values ranged from 3.0 to 6.0. All analyses were done under standard conditions. Data was fitted to Gaussian and Lorentzian models, and the optimal pH and temperature were estimated using the SigmaPlot 9.0 software package (Systat Software Inc., San Jose, CA, USA).

The stabilities of the enzymatic complex were determined by incubating the crude extracts at three different temperatures (37, 50 and 60°C). Enzymatic quantifications were done under standard conditions.

To estimate the $\beta$-glucosidase parameters, solutions of 1–40 mM cellobiose were used, and to estimate the endoglucanase parameters, medium-viscosity 1–20 g $L^{-1}$ CMC solutions were considered. Reactions were carried out at 50°C, pH 4.8, for 5–30 min. Parameters were estimated using nonlinear regression (Levenberg–Marquardt method) using the Statistica® 6.0 software package (Statsoft Inc., Tulsa, OK, USA), and were expressed along with their 95% confidence interval ranges.

### Application of cellulases to corn cob hydrolysis

Crude extracts from *P. funiculosum* that had been concentrated five times in a rotoevaporator (at about 37°C and 6.3 kPa), as well as commercial preparations, were used to hydrolyze cellulose in milled untreated corn cob. Enzymes were added to make 11 U $g^{-1}$ raw material (assayed under filter paper). Experiments were carried out in 50 mL conical flasks containing 1 g of corn cob and a total liquid volume of 16 mL (i.e., crude extracts or enzymatic preparations diluted in citrate buffer of pH 4.8) for 48 h at 45°C in a rotary shaker at 150 rpm. At periodic time intervals, glucose and TRS were measured.

## Results and discussion

*Penicillium funiculosum* was cultured in conical flasks in the presence of several carbon sources, both pure (CMC, Avicel) or lignocellulosic. The latter were obtained from different pretreatments of sugarcane bagasse, which were performed in order to remove the hemicellulose (diluted acid pretreatment) and lignin (alkali pretreatment) fractions. The pretreatments caused a reduction in particle size, which in turn meant an increase in the superficial area exposed to microbial attack. For example, the SB showed a mean particle size of 1,163 μm. After acid pretreatment, the obtained cellulignin contained particles measuring an

**Table 1** Partial characterization of sugarcane bagasse and sugarcane bagasse-derived materials

| Raw material | Glucan content (%) | Xylan content (%) | Lignin (%) | Ash (%) |
|---|---|---|---|---|
| SB | 38.0 ± 0.8 | 24.0 ± 2.5 | 19.4 ± 0.4 | 7.9 ± 1.1 |
| PDSB | 58.8 ± 0.9 | 14.5 ± 0.3 | 11.2 ± 0.7 | 8.7 ± 1.0 |
| Cellulignin | 51.9 ± 2.2 | ND | 26.1 ± 0.6 | 4.1 ± 0.4 |
| PDC | 68.8 ± 1.2 | ND | 9.3 ± 0.6 | 3.5 ± 0.4 |

*ND*, not detected

average of 509 μm. When submitted to the other pretreatment (alkali), the raw material generated (PDC) measured about 426 μm. Additionally, HPLC analysis revealed that the acid pretreatment completely removed the xylan fraction from the bagasse, thus increasing its glucan content by 37% (considering glucose from both the cellulose and the hemicellulose fractions). Following a subsequent alkaline pretreatment of the obtained cellulignin, the remaining solids (PDC) were enriched by a further 33% in glucan and the lignin content dropped by 64% (Table 1).

During the experiments, samples were withdrawn periodically and analyzed according to their FPase (the global synergistic activity)—predominantly their endoglucanase (under CMC) and β-glucosidase (under cellobiose) activities. The maximum values obtained for each assay are shown in Table 2.

