Sugarcane bagasse as feedstock for cellulase production by *Trichoderma harzianum* in optimized culture medium

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Abstract

This work aimed at the production of cellulases from pretreated sugarcane bagasse by the filamentous fungus *Trichoderma harzianum* IOC 3844 and their application in the hydrolysis of this same substrate, for a future use in second-generation ethanol production. The production of cellulases was optimized, which resulted in high enzymatic activities after 42 hrs of process in an instrumented bioreactor (CMCase 27,017 U x L^{-1}; FPase 1,225 U x L^{-1}; and β-glucosidase 609 U x L^{-1}). The enzymatic extract was concentrated by using a hollow fiber membrane filtration system. The concentrated extract was applied in the hydrolysis of pretreated sugarcane bagasse, after 28 hrs of enzymatic reaction, displaying a similar catalytic performance of that attained with a commercial enzymatic preparation (hydrolysis efficiency of roughly 50%). Finally, the enzymatic extract was partially characterized in terms of the molecular weights of the main activities of the enzymatic pool. Electrophoretic analysis identified eleven protein bands; six of them were related to CMCase activity and revealing molecular weights that varied from 48 to 78 kDa, and two bands were associated with β-glucosidase activity and having molecular weights of 75 and 85 kDa.

Keywords: electrophoresis, enzymatic hydrolysis, second generation ethanol, *Trichoderma harzianum*.

INTRODUCTION

Cellulases are enzymes that hydrolyze the cellulose β(1,4) bonds, act synergistically in its degradation, and yield glucose as the main final product, which is readily fermented to ethanol and other molecules. Three main groups of these enzymes are described: CMCases, exoglucanases (cellobiohydrolase I and cellobiohydrolase II) and β-glucosidases. The CMCases act on the amorphous region of the fiber and generate non-reducing and reducing terminals for the subsequent action of exoglucanases (CBH I and CBH II). CBH I and CBH II act simultaneously on the reducing and non-reducing ends to release cellobiose. The β-glucosidases hydrolyze cellobiose to produce glucose. This synergistic action of cellulases promotes a higher yield of the cellulolytic complex when compared to the sum of individual yields (Lynd et al. 2002). One of the most recent challenges to Brazil's Biotechnology is related to the technological development for the production of cellulolytic preparations aimed at reducing the production costs in the second-generation bioethanol technology. The sugarcane bagasse is the most abundant agricultural residue generated in Brazil (approximately 170 million tons/year in 2011) and represents a potential energy source for the production of second-generation ethanol because it is a biomass rich in hydrolysable polysaccharides that is generated in industrial units (UNICA, 2012). This lignocellulosic residue is considered to be an attractive feedstock for ethanol production due to its availability in large amounts and at low costs. Furthermore, the use of waste materials for the

production of fuels and chemicals adds economic value and reduces environmental impacts (Pereira et al. 2008). Fungi are the most important microorganisms used for cellulase industrial production (Lynd et al. 2002). Most commercial cellulases are produced by the filamentous fungi *Trichoderma reesei* and *Aspergillus niger*. Recent studies have shown that *T. harzianum* has an efficient cellulase production system (Ahmed et al. 2009, Castro et al. 2010). The current study aimed at evaluating the ability of the fungus *T. harzianum* IOC 3844 to produce cellulases from pretreated sugarcane bagasse in an instrumented bioreactor containing optimized medium and to apply the concentrated enzymatic extract in the hydrolysis of this substrate. Furthermore, this study aimed to partially characterize by electrophoresis the molecular weights of the main activities of the enzymatic pool. For these reasons, we aim at improving the knowledge about this hyper-cellulolytic fungus.

MATERIALS AND METHODS

Sugarcane bagasse pretreatments and cellulignin composition analysis

The cellulignin used in this study was obtained from sugarcane bagasse (*Saccharum* spp., Dedini Plant, Piracicaba, São Paulo, Brazil) by using two consecutive pre-treatments (acid and alkaline). The acid pretreatment was conducted with sulphuric acid solution (1% v/v) at a solid:liquid ratio of 1:2 (33.3% w/w) at 121ºC for 45 min, as reported by Betancur and Pereira (2010). After the acid pretreatment, two fractions were obtained: a liquid phase, containing the hemicellulose hydrolyzate, and a solid fraction, composed mainly of cellulose and lignin. These two fractions were separated by filtration in a hydraulic press; the remaining solid was repeatedly washed with water and pH adjusted to 5 with 2 M HCl. The washed pretreated residue underwent an alkaline pretreatment with sodium hydroxide (4% w/v), with a solid: liquid ratio of 1:20 at 121ºC for 30 min, as described by Vásquez et al. (2007). The resulting solid material was called partially delignified cellulignin (PDC). The compositional determination of sugarcane bagasse and PDC was conducted by chemical hydrolysis with H_2SO_4 performed in two stages, according to Sluiter et al. (2005) and Ververis et al. (2007).

Microorganism and cultivation conditions

The microorganism used in this study was *T. harzianum* IOC 3844, characterized and catalogued by the database of the Oswaldo Cruz Institute (Fiocruz), Rio de Janeiro, Brazil. Spores were stored in glycerol (20% v/v) at 4ºC in microtubes. In each experiment, the strain was grown on plates containing PDA medium, at 30ºC for 9-10 days before inoculation.

