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Use of a Mixture of Thermophilic Enzymes Produced by the Fungus *Thermoascus aurantiacus* to Enhance the Enzymatic Hydrolysis of the Sugarcane Bagasse Cellulose

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Abstract: Problem statement: The production of hydrolytic enzymes by *T. aurantiacus* has been performed under solid-state fermentations using lignocellulosic materials. The influences of the inoculum size and of the fermentation medium on the production of hemicellulases and cellulases were studied. Filtrates from the cultures were used to hydrolyze a pulp of sugarcane bagasse and the produced enzymes were shown to be candidates for use as co-adjuvants in plant saccharification. **Aproach:** The present study focuses on the effect of different culture conditions on production of cellulases and hemicellulases by *T. aurantiacus*. It is also provides a possible application of *T. aurantiacus* enzymes in the degradation of sugarcane bagasse pulp, considering that this thermophilic fungus is a potential source of thermostable enzymes. **Results:** *T. aurantiacus* was cultivated on four different agricultural residues: sugarcane bagasse, sugarcane straw, wheat straw and corn cob. Xylanase was produced with much more expressive activity than cellulases. The highest titre of xylanase was obtained on sugarcane straw at 9 days (1679.8 IU g^{-1}); the same was observed for β-glucosidase (29.9 IU g^{-1}) at 6 days. With an inoculum