As expected, faster kinetics were observed when *P. funiculosum* was cultivated in the presence of the synthetic soluble substrate CMC. Endoglucanase was the main enzyme produced, since its natural inductor (amorphous cellulose) was used as the substrate in the fermentation. Among the sugarcane bagasse-derived carbon sources, the lowest FPase and β-glucosidase activities were achieved using the SB. Because no fraction was intentionally removed before fermentation, it is possible that the cellulosic chain was partially covered by lignin and hemicellulose. PDC induced the highest FPase and β-glucosidase activities, even when compared to pure substrates such as CMC and Avicel. The results shown in Table 2 clearly indicate that the acid pretreatment positively influenced endoglucanase induction in *P. funiculosum*, reaching 3,588 U $L^{-1}$ when the cells were incubated with cellulignin. Nevertheless, as the main goal of this study is to produce a cellulolytic complex that performs complete cellulose hydrolysis most efficiently, a high level of β-glucosidase activity in the pool is also fairly important for glucose release. PDC shows a good balance of endoglucanase and β-glucosidase activities and the highest observed FPase activity, justifying its selection as the most suitable material for cellulase production, and so this raw material was chosen for further investigations.

Figure 2 shows the kinetics of the cellulases as well as the total extracellular protein production during the incubation of *P. funiculosum* with PDC. Data were fitted to polynomial or Boltzmann models depending on which of them resulted in the highest $R^2$. Endoglucanase was induced earlier than β-glucosidase, since it acts in the polymeric cellulose, which is abundant at the beginning of fermentation. As this substrate was hydrolyzed into short-chain oligosaccharides, β-glucosidase induction increased, even after the endoglucanase activity became stable. The maximum FPase, endoglucanase and β-glucosidase activities observed were 354, 1,835 and 1,836 U $L^{-1}$, respectively, corresponding to 76, 396 and 396 U $g^{-1}$ cellulose.

**Table 2** Cellulase production by *P. funiculosum* in several raw materials

| Raw material | Maximum activity[a] (U $L^{-1}$) | | |
|---|---|---|---|
| | FPase | Endoglucanase | β-glucosidase |
| CMC | 70 ± 16 (9) | 446 ± 38 (57) | 316 ± 34 (98) |
| Avicel | 190 ± 26 (169) | 2,732 ± 226 (57) | 926 ± 94 (192) |
| SB | 38 ± 15 (126) | 1,169 ± 32 (169) | 555 ± 5 (169) |
| PDSB | 164 ± 28 (169) | 514 ± 27 (81) | 938 ± 0 (192) |
| Cellulignin | 128 ± 1 (81) | 3,588 ± 118 (81) | 1,224 ± 32 (192) |
| PDC | 354 ± 29 (182) | 1,835 ± 269 (126) | 1,835 ± 111 (276) |

[a] Values in parentheses correspond to the fermentation times (hours) at which the maximum activities were obtained

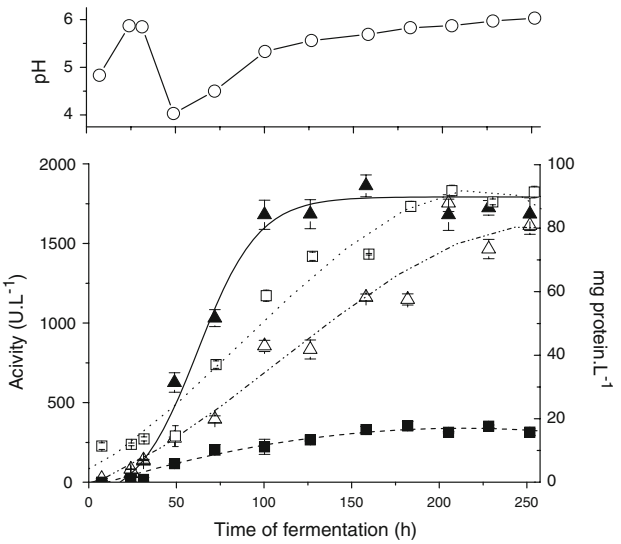


**Fig. 2** Cellulase production by *P. funiculosum* from PDC. FPase activity (*closed squares*, *dashed line*); β-glucosidase activity (*open triangles*, *dash-dot line*); endoglucanase activity (*closed triangles*, *solid line*); protein content (*open squares*, *dotted line*); pH (*open circles*, *solid line*)