Optimization of cellulase production

Resuspended spores were inoculated (10^6 conidia x mL^{-1}) in 200 mL of Mandels and Weber medium (Mandels and Weber, 1969) containing 15 g x L^{-1} of PDC as the carbon source in a 500-mL conical flask and incubated at 200 rpm at 30ºC for 48 hrs (preculture media). Optimization of cellulase production was conducted through a central composite rotational design (CCRD) for which study ranges are shown in Table 1. The CCRD was composed of a complete planning 2^3 (8 experiments) with 6 axial points and 3 repetitions of the center point, which resulted in 17 assays. The cellulase production was carried out in 500-mL conical flasks containing 200 mL of medium, 15 g x L^{-1} of PDC and inoculated with preculture media (10% v/v). The other elements of the culture medium were kept in the same concentrations established by Mandels and Weber (Mandels and Weber, 1969): 2 mg x L^{-1} KH_2PO_4, 0.4 mg x L^{-1} $CaCl_2$ x 2 H_2O, 0.3 mg x L^{-1} $MgSO_4$ x 7 H_2O, 5 mg x L^{-1} $FeSO_4$ x 7 H_2O, 1.6 mg x L^{-1} $MnSO_4$ x 4 H_2O, 1.4 mg x L^{-1} $ZnSO_4$ x 7 H_2O, 2 mg x L^{-1} $CoCl_2$ x 6 H_2O. The flasks were incubated at 200 rpm at 30ºC during 72 hrs. After the period of fermentation, the medium was centrifuged at 2,500 x g for 10 min, and the crude enzymatic extracts was used to quantify the cellulase activities (FPase, CMCase and β -glucosidase).

To maximize the three response variables simultaneously, an optimization using the global desirability function (D), which consists of converting each response into a single desirability function (di) that ranges from 0 to 1 (0< di < 1) (Derringer and Suich, 1980), was performed. For a function with three independent variables, the global desirability function is expressed as follows: $D = (d_1.d_2.d_3)^{1/3}$. The predicted values were validated experimentally in the optimized conditions. The activity measurements were assayed in triplicate. The statistical treatment of the results was performed by the software Statistica version 6.0 (StatSoft, Inc.).

Table 1. Factors and levels used in the central composite rotational design (CCRD) for the optimization of cellulase production.

Factor	Axial -1.68	Min -1	CP 0	Max 1	Axial 1.68
urea (g x L^{-1})	0.00	0.81	2.00	3.19	4.00
yeast extract (g x L^{-1})	0.00	0.40	1.00	1.60	2.00
ammonium sulfate (g x L^{-1})	0.00	0.81	2.00	3.19	4.00

Min: minimum value, CP: center point, Max: maximum value.

Cellulase production in an instrumented bioreactor and the concentration of the crude extract

The cellulolytic enzyme production was carried out in a 10 L stirred tank bioreactor (BIOSTAT® B, Sartorius AG, Gottingen, Germany) with an operating volume of 8 L. The medium was prepared under the same conditions used in flasks (15 g x L^{-1} of PDC, 2 mg x L^{-1} KH$_2$PO$_4$, 0.4 mg x L^{-1} CaCl$_2$ x 2 H$_2$O, 0.3 mg x L^{-1} MgSO$_4$ x 7 H$_2$O, 5 mg x L^{-1} FeSO$_4$ x 7 H$_2$O, 1.6 mg x L^{-1} MnSO$_4$ x 4 H$_2$O, 1.4 mg x L^{-1} ZnSO$_4$ x 7 H$_2$O, 2 mg x L^{-1} CoCl$_2$ x 6 H$_2$O, and inoculated with 10% v/v preculture media). The concentrations of urea, yeast extract and ammonium sulphate were the one optimized according to CCRD (described in the results). Temperature, agitation, pH, aeration and antifoam were automatically monitored. The process was maintained at 30ºC, pH 5.0 with a stirring speed of 200 rpm, according to previous methodology used by Castro et al. (2010). Dissolved oxygen (DO) was kept at 10% saturation. The pH was set to 5.0 ± 0.2 and it was kept by the addition of 2 M HCl or 2 M NaOH. The foam was controlled by addition of antifoaming agent (Sigma). The total culture period was 42 hrs. Samples were taken every 12 hrs, and each sample was centrifuged at 4,000 rpm for 10 min at 10ºC. The supernatants were separated to quantify cellulase activities. The fermented medium produced in the bioreactor was filtered through glass wool with the use of a vacuum pump to remove the cells. The enzymatic extract was concentrated 48-fold by a hollow fiber system (QuixStand® model, a GE Healthcare) with a polysulfone cartridge that had a cut-off of 5 kDa in order to remove the excess of water and increase the enzyme activity concentration. Glicerol (10%) was added like enzyme stabilizer and the concentrated enzyme was kept frozen until the application on the hydrolysis of pretreated sugarcane bagasse.

Cellulase and protein assays

FPase, CMCase and β-glucosidase activities were measured with filter paper, carboxymethylcellulose and cellobiose as substrates, respectively, according to the standard methodologies described by Eveleigh et al. (2009) and Ghose (1987). Cellulase activities were expressed as international unit (U), for which one unit of activity is the amount of crude enzymatic extract able to release 1 µmol of sugar (glucose equivalent) per minute. The protein concentrations of the cellulase preparations were measured with the Bradford Protein Assay (Bio-Rad) with bovine serum albumin as a standard (5-30 mg x mL^{-1}) (Bradford, 1976).

Enzymatic hydrolysis

The concentrated enzymatic preparation from *T. harzianum,* coded as "*Ladebio Th*", was applied to the hydrolysis of PDC to evaluate its catalytic power in comparison with the enzymatic preparation *Multifect*® produced by Genencor. Its performance was also evaluated on another substrate (microcrystalline cellulose - Avicel®). The hydrolysis was performed with a standard enzymatic load of 25 FPU x g^{-1} and a substrate concentration of 40 g x L^{-1}. A control experiment was made without enzyme at the same conditions that the samples. The experiments were performed in triplicate in conical flasks at 50ºC, shaken at 200 rpm for 96 hrs. During this procedure, samples were periodically taken for a kinetic profile. The samples were centrifuged at 10,000 x g for 10 min. The variables that were analyzed were glucose, hydrolysis yield (glucose in relation to cellulose) and cellobiose. For cellulignin, the hydrolysis yield was calculated based on the presence of 68% cellulose in the substrate (data shown in the Results section).

