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Aspergillus fumigatus Thermophilic and Acidophilic Endoglucanases

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Abstract This study evaluated the production of cellulolytic enzymes by an *Aspergillus fumigatus* strain, isolated from sugar cane bagasse, according to its ability to grow on microcrystalline cellulose as the sole carbon source. The effect of the carbon source (brewer's spent grain, sugarcane bagasse, and wheat bran) and of the nitrogen source (corn steep liquor and sodium nitrate) on cellulase production was studied using submerged and solid state cultivations at 30 °C. The highest levels of endoglucanase (CMCase) corresponded to 365 U L^{-1} and was obtained using sugarcane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation within 6 days of cultivation. This supernatant was used to run a sodium dodecyl sulfate polyacrylamide gel electrophoresis that showed six bands with endoglucanase activity. CMCase activity was higher at 65 °C and pH 2.0, indicating that this microorganism produces a thermophilic and acid endoglucanase. Solid state cultivation favored FPase production, that reached 47 U g^{-1} of dry substrate (wheat bran and sugarcane bagasse) within 3 days.

Keywords *Aspergillus fumigatus* · Thermophilic endoglucanase · Acidophilic endoglucanase · CMCase · Agro-industrial by-products

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Introduction

Lignocellulosic residues from agriculture and forestry have potential as cheap and renewable feedstocks for large-scale production of fuels and chemicals. The biodegradation of cellulose to soluble sugar is a process which is only possible after the action of complex cellulolytic pools produced by cellulolytic microorganisms. In recent years, more scientific attention is given to this process due to its environmental and economical significance [1–3].

Cellulolytic microorganisms produce a complex array of glycosyl hydrolases. Filamentous fungi, typically *Trichoderma* and *Aspergillus* species, are the main source for industrial cellulase preparations. Their enzyme profiles are dominated by endoglucanases (1,4-β-D-glucan glucanohydrolases) and exoglucanases (1,4-β-D-glucan cellobiohydrolases) but also include a range of accessory enzymes, including β-glucosidase, hemicellulases, and pectinases. Irrespective of the recent gains on the reported enzyme production costs, enzymatic hydrolysis of biomass is still uneconomical. Further cost reductions are needed for full-scale process commercialization, and efforts to further improve enzyme performance for lignocellulosic feedstocks continue [1, 4, 5].

Aspergillus fumigatus, one of a wide range of cellulase-producing organisms, is ubiquitous in Brazil, being commonly found in lignocellulosic residues. The present work reports on the cellulolytic enzymes production by a strain, identified in our laboratory as FBSPE-05, that was isolated from sugarcane bagasse. We studied growth media composition (sugar cane bagasse, wheat bran, brewer's spent grain, and corn steep liquor) that favored enzyme production as well as evaluated the diversity of protein bands showing endoglucanase activity and its molecular mass. Crude enzyme preparations (culture supernatants) were used to perform preliminary studies for the temperature and pH activity profile.

Materials and Methods

Isolation, Selection, and Inoculum Preparation of the Fungal Strain

A. fumigatus FBSPE-05 was isolated from sugar cane bagasse collected in Ribeirão, PE, Brazil. The crude sample was diluted in 0.85% (*w*/*v*) saline solution (1:10), stirred at 150 rpm for 45 min, and serially diluted. Dilutions were plated on carboxymethylcellulose (CMC)—salt mineral medium agar consisting of (in g L^{-1}) 2.6 $(NH_4)_2SO_4$; 2.0 NaCl; 3.0 KH_2PO_4; 6.0 K_2HPO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.02 $CaCl_2 \cdot 2H_2O$; 10.0 CMC (Sigma); 15.0 agar; 1.0 mL trace solution (0.64 g $CuSO_4 \cdot 5H_2O$; 0.15 g $ZnSO_4 \cdot 7H_2O$; 0.11 g $FeSO_4 \cdot 7H_2O$; 0.79 g $MnCl_2 \cdot 4H_2O$; 100 mL distilled water). The inoculated plates were incubated for 10 days at 30 °C, and the grown fungi cultivated in pure culture.