**Table 3** Maximum values for volumetric productivity and specific activity obtained by *P. funiculosum* during cellulase production in PDC

| Response factor | FPase | β-Glucosidase | Endoglucanase |
|---|---|---|---|
| Volumetric productivity (U $L^{-1}$ $h^{-1}$) | 2.83 ± 0.46 (72) | 8.54 ± 0.35 (100) | 16.76 ± 0.91 (100) |
| Specific activity (U $mg^{-1}$ protein) | 7.98 ± 0.07 (49) | 19.06 ± 0.47 (49) | 43.14 ± 4.22 (49) |

Values in parentheses correspond to the fermentation times (hours) at which the maximum activities were obtained

The response factors shown in Table 3 demonstrate that, although the maximum activities observed for endoglucanase and β-glucosidase were the same, the volumetric productivity and specific activity of the former (17 U $L^{-1}$ $h^{-1}$ and 43 U $mg^{-1}$, respectively) were higher. This is due to the faster kinetics of endoglucanase and the fact that the protein content of the medium had not reached its maximum value when the maximum activity was achieved.

Strains of the genus *Penicillium* have been investigated previously for cellulase production [12, 13, 25]. A study of about twelve strains, cultivated with solka floc under submerged fermentation, demonstrated that *P. simplicissimum* IBT 15303 was the best β-glucosidase producer (maximum 120 IU $g^{-1}$ cellulose), while *P. brasilianum* (IBT 20888) achieved the highest FPase activity (34 IU $g^{-1}$ cellulose) [13]. This latter strain was incubated longer with several lignocellulosic and pure raw materials, and the best β-glucosidase and FPase activities (175 and 29 IU $L^{-1}$, respectively) were obtained after 165 h of fermentation. Wheat bran and straw were used as carbon sources for cellulase production by *P. decumbens* in bioreactors. When optimization steps were performed beforehand, the maximum FPase and β-glucosidase activities achieved were 15 and 51 U $g^{-1}$, respectively [25]. Comparing these results with those obtained in the present work, it is clear that *P. funiculosum* is a promising strain for cellulase production.

The effect of alkali pretreatment on cellulases production was studied by Aiello et al. [26], using sugarcane bagasse as the raw material. The FPase activities obtained by culturing *T. reesei* QM9414 with native and pretreated materials were 85 and 90 IU $L^{-1}$, respectively. Both of these values were lower than that observed when the control carbon source, Avicel, was considered (163 IU $L^{-1}$). Recently, a biological pretreatment was reported for cellulase production by *P. echinulatum* using sugarcane bagasse as the raw material [27]. The FPase, endoglucanase and β-glucosidase activities were higher when untreated sugarcane bagasse was used instead of the pretreated material, resulting in 950, 1,600 and 210 U $L^{-1}$, respectively, for the former source. In addition, this strain exhibited low β-glucosidase production compared to the other group of cellulases, which is unfavorable to complete cellulose hydrolysis. In the present work, the incubation of *P. funiculosum* in PDC resulted in high levels of cellulase production, as well as significant β-glucosidase production.

In order to partially characterize the *P. funiculosum* cellulases, the crude extract obtained at the end of fermentation using PDC as a substrate was investigated in order to establish suitable applications for the enzymes present.

Figure 3 shows 3D plots obtained after simultaneously investigating the influences of pH and temperature on cellulase activity. The dots represent the averages of three replicates. In the upper right corners of the graphs, the pH ($pH_o$) and temperature ($T_o$) corresponding to optimum enzyme performance are presented, along with their corresponding SDs.

Considering the conditions that led to the greatest activities, the cellulases were more susceptible to decreases than increases in pH; that is, activity losses were higher in the acid pH range, especially for β-glucosidases. When the enzymes were subjected to pH and temperature variations, they exhibited unimodal activity curves, similar to convex parabolas. At low pH, the higher the temperature the higher the cellulase activity, until optimal activities were reached between 52 and 58°C. Also, the evaluated factors influenced the activity of β-glucosidase more than those of endoglucanase and FPase. This means that there are advantages to the cellulolytic complex produced when cellulose liquefaction (primary hydrolysis stage) is desired under mild conditions, such as those used in the SSF process in the production of ethanol [28, 29] or some organic acids [30].