The hydrolysis yield was determined by using the following equation:

$$HY = \frac{\text{released glucose (g x L}^{-1})}{40 \times 0.68 \times 1.10} \times 100$$

In which HY is the hydrolysis yield, 40 is the cellulignin concentration (g x L^{-1}), 0.68 is the cellulose content in the cellulignin (g x g^{-1}) and 1.10 is a correction factor that accounts for the addition of water molecules to the anhydroglucose residues in cellulose.

The quantification of sugars by high performance liquid chromatography (HPLC)

The sugars produced in the enzymatic hydrolysis (glucose and cellobiose) were quantified by high performance liquid chromatography (Waters 2414, refractive index detector, column HPX87P). The mobile phase was MilliQ water at a flow rate of 0.6 mL/min. Glucose and cellobiose were used as standards.

SDS-PAGE electrophoresis and zymograms of the enzymatic preparation

Discontinuous SDS-PAGE was conducted in a vertical system, the Mini-Protean Tetra model (Bio-Rad®). For protein identification, the electrophoretic run was conducted in polyacrylamide gel (15%) by applying a potential difference of 120 V and a current of 20 mA/gel at a temperature of 5ºC. For the CMCase zymograms, carboxymethylcellulose (0.2% w/v) was added to the polyacrylamide gel composition. The β-glucosidases gel preparation followed the same procedures of the protein SDS/PAGE gel (Alfenas, 1998). Cellulase and protein from crude enzyme extract produced by *T. harzianum* IOC 3844 in a bioreactor and from enzymatic extract concentrated in a hollow fiber were evaluated. The characterization was performed by comparing the revealed bands with molecular weight standards: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), bovine carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14 kDa). For all the samples were applied 10 μg of proteins per well in the gel. The proteins were revealed in denaturing conditions, whereas the CMCase and β-glucosidase were revealed in native conditions. The gels were analyzed with the software "Gel-Pro Analyzer 4.0".

Electrophoretograms revelation

To visualize the protein bands, the gel was stained in coomassie brilliant blue R-250 (0.1% w/v) and then bleached in bleach fixer solution through successive washes every 3 hrs until the bands' appearance. With regards to the CMCases, the gel was immersed in 1% Triton X-100 solution and kept under constant stirring for 20 min to remove the SDS. Then, the gel was washed twice in 50 mM sodium citrate buffer (pH 5.0) for 20 min and was incubated in the same buffer at 50ºC for 20 min. Finally, the gel was immersed in Congo red solution (0.1% w/v) for 30 min and was then washed successive times with 1 M NaCl solution until the bands were revealed. After complete visualization of the bands, the gel was washed with acetic acid (5% v/v). For β-glucosidases identification, the gel was immersed in 1% Triton X-100 solution and kept under constant stirring for 20 min to remove the SDS. Then, the solution was replaced by 50 mM sodium citrate buffer (pH 5.0) and kept under constant stirring for 20 min. The washing procedure was repeated twice. Finally, the gel was immersed in esculin solution (0.1% w/v) with ferric chloride (0.033% w/v) dissolved in 50 mM sodium citrate buffer (pH 5.0). The reaction was conducted in a water bath at 50ºC for 30 min (Alfenas, 1998).

RESULTS AND DISCUSSION

Composition of sugarcane bagasse and cellulignin

In addition to disorganizing the lignocellulosic complex, the aim of acid and alkaline pretreatments is to fractionate the hemicellulose and lignin components to reduce the cellulose crystallinity and increase

the porosity of the remaining solid fraction. Therefore, the pretreatment contributes with amorphogenesis that is initial stage in enzymatic saccharification of cellulose because increase the cellulose surface area and making it more accessible to the cellulase enzyme complex (Arantes and Saddler, 2010; Barcelos et al. 2012). The lignin can form unproductive bonds with cellulases, which requires more enzymatic loads when feedstocks with high lignin content are used (Kumar et al. 2012). In the present study, the acid and alkaline pretreatments were efficient in reducing the hemicellulose and lignin contents (28.83% to 12.60% and 18.94% to 8.97%, respectively). Simultaneously, there was an increase in the cellulose content (35.02% to 68.04%). These results are showed in Table 2.

Table 2. Composition of sugar cane bagasse and PDC.

Components	Cellulose	Hemicellulose	Lignin	Ash	Moisture
Sugar cane bagasse (% w/w)	35.02 ± 1.40	28.83 ± 1.22	18.94 ± 0.32	8.01 ± 1.13	4.47 ± 0.15
PDC (% w/w)	68.04 ± 1.27	12.60 ± 0.87	8.97 ± 0.62	3.47 ± 0.36	3.84 ± 0.17

Central composite rotational design (CCRD)

The matrix used for the cellulase optimization design and the results of the response variable can be observed in Table 3. Enzyme activities of the center points were well differentiated from the axial points. The ranges of values were: FPase (79.02 to 459.63 U x L^{-1}), CMCase (2,011.33 to 13,475.30 U x L^{-1}) and β-glucosidase (2.06 to 485.25 U x L^{-1}). It was observed that most of the cellulase activities were very close to the center point values, with the exception of the axial points, which indicates that the evaluated conditions were in the optimum range of this bioprocess.