Cellulase-producing capacity of the strain was carried out using microcrystalline cellulose in a salt mineral medium agar, same as above, except that microcrystalline cellulose was placed instead of CMC. Identification as *A. fumigatus* was performed by Instituto Oswaldo Cruz (Rio de Janeiro, Brazil).

For inoculum production, fungal spores of culture grown on potato dextrose agar were harvested in sterile saline solution (0.85% *w*/*v*), as described by Hopwood et al. [6].

Fermentation Conditions

A. fumigatus was cultivated in two conditions: submerged and solid state fermentation. Submerged fermentation (SF) was carried out by seeding 25 μL of a spore suspension (final

concentration in the medium of 10^6 spores mL^{-1}) in Erlenmeyer flasks (125 mL) containing 25 mL of the salt mineral broth (as described above, without agar, $(NH_4)_2SO_4$ and CMC, and cited as so throughout this text) supplemented with either 1% (*w*/*v*) sugar cane bagasse (SCB, pre-treated by steam explosion) or wheat bran (WB), as main carbon sources, and 1.2% (*w*/*v*) corn steep liquor (CSL), as main nitrogen source, pH 4.8. After incubation at 30 °C, under agitation (200 rpm), the content of an entire flask was withdrawn daily and filtered (glass microfiber filter, Whatman GF/A). The culture filtrate thus obtained was used for determination of cellulase activity.

Solid state fermentation (SSF) of lignocellulosic residues, as sugar cane bagasse, wheat bran, and brewer's spent grain (BSG), was carried out in Roux bottle containing 20 g of each substrate and the salt mineral broth added in solid/liquid ratios of 1:1, 1:2, and 1:3, respectively. Two different nitrogen sources were used, added to the salt mineral broth as 0.7% (*w*/*v*) for sodium nitrate (SN) or 1.4% (*w*/*v*) for corn steep liquor. Twenty microliters of a spore suspension (10^8 UFC mL^{-1}) was also added to the salt mineral broth which was uniformly distributed on substrate surface in the Roux bottle. Fermentations were carried out for 8 days at 30 °C. After 2-day intervals, the entire bioconverted lignocellulosic residues were withdrawn. Cellulotytic enzymes were extracted with distilled water at a ratio of 10:1 (water/solid medium), under agitation at 200 rpm for 30 min at room temperature. The suspended material was separated by centrifugation (8,000×*g* for 10 min) and filtered through a glass microfiber filter (Whatman GF/A). This process was repeated twice for a second extraction. The clarified filtrates were combined and assayed for cellulase activity.

All experiments were carried out in duplicates.

Enzyme Assays

Endoglucanase activity as carboxymethylcellulase (CMCase) was determined by measuring the release of reducing sugars in a reaction mixture of 1.0 ml of the crude enzyme and 1.0 ml of 2% (*w*/*v*) CMC solution in 50 mM sodium citrate buffer (pH 4.8) incubated at 50 °C for 10 min. Reducing sugars were assayed by the dinitrosalicylic acid method [7]. One unit (U) of CMCase activity corresponded to 1 μmol of glucose equivalents released per minute under the assay conditions [8].

Filter paper cellulase (FPase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman no. 1 filter paper (1.0×6.0 cm≅50 mg) as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50 °C, after 30 min. One unit (U) of FPase activity corresponded to 1 μmol of glucose equivalents released per minute under the assay conditions [8].

All assays were conducted in duplicates and results expressed as average values. Variations in the multiple assays were <5%.