The cellulases were incubated at three temperatures to investigate the thermal stability of the crude extract produced: 37°C in order to predict the behavior of the enzymes under SSF conditions, 50°C for the SHF process, and 60°C to determine the applicability of the enzymes to processes where higher temperatures are required. Thus, Fig. 4 shows the relative activities that were periodically determined over the course of 8 h, along with an additional measurement at 23 h. The cellulases were highly stable at 37°C throughout the incubation period. Increasing the temperature to 50°C reduced enzyme activity, especially for β-glucosidase and endoglucanase, which exhibited 56 and 53%, respectively, of their initial activities at the end of the incubation time (23 h) at this temperature. At 60°C, the

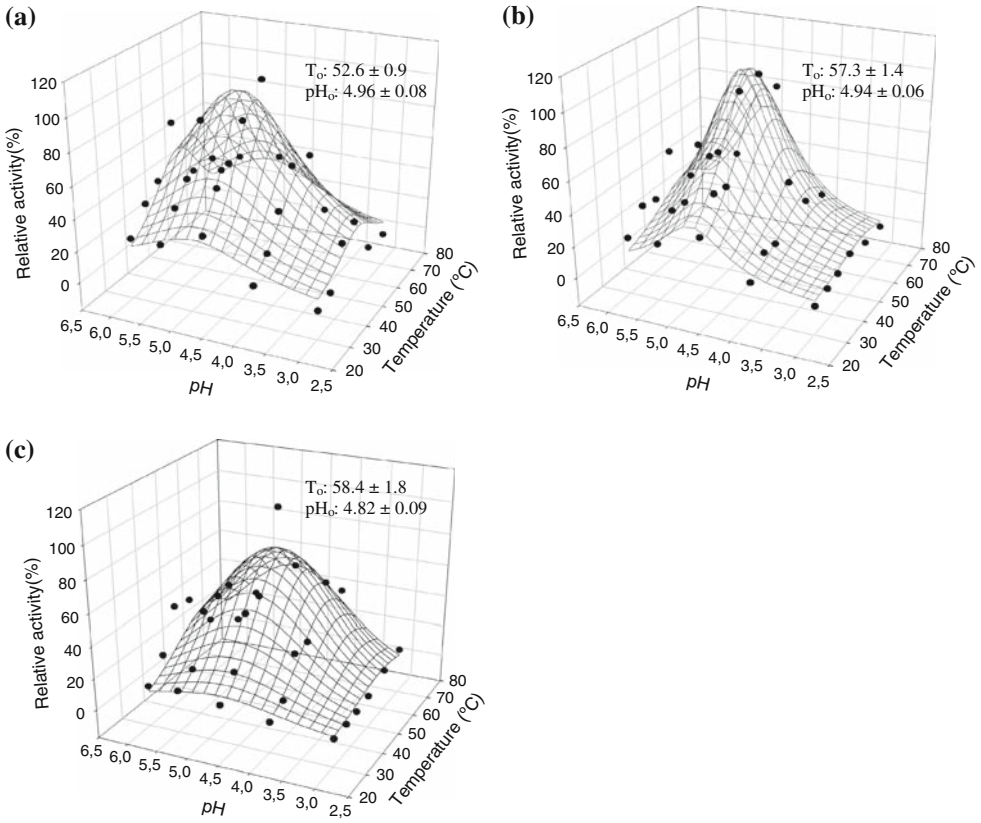


**Fig. 3** Influence of pH and temperature on the enzymatic activity of: **a** FPase; **b** $\beta$-glucosidase; **c** endoglucanase

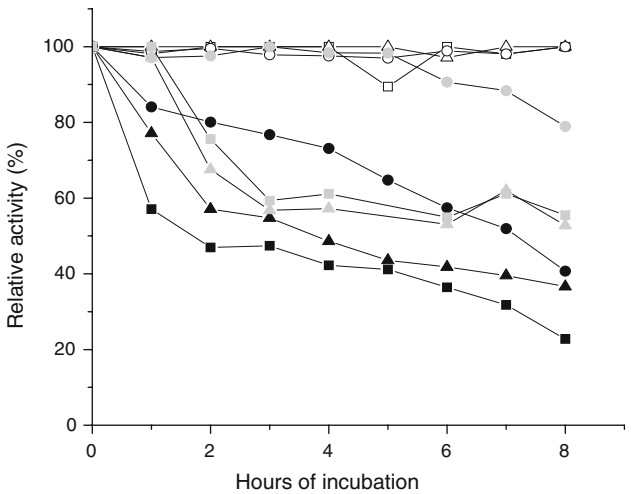


**Fig. 4** Thermal stability of FPase (*circles*), $\beta$-glucosidase (*triangles*), and endoglucanase (*squares*) at 37°C (*white symbols*), 50°C (*gray symbols*) and 60°C (*black symbols*)