Table 3. CCRD matrix and corresponding results of cellulase activities.

Assay	Urea	Yeast extract	Ammonium sulphate	FPase (U x L^{-1})	CMCase (U x L^{-1})	β-glucosidase (U x L^{-1})
1	-1	-1	-1	288.14	8,041.21	92.59
2	-1	-1	1	331.32	10,126.50	91.22
3	-1	1	-1	384.99	10,845.57	150.89
4	-1	1	1	409.67	11,821.45	262.00
5	1	-1	-1	296.47	10,455.22	184.50
6	1	-1	1	363.40	11,708.45	294.92
7	1	1	-1	105.86	6,705.80	2.06
8	1	1	1	456.86	13,321.21	409.12
9	-1.68	0	0	79.02	2,011.33	51.10
10	1.68	0	0	403.19	11,893.35	273.66
11	0	-1.68	0	312.51	11,246.19	207.48
12	0	1.68	0	331.63	12,283.70	332.99
13	0	0	-1.68	393.01	11,872.81	193.04
14	0	0	1.68	454.39	12,766.50	485.25
15	0	0	0	450.38	13,454.75	420.78
16	0	0	0	459.63	13,352.03	416.32
17	0	0	0	452.54	13,475.30	427.64

In response surface graphs, it was possible to verify the strong influence of the urea and ammonium sulphate in the cellulase production by *T. harzianum* IOC 3844 (Figure 1).

The range study of this work allowed obtaining optimized results for the 3 variables: CMCase, FPase and β-glucosidase. There was greater abundance in CMCase production, which is a characteristic of this microorganism, as reported in other studies (Ahmed et al. 2009; Castro et al. 2010).

Optimization of cellulase production with desirability function and experimental validation

The global desirability value to simultaneously reach the optimum for the three enzymatic activities was 1.00, which means that the optimization by this function achieved the maximum value that was obtainable for each response variable. According this function, the predicted optimal concentrations of nutrients were the following: urea (2.24 g x L^{-1}), yeast extract (1.14 g x L^{-1}) and ammonium sulphate (3.42 g x L^{-1}), whereas the predicted optimum cellulase activities were: CMCase 14,446.14 U x L^{-1}; β-glucosidase 494.71 U x L^{-1}; and FPase 509.07 U x L^{-1}. These conditions were validated by tests in conical flasks with the other nutrients kept in the same concentrations described in the methodology. CMCase and FPase activities were on the margin of 95% confidence, whereas the β-glucosidase activity value was slightly higher.

Cellulase production in an instrumented bioreactor and concentration of the crude extract

T. harzianum IOC 3844 had the ability to produce cellulases in an instrumented bioreactor in the optimized conditions. The kinetics of cellulase production in submerged fermentation depicted high enzymatic activity after 42 hrs of fermentation (CMCase, 27,017.56 U x L^{-1}; FPase, 1,225.87 U x L^{-1}; and β-glucosidase, 609.43 U x L^{-1}) (Figure 2).

The current study showed significant CMCase and FPase activities compared with other works. There were increases of 4- and 3-fold for CMCase and FPase activities, respectively, in relation to the results obtained with Mandels and Weber medium (Mandels and Weber, 1969) for the same fungi according to Castro et al. (2010), which enzyme activities were: CMCase 6,358 U x L^{-1}, FPase 445 U x L^{-1} and β-glucosidase 742 U x L^{-1}. CMCase activity from *T. harzianum* obtained in the present work (27,017 U x L^{-1}) surpassed that reported by Li et al. (2005) after 8 days of culture of *T. reesei* QM 9414 in pretreated corn fiber (9,529 U x L^{-1}). Roussos and Raimbault (1982) reported that the maximum CMCase activity produced by *T. harzianum* in microcrystalline cellulose was 3,000 U x L^{-1}. With regards to FPase activity, the maximum value achieved in this work (1,225 U x L^{-1}) was higher than some of the results reported in literature in which several cellulosic substrates, such as pretreated corn fiber (280 U x L^{-1}) and microcrystalline cellulose (400 U x L^{-1}) (Roussos and Raimbault, 1982; Li et al. 2005), have been used. In other studies, were obtained FPase activities close to this study. Delabona et al. (2012) founded a maximum FPase activity of 1,210 U x L^{-1} by *T. harzianum* P49P11 using pretreated sugarcane in 120 hrs of process. Juhász et al. (2005) reported 1,200 FPU x L^{-1} produced by *T. reesei* RUT C30 (ATCC 56765) during 168 hrs. For the β-glucosidase activity, Kocher et al. (2008) has found a maximum of 1,650 U x L^{-1} in submerged fermentation of rice straw by *T. harzianum* RUT-C 8230 after 10 days of culture. With regards to the protein content, the concentration obtained in the current work after 42 hrs was 261.56 mg x L^{-1}. The specific activities of the enzymes were the following: 103.29 U x mg^{-1} protein (CMCase); 4.69 U x mg^{-1} protein (FPase); and 2.33 U x mg^{-1} protein (β-glucosidase). This CMCase specific activity was so higher than those observed for the model fungus *T. reesei* RUT C30 in bioreactor (Ahamed and Vermette, 2008), which value was 4.20 U x mg^{-1}, whereas the FPase specific activity was similar (5.02 U x mg^{-1}) to the present work. In terms of volumetric productivity, our study obtained the highest values registered in the literature for CMCase and FPase activities from *T. harzianum* (Table 4). An important characteristic observed in the profile of cellulase production by *T. harzianum* IOC 3844 was the fast kinetics of the process, which reached high rates in only 42 hrs of fermentation. This is an advantageous aspect of this microorganism, which is able to produce these enzymes in short periods of time to result in high volumetric productivity, especially for CMCase (643.28 U x L^{-1} x h^{-1}). For FPase and β-glucosidase, the volumetric productivity were, respectively, 29.19 and 14.51 U x L^{-1} x h^{-1}. The optimized conditions evaluated in this study allowed an increase the volumetric productivity (7-fold for CMCase, 5-fold for FPase and 1,4-fold for β-glucosidase) compared with a non-optimized medium for the same strain (Castro et al. 2010). By using the known fungus *T. reesei* RUT-C30 from IOGEN Corporation (Ottawa, Canada) in a stirred tank bioreactor, Ahamed and Vermette (2008) obtained the following volumetric productivity: 29.17 U x L^{-1} x h^{-1} (for CMCase) and 34.86 U x L^{-1} x h^{-1} (for FPase). With regards these results described for this