Partial Crude Enzyme Characterization: Influence of pH and Temperature and Thermal Stability

Temperature profile for CMCase activity (supernatant corresponding to the best production condition on SSF) was determined by assaying activity at different reaction temperatures (20, 35, 50, 65, 80 and 95 °C) in 50 mM sodium citrate buffer (pH 4.8). Accordingly, cellulase activity was assayed in different reaction buffers (50 mM glycine-HCl for pH 1.5–3.0; 50 mM sodium citrate for pH 3.0–6.0; 50 mM citrate phosphate for pH 6.0–7.0; 50 mM phosphate for pH 7.0–8.0; 50 mM Tris–HCl for pH 8.0–10.0) at 65 °C in order to determine pH profile. For thermal stability determination, the crude supernatant obtained in

the best production condition was incubated at different temperatures (50°C and 65°C) and the residual CMCase activity measured at different periods of time.

Zymograms

The culture supernatant from cells grown on the best conditions was analyzed by electrophoresis on denaturing 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel, with 0.2% (*w*/*v*) copolymerized CMC (Sigma) as substrate. Electrophoresis was performed at constant voltage (100 V) at 4 °C for 3 h. Samples containing 500 mU of activity were loaded. After electrophoresis, gel was incubated with Triton X-100 sodium acetate 1% buffer for 30 min in ice-bath, for SDS removal, and then incubated with 50 mM glycine–HCl buffer pH 1.5 for 10 min at 65 °C. For the CMCase activity detection, the gel strip was submerged in 0.1% (*w*/*v*) Congo Red solution for 10 min, and then washed with 1 M NaCl until visualization of enzyme bands. Molecular masses were calculated from mobility of standards ranging from 14 to 200 kDa (Electrophoresis Calibration Kit from Pharmacia), and the corresponding gel was stained using the Coomassie blue staining method [9, 10].

Results and Discussion

When *A. fumigatus*, strain FBSPE-05, was grown in submerged fermentation, best results for CMCase accumulation (365 U L^{-1}) were observed within 6 days cultivation in medium containing 1.0% (*w*/*v*) SCB and 1.2% CSL (Fig. 1). Experiments using WB showed best CMCase activity at lower levels (153 U L^{-1}) although in a shorter time (within 3 days). There are very few data in literature concerning cellulase production by *A. fumigatus*. Dahot and Noomrio [11] observed levels of CMCase activity of 0.225 μmol mL^{-1} when using wheat bran pre-treated with H_2SO_4 for cellulase production. Hamilton and Wase [12] established a comparison between two different strains of cellulase-producing *A. fumigatus*, the highest level of CMCase activity being around 1,800 U L^{-1} but using a high-cost medium containing peptone and urea, among other nutrients. Concerning other fungi, Pothiraj et al. [5] observed maximum values for CMCase of 120 U L^{-1} after 8 days fermentation for *Aspergillus niger*, using cassava waste as carbon source. For *Aspergillus terreus*, during the same period, lower values (100 U L^{-1} of CMCase) were observed [5].

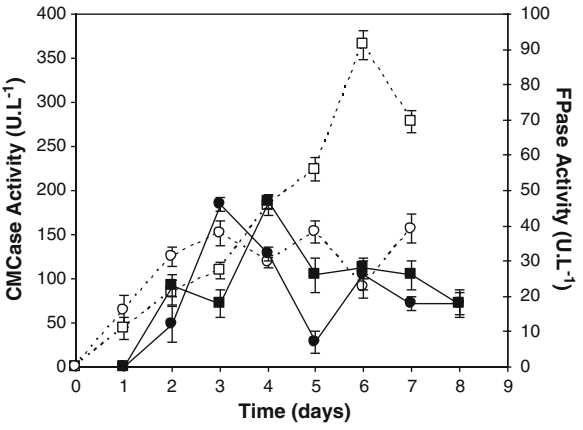

Fig. 1 Fermentation time-course for CMCase and FPase production by *A. fumigatus* FBSPE-05, in submerged fermentation using sugar cane bagasse (*open squares*, CMCase; *closed squares*, FPase activity) and wheat bran (*open circles*, CMCase; *closed circles*, FPase activity) as main carbon source