activity of FPase was the most stable, since it maintained 44% of its initial activity after 8 h, while $\beta$-glucosidase and endoglucanase showed 42 and 18%, respectively, at the same time point. These results suggest that the extract produced shows the greatest potential for the SSF process (due to its high stability at 37°C) as well as for applications that require that enzyme activity is maintained at higher temperatures for a short period (a few hours), such as processes used in the textile industry [31].

**Table 4** Kinetic parameters of endoglucanase and $\beta$-glucosidase from *P. funiculosum*

| Kinetic parameters | Endoglucanase | $\beta$-Glucosidase |
|---|---|---|
| $K_M^a$ | 17.691 ± 0.126 | 1.653 ± 0.046 |
| $V'_{max}$ (mmol $L^{-1}$ $min^{-1}$) | 0.780 ± 0.006 | 0.024 ± 0.001 |

[a] Expressed as g $L^{-1}$ for endoglucanase, and expressed as mM for $\beta$-glucosidase

Kinetic parameters were estimated by the incubating the produced cellulases with cellobiose and CMC. The results are shown in Table 4. Up to the maximum evaluated concentrations (40 mM of cellobiose and 20 g $L^{-1}$ of CMC), the $\beta$-glucosidase and endoglucanase were not inhibited by their respective substrates. The parameter $V'_{max}$ was much higher for endoglucanase than for $\beta$-glucosidase. This is probably because the enzymes differ in terms of the substrates they can act upon: while $\beta$-glucosidase exhibits major activity towards cellobiose, and the analytic method used for **their** quantification specifically determines glucose concentration, endoglucanase hydrolyzes oligosaccharides with varying degrees of polymerization. The standard methodology reported by Ghose [20], which was used in this work, quantifies all products with

reducing power. This means that, during the kinetic assays, $\beta$-glucosidase acted on only one molecule (just one reaction rate was possible), but endoglucanase, because of its particular mechanism of action, recognized and could act on various substrates, meaning that more than one reaction could be processed and accounted for simultaneously.

Incubating the cellulases produced with some substrates resulted in low endoxylanase activity ($148 \pm 21$ U $L^{-1}$) and no protease activity when azocasein was used. Endoglucanase activity decreased as the viscosity of the CMC solution increased, probably due to diffusivity problems. Activities in high, medium and low viscosity CMC were $2{,}909 \pm 260$, $4{,}383 \pm 358$ and $5{,}451 \pm 350$ U $L^{-1}$, respectively. When the three major groups of cellulases (endoglucanase, exoglucanase and $\beta$-glucosidase) were classified (Fig. 5), the extract produced by *P. funiculosum* showed well-balanced activities compared to commercial preparations (Celluclast®, GC-220® and Spezyme®). In Fig. 5, the activities are normalized to 100% to highlight the contribution of each group of enzymes to the total pool activity. Spezyme® showed the lowest percentage of $\beta$-glucosidase activity, since it represented only 1.5% of the total activity detected, which indicates the low potential of this preparation for glucose release. 11.9% of the total cellulose activity for *P. funiculosum* extract was due to exoglucanase, while this cellulase contributed only 2.7, 5.8 and 2.7% of the total cellulase activities of Celluclast®, Spezyme® and GC-220®, respectively. If we consider the endoglucanase activity of each sample to be 1, the ratio of this enzyme to exoglucanase and $\beta$-glucosidase was 1:0.23:0.16 for the *P. funiculosum* extract, while these ratios for Celluclast®, Spezyme® and GC-220® were 1:0.03:0.02, 1:0.02:0.06 and 1:0.06:0.03, respectively. The very low contributions of glucose- and cellobiose-liberating enzymes ($\beta$-glucosidase and exoglucanase, respectively) in the commercial formulations indicate that they are more suitable for applications that require cellulose depolymerization than for uses in which cellulose saccharification is desired.