model fungus, in the present study there was a significant CMCase volumetric productivity. Table 4 shows the values of cellulase activities and the volumetric productivity from *T. harzianum* reported in different studies.

Table 4. Cellulases activities and volumetric productivity from *T. harzianum* in different studies.

Microorganism	Activity (U x L^{-1})			Volumetric Productivity (U x L^{-1} x h^{-1})		
	FPase	CMCase	β-glucosidase	FPase	CMCase	β-glucosidase
T. harzianum IOC 3844 in optimized medium (a)	1,225	27,017	609	29.19	643.28	14.51
T. harzianum IOC 3844 in Mandels and Weber medium (b)	445	6,358	742	6.18	88.31	10.31
T. harzianum (c)	400	3,000	NR*	8.33	0.06	NR*
T. harzianum (d)	NR*	790	920	NR*	6.58	7.67
T. harzianum RUT-C 8230 (e)	127	150	1,650	0.53	0.63	6.88

(a) This study; (b) Castro et al. 2010; (c) Roussos and Raimbault, 1982; (d) Ahmed et al. 2009; (e) Kocher et al. 2008. NR*: non reported.

The produced enzymatic extract was concentrated 48-fold by using a hollow fiber membrane system, and cellulolytic activities of 46,661.49 U x L^{-1} (FPase), 1,034,801.14 U x L^{-1} (CMCase) and 23,750.73 U x L^{-1} (β-glucosidase) were obtained. There was a small loss of CMCase activity after concentration due to the passage of molecules to the permeate fraction, in which cellulase activities were observed (data not shown). Although the filtration membrane porosity was 5 kDa, the passage of larger proteins may occur due to the pressure applied during the concentration. The proportion of FPase:CMCase:β-glucosidase activities was 1:22:0.5 in the concentrated extract from *T. harzianum*, coded by "*Ladebio Th*". When considering the proportions of cellulase activities of the commercial preparation *Multifect*® from Genencor, the ratio 1:32:0.6 for FPase:CMCase:β-glucosidase activities was attained. Thus, there is a similar proportion of FPase and β-glucosidase in the *Ladebio Th* enzymatic preparation when compared to the commercial enzymatic pool. The cellulolytic activities of the preparations of *Ladebio Th* and *Multifect*® are presented in Table 5.

Table 5. Cellulolytic activities of the preparations of *Ladebio Th* and *Multifect*®.

Enzymatic preparation	FPase (U x L^{-1})	CMCase (U x L^{-1})	β-glucosidase (U x L^{-1})
Ladebio Th	46,661	1,034,801	23,751
Multifect®	200,000	6,500,000	125,000

Enzymatic hydrolysis

Figure 3 illustrates the release of glucose during enzymatic hydrolysis by *T. harzianum* (*Ladebio Th*) and commercial (*Multifect*®) cellulase. At the first moment, we can observe in the results that there is no free glucose in the PDC. It probably occurs because the free glucose is washed during the washes in PDC pretreatment. The kinetic profiles clearly show that both enzymatic preparations depicted similar catalytic performance. After 28 hrs of hydrolysis, glucose concentrations were as follows:

Ladebio Th in PDC, of 14.39 g x L^{-1}; *Multifect*$^{®}$ in PDC, of 14.85 g x L^{-1}; *Ladebio Th* in Avicel$^{®}$, of 12.79 g x L^{-1}; and *Multifect*$^{®}$ in Avicel$^{®}$ of 10.40 g x L^{-1}.

The hydrolysis yield values indicated that *T. harzianum* has a competitive cellulolytic complex for PDC hydrolysis compared with the commercial enzymatic preparation *Multifect*$^{®}$. After 28 hrs, the hydrolysis yields were 48% (*Ladebio Th* in PDC) and 49% (*Multifect*$^{®}$ in PDC). However, there were lower values of hydrolysis yield in Avicel$^{®}$ (microcrystalline cellulose) after 28 hrs: 29% (using *Ladebio Th*) and 23% (using *Multifect*$^{®}$). This pattern was expected because crystalline cellulose is more difficultly attacked by enzymes than amorphous cellulose. Some studies have showed that the highly ordered and tightly packed fibrillar architecture of the cellulose microfibrils is one of the major barriers faced by cellulose enzymes which hinder their access to much of the cellulose. In some regions, the cellulose chains are packed so tightly that small molecules like water cannot penetrate these structures (Arantes and Saddler, 2010; Kumar et al. 2012). Limited access to these regions leads to changes in its reactivity to macromolecules such as cellulases and contributes to the cellulose degradation resistance. With this type of structure, only cellulose molecules located on the surface would be susceptible to the action of degrading enzymes. The literature have related that the ability of *T. reesei* cellobiohydrolase to access the cellulose chains within the microfibrils is significantly limited, possibly due to the accessibility of the enzymes only to the superficial layers of the microfibrils. When hydrolysis occurs only at the cellulose surface, the available superficial area determines the maximum hydrolysis rate that can be achieved (Arantes and Saddler, 2010). The hydrolytic performance of the enzymatic preparation *Ladebio Th* was higher or close to that obtained in other studies using *T. reesei* cellulases or commercial enzymatic preparations (Juhász et al. 2005; Li et al. 2005). With regards to cellobiose, as well as to glucose results, there was not free sugar from PDC in the reactional medium, which we can observe in the control experiment. For the others samples, in the first 12 hrs of hydrolysis a slight accumulation of this disaccharide took place in the experiments: *Ladebio Th* in PDC (0.84 g x L^{-1}), *Ladebio Th* in Avicel$^{®}$ (0.43 g x L^{-1}), *Multifect*$^{®}$ in PDC (1.40 g x L^{-1}) and *Multifect*$^{®}$ in Avicel$^{®}$ (0.31 g x L^{-1}). However, these molecules were rapidly cleaved by the action of β-glucosidases and did not remain available in the medium (Figure 4). The experiments in which the enzymes were applied on PDC showed the highest amounts of cellobiose. A factor that may have influenced the cellobiose accumulation is the possible presence of lignin residues in the substrate (PDC) that generated unproductive linkages of β-glucosidase onto lignin. Kumar et al. (2012) reported that the lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. This work showed that the lignin plays in restricting cellulose hydrolysis through either binding of the cellulases or limiting swelling of the cellulose and therefore limiting accessibility. In some cases, during the enzymatic hydrolysis of cellulignin, β-glucosidases have more binding affinity with lignin than with carbohydrates. Regarding the results of cellobiose, Maeda et al. (2011) noted this same pattern of cellobiose accumulation during the first hours of hydrolysis when they used the enzyme *Multifect*$^{®}$ on pretreated sugarcane bagasse. In general terms, cellulases from *T. harzianum* IOC 3844 are shown to be able to hydrolyze PDC; thus, these cellulases have great potential for application in the conversion of cellulose into fermentable sugars for the production of second-generation bioethanol.

SDS-PAGE electrophoresis and zymograms of the enzymatic preparation

The protein SDS-PAGE and the CMCase and β-glucosidases zymograms from *T. harzianum* IOC 3844 are shown in Figure 5. In all of the gels, the first column corresponds to the molecular weight standards and the other columns refer to the crude and concentrated enzymatic extracts. In relation to the proteins gel (Figure 5a), in the concentrated enzymatic extract were located 11 bands with the following molecular weights: 14 kDa, 19 kDa, 28 kDa, 43 kDa, 48 kDa, 50 kDa, 54 kDa, 62 kDa, 74 kDa, 80 kDa and 86.5 kDa. Some proteins were found simultaneously in the crude and concentrated extracts (19 kDa, 43 kDa and 62 kDa). These proteins are possibly the most abundant among those produced by *T. harzianum* IOC 3844. In the CMCase zymograms (Figure 5b), six enzyme bands with CMCase activity were identified. The CMCase molecular weights were approximately 48 kDa, 55 kDa, 59 kDa, 62 kDa, 69 kDa and 78 kDa. In the concentrated enzyme, it was possible to detect regions of high density of CMCase. There was a prominent 62 kDa band in the protein and CMCase gels; this is probably one of the most abundant CMCase produced by this strain. The high CMCase content is characteristic of this microorganism, as previously highlighted in the results of cellulase production in this study. The literature cited that the genus *Trichoderma* is a potential producer of CMCases. Kleywegt et al. (1997), for example, related that the filamentous fungus *T. reesei* secretes a very efficient cellulolytic system that consists of at least four CMCases (EG I, EG II, EG III and EG V), two cellobiohydrolases (CBH I and CBH II), and one β-glucosidase. In another study, Sadia et al. (2005) identified the presence of CMCase in the strain *T. harzianum* E 58 by electrophoresis. With regards to

the zymograms to identify β-glucosidases were found two bands with molecular weights of 75 kDa and 85 kDa in the concentrated enzymatic extract (Figure 5c). Due to the low amounts of these enzymes, the bands of the crude extract were not observed. The small amount of β-glucosidases identified through the gel is consistent with the results from the reduced production of these enzymes by *T. harzianum* IOC 3844 verified in this work, as mentioned earlier. A similar profile was observed by Kwon et al. (1994). They identified only one band of β-glucosidase from *T. koningii* ATCC 26113 and three bands from *T. reesei* ATCC 2692. In general, the cellulase activities revealed by electrophoresis are in accordance to the previous results of cellulase kinetics, which showed high CMCase activity by *T. harzianum*.

CONCLUDING REMARKS

The central composite rotational design (CCRD) combined with the use of the desirability function proved to be an important tool for maximizing cellulase production by *T. harzianum* IOC 3844. This strain showed to be a potential producing agent of cellulases when grown in an instrumented bioreactor under optimal conditions with high enzymatic production (CMCase, 27,017 U x L^{-1}; FPase, 1,225 U x L^{-1} and β-glucosidase, 609 U x L^{-1} in 42 hrs). A comparison of the results obtained in the bioreactor containing optimized medium with those obtained with non-optimized media reveals that there was an expressive increase in enzymatic activities of 4- and 3-fold for CMCase and FPase activities, respectively, in the optimized media. Among the evaluated nutrients, urea and ammonium sulphate were of greater importance to cellulase production by this filamentous fungus. Hydrolysis yield values indicated that *T. harzianum* has enzymatic machinery competitive with currently available commercial preparations. After 28 hrs, the cellulignin hydrolysis yields were 48% (enzymatic preparation from *T. harzianum*) and 49% (*Multifect*®). Electrophoretic analysis identified eleven protein bands, six bands of enzymes with CMCase activity of molecular weights from 48 to 78 kDa and two bands with β-glucosidase activity of molecular weights of 75 and 85 kDa. Our work shows that the strain *T. harzianum* IOC 3844 is a hyper-cellulolytic fungus and that its concentrated enzymatic extract has great potential to be applied in the conversion of cellulose into fermentable sugars for the production of second-generation bioethanol.