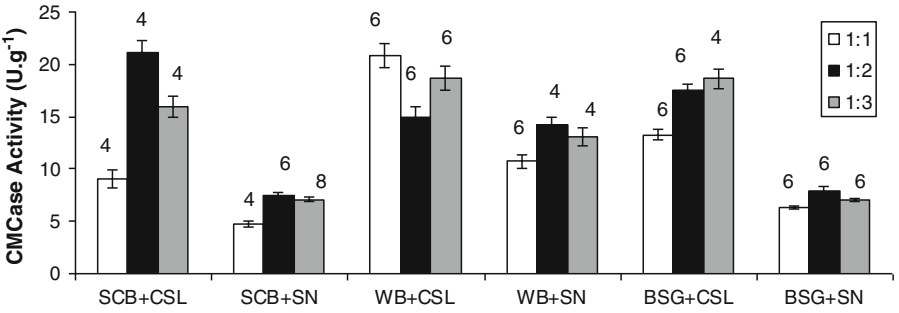

Fig. 2 Maximum values for CMCase production by *A. fumigatus* FBSPE-05, in solid state fermentation, using different substrates and different solid/liquid ratio (*SCB* sugar cane bagasse, *SN* sodium nitrate, *WB* wheat bran, *CSL* corn steep liquor, *BSG* brewer's spent grain). *Numbers in each bar* correspond to incubation period, in days

However, a comparison between results described in literature and ours is difficult, since the conditions for measuring CMCase activity and enzyme production were different.

Values obtained for FPase activity in submerged fermentation (Fig. 1) using both substrates, SCB and WB, were equally interesting, with levels of 47 U L^{-1}; however, in WB, best production occurred earlier (2 days) as compared to SCB (3 days). Pothiraj et al. in the same research cited above [5] observed maximum values for FPase activity of 40 U L^{-1} for both fungi. *A. fumigatus* FBSPE-05 was able to produce more activity in a shorter time, when compared to *A. niger* and *A. terreus*.

Experiments in solid state fermentation have shown that SCB, WB, and BSG were equally good carbon sources for CMCase activity (Fig. 2). The combination of SCB and CSL, in solid/liquid ratio 1:2, have shown the best activity (21.06 U g^{-1}) after 4 days-fermentation. However, very similar values were observed when WB and BSG were used in different solid/liquid ratios and different incubation times, which indicates that these three low-cost lignocellulosic residues can be equally useful in CMCase production.

Fermentation time course for FPase production in solid state fermentation have shown maximal activity in solid/liquid ratio of 1:3, using WB (5.35 U g^{-1}), BSG (5.03 U g^{-1}), or SCB (4.4 U g^{-1}) as carbon source, and CSL as nitrogen source, after 4 days growth (Fig. 3). Enzyme activity was lower when using 1:2 and 1:1 solid/liquid ratios, where values for

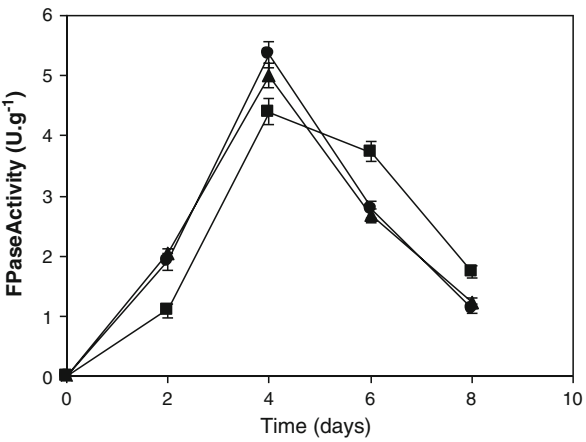

Fig. 3 Fermentation time-course for FPase production by *A. fumigatus* FBSPE-05, in solid state fermentation, using different substrates: SCB + CSL (*closed squares*), WB + CSL (*closed circles*), and BSG + CSL (*closed triangles*) at 1:3 solid/liquid ratio (*SCB* sugar cane bagasse, *SN* sodium nitrate, *WB* wheat bran, *CSL* corn steep liquor, *BSG* brewer's spent grain)

FPase activity were around 3.3 and 4.0 U g^{-1} (data not shown). In addition, FPase production using SN as nitrogen source was very low (data not shown) in comparison to CSL source.