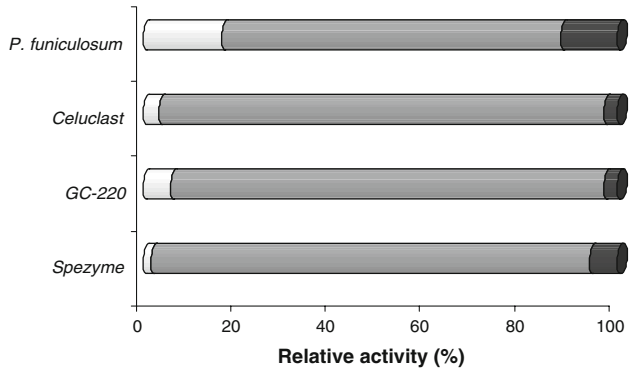


**Fig. 5** Distribution of endoglucanase (*gray*), exoglucanase (*black*) and $\beta$-glucosidase (*white*) activities in the crude extract from *P. funiculosum* and in commercial preparations

Finally, in an attempt to evaluate the performances of cellulases from *P. funiculosum* in cellulose hydrolysis, milled untreated corn cobs were used. Over 48 h, glucose and TRS were measured and glucose production rates were determined. The results are shown in Table 5. Since the hydrolysis exhibited Michaelis–Menten behavior, the highest rates were observed at the beginning of each enzymatic reaction (in the first 8 h). Cellulases from *P. funiculosum* yielded the highest glucose production rate (471 mg $L^{-1}$ $h^{-1}$), which was ca. 51% higher than the lowest observed rate. The cellulases obtained in this work also gave the highest glucose/TRS ratio (0.867), which reinforces the results shown in Fig. 5 concerning the significant activities of cellulose-end hydrolases. The enzymatic complex produced by *P. funiculosum* was shown to be more effective at causing the complete hydrolysis of cellulose to its monosaccharide (liberating more glucose and, consequently, less oligosaccharide with reducing power).

## Conclusions

The present work showed that cellulase production by *Penicillium funiculosum* ATCC 11797 was induced by culturing this strain in sugarcane bagasse-derived material. Acid pretreatment followed by alkali incubation produced the greatest particle size reduction and resulted in a material, termed "PDC," that presented the best fermentation results. The crude extract obtained showed similar levels of activity from each type of cellulase (endoglucanase, $\beta$-glucosidase and exoglucanase) and no proteolytic activity under azocasein. The enzymes showed more catalytic power at temperatures ranging from 52.6 and 58.4°C and at pH values of between 4.82 and 4.96. At 37°C the enzymes exhibited no significant activity loss during the period of incubation (23 h), while FPase showed the best stability at higher temperatures (e.g., 50 and 60°C).

**Table 5** Application of cellulases from *P. funiculosum* and from a commercial preparation to the hydrolysis of corn cob

| Source of cellulases | Maximum glucose production rate (mg $L^{-1}$ $h^{-1}$) | Ratio glucose/TRS[a,b] |
|---|---|---|
| *P. funiculosum* ATCC 11797 | $471.2 \pm 1.2$ | $0.867 \pm 0.002$ |
| GC 220® | $453.7 \pm 7.1$ | $0.824 \pm 0.021$ |
| Spezyme® | $399.7 \pm 0.8$ | $0.711 \pm 0.008$ |
| Celluclast® | $311.6 \pm 2.0$ | $0.668 \pm 0.008$ |

[a] After 8 h of hydrolysis

[b] Total reducing sugars

The performances of the cellulases in corn cob hydrolysis proved that those from *P. funiculosum* act more synergistically than those in the commercial preparations Celluclast®, GC-220® and Spezyme®, resulting in a higher glucose production rate. Thus, cellulases produced by this strain show great potential for applications that aim for complete cellulose saccharification and require temperatures near to 37°C, such as the SSF process.

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