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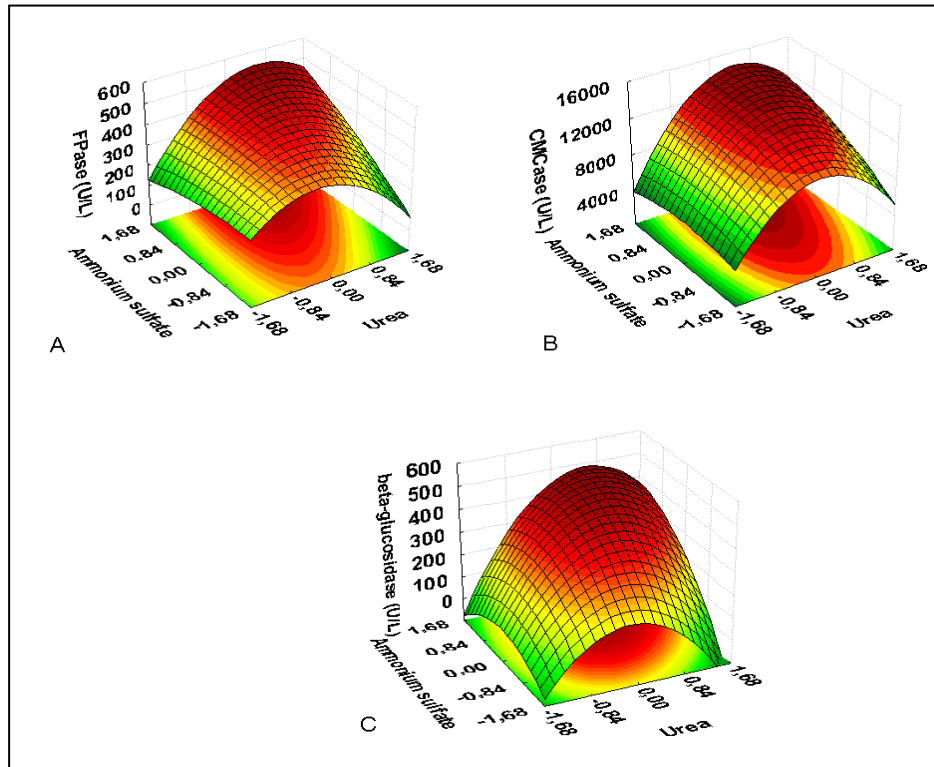
Figures

Fig. 1 The response surface of the CMCase, FPase and β-glucosidase activities (U x L^{-1}) according to the urea and ammonium sulphate (coded values) in the conditions evaluated in this study.
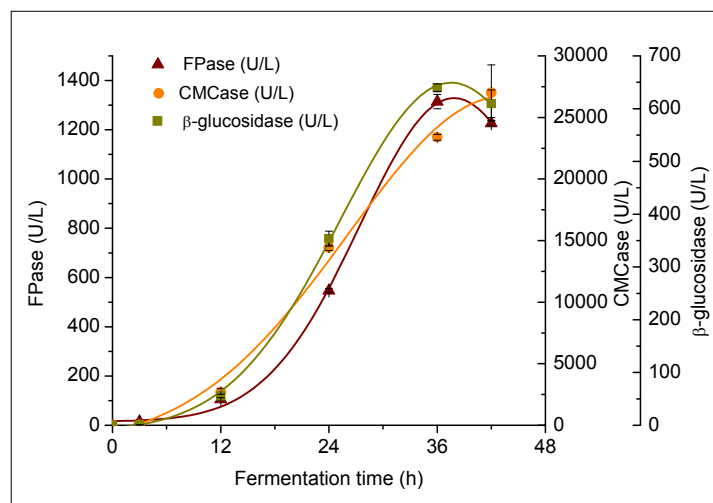