Results concerning the nitrogen source indicate the good performance of CSL. According to Gao et al. [13], cellulase activities produced by *A. terreus* was higher with organic nitrogen, being maximal when yeast extract was added. CSL is a very low-cost substrate and has already been suggested as an alternative to yeast extract for cellulase production by *Streptomyces drozdowiczii* [14]. Also, in protease synthesis, by *Streptomyces* sp 594, CSL have enhanced production in 86% and 39%, respectively, for SF and SSF, when compared with yeast extract [15].

There are no data in literature concerning FPase production by *A. fumigatus* in SSF. However, when using a mutant strain of *Trichoderma reesei* MCG77 and rice bran as carbon source, Latifan et al. [16] observed maximum FPase activity of 2.3 U g^{-1} at pH 5.0, with a moisture content of 70%, after incubation at 30 °C. Also, a strain KK2 of *A. niger* was able to produce 19.5 U g^{-1} of FPase when grown in rice straw [17].

CMCases present in the liquid extract obtained from *A. fumigatus* FBSPE 05 grown in SCB and CSL in SSF presented maximal activity at 65 °C (Fig. 4a). This supernatant was

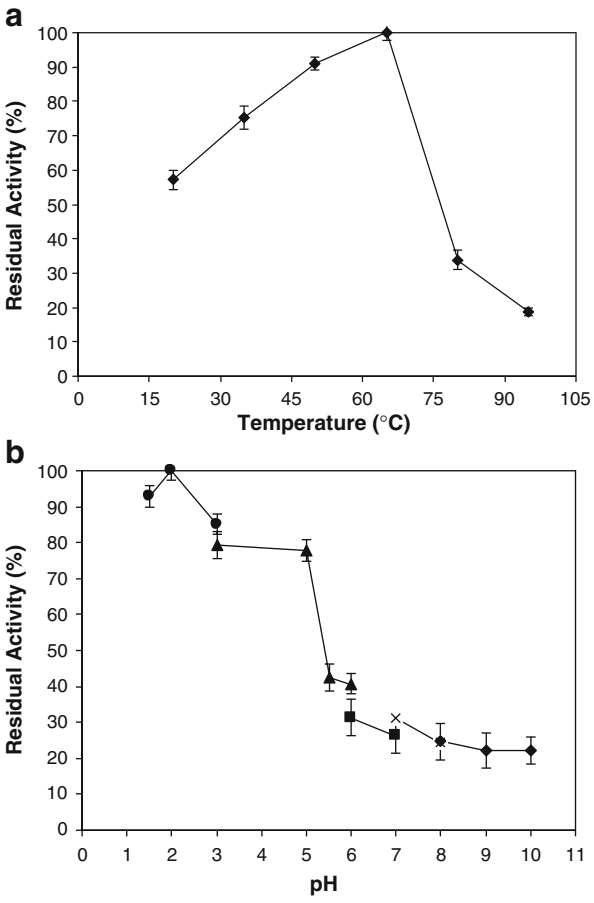

Fig. 4 Effect of temperature (at pH 4.8) (**a**) and pH (at 65 °C) (**b**) on CMCase activity produced by *A. fumigatus* FBSPE-05 grown on solid state fermentation in 1.0% (*w*/*v*) SCB and 1.2% (*w*/*v*) CSL. The ionic strength for all buffers was 50mM: (*closed circles*), glycine-HCl; (*closed triangles*), sodium citrate; (*closed squares*), phosphate citrate; (*X mark*), phosphate; (*closed diamonds*), glycine-NaOH. Residual activity is expressed as a percentage of the maximum activity

able to retain 100% residual activity at 50 °C during 2 h and 70% after 3 h incubation, half-life of crude enzyme being of 3 h at 65 °C (data not show). Other studies using *A. niger* [18] have shown a residual activity around 100% for cellulase activity at temperatures between 50° and 60 °C and half-lives of 2 h at 60 °C or 1 h at 70 °C, very similar to our results. Also, *A. terreus* has shown optimal temperature for endoglucanase activity around 70 °C [13]. Our results strongly suggest that cellulases in this supernatant seem to be thermophilic, considered ideal for many biotechnological processes.

The pH profiles (Fig. 4b) have shown more than 80% activity in the acidic pH range (1.5 to 3.0), with optimal activity occurring at pH 2.0. Values in the alkali range (8.0 to 10.0.) were very low, around 25% of residual activity According to Gao et al. [13], this remarkable characteristic was detected also in a thermoacidophilic fungus, *A. terreus* M11. This biochemical characteristic is very interesting for processes that require acid conditions, and considering also the optimal temperature, strain FBSPE-05 can be considered as producer of a thermoacidophilic endoglucanase.

Zymogram of culture supernatant of *A. fumigatus* FBSPE-05 have shown the synthesis of multiple cellulases when the fungus was grown in 1.0% SCB and 1.2% CSL by SSF, after 4 days fermentation (Fig. 5). In these conditions, six bands of cellulase activity were

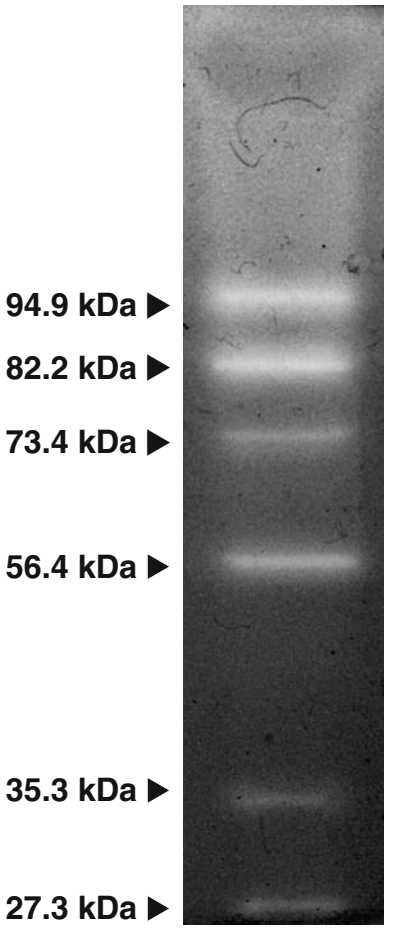

Fig. 5 Zymogram analysis of cellulase activity in the supernatant of *A. fumigatus* FBSPE-05 grown on 1.0% (*w*/*v*) SCB and 1.2% (*w*/*v*) CSL in SSF. The calculated molecular masses (in kDa) of the CMCases are indicated in the *left side of the figure*

observed with estimated molecular masses of 94.9, 82.2, 73.4, 56.4, 35.3, and 27.3 kDa, respectively. There are no reports in literature about molecular masses of cellulase enzyme produced by *A. fumigatus*.

In conclusion, *A. fumigatus* FBSPE-05 was able to grow and produce CMCase and FPase using agro-industrial by-products as main sources of carbon and nitrogen, in both submerged and solid-state cultivations. The use of sugarcane bagasse, wheat bran, or brewer's spent grain as carbon source and corn steep liquor as nitrogen source showed to be advantageous and will be used in further studies. Characterization of the crude enzyme has shown *A. fumigatus* FBSPE-05 endoglucanase to be active in the acidic pH range [1–5], with maximal activity at pH 2, and 65 °C, being so a promising organism for production of acidophilic and thermophilic cellulase.

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