Fig. 2 The kinetic profile of cellulase production in an instrumented bioreactor (optimized medium, 10% v/v pre-culture media, temperature 30⁰C, pH 5.0, agitation 200 rpm, dissolved oxygen (DO) at 40% of saturation, 15 g x L^{-1} cellulose).
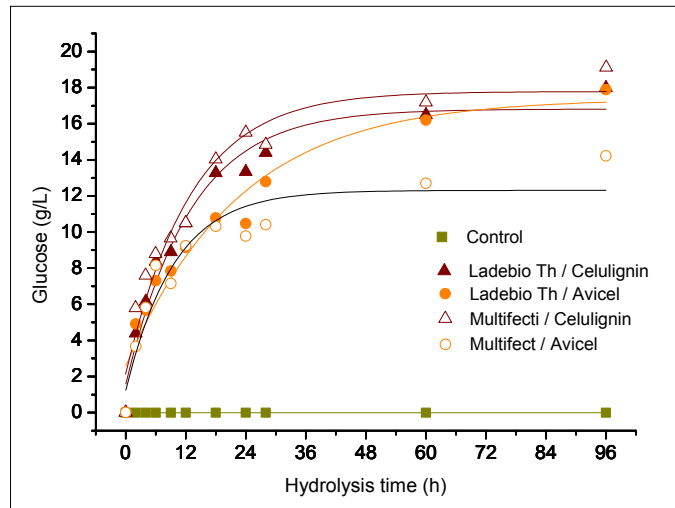
Fig. 3 Released glucose during the hydrolysis of PDC and Avicel® using cellulases from *T. harzianum* (*Ladebio Th*) and commercial enzymatic preparations (substrate concentration 40 g x L^{-1}; enzyme load 25 FPU/g, temperature 50ºC, agitation 200 rpm).
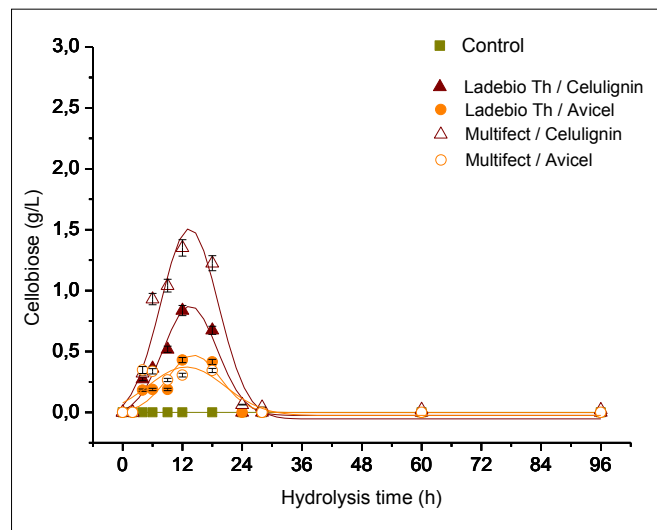

Fig. 4 Released and consumed cellobiose during PDC and Avicel® hydrolysis using cellulases from *T. harzianum* (*Ladebio Th*) and commercial enzymatic preparations (substrate concentration 40 g x L^{-1}; enzyme load 25 FPU/g, temperature 50ºC, agitation 200 rpm).
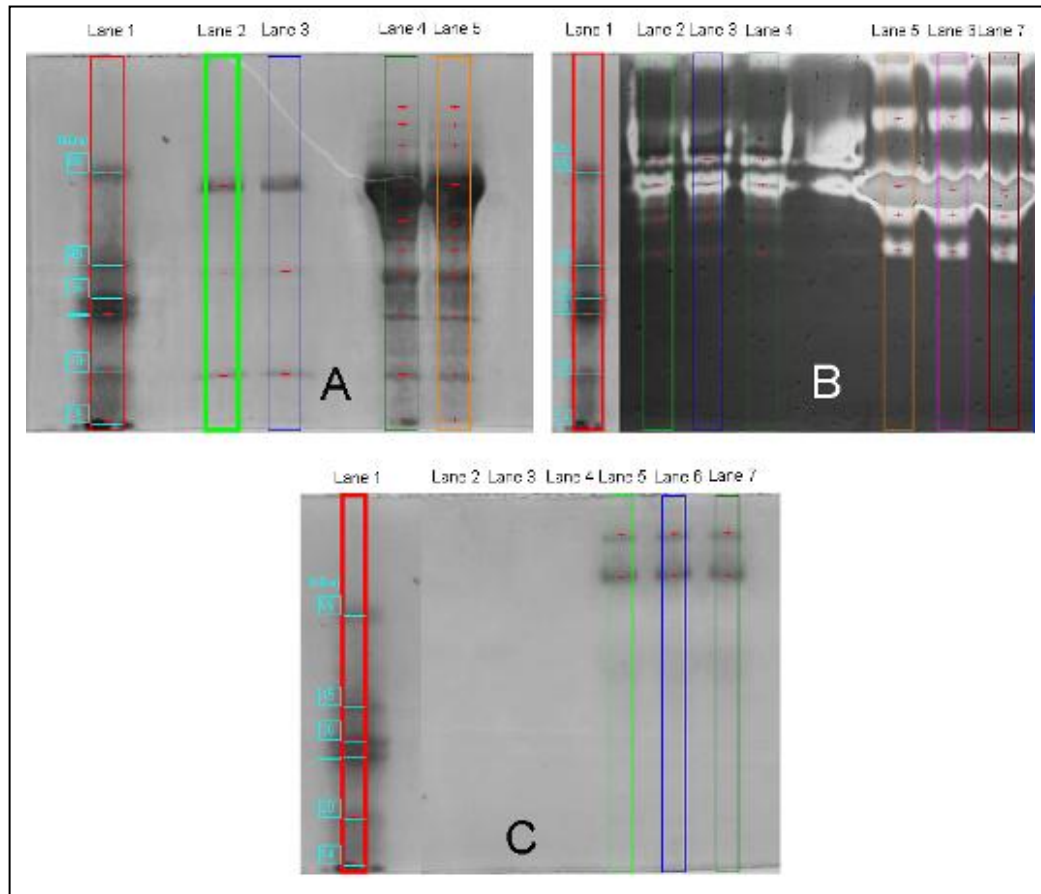
Fig. 5 (a) Protein SDS-PAGE gel stained in coomassie blue dye. Lane 1 (molecular weight standards), lanes 2 and 3 (crude enzymatic extract), lanes 4 and 5 (concentrated enzymatic extract).
(b) CMCase zymograms revealed in Congo red dye. Lane 1 (molecular weight standards), lanes 2, 3 and 4 (crude enzymatic extract), lanes 5, 6 and 7 (concentrated enzymatic extract).
(c) β-glucosidases zymograms revealed in esculin solution (0.1%). Lane 1 (molecular weight standards), lanes 2, 3 and 4 (crude enzymatic extract), lanes 5, 6 and 7 (concentrated enzymatic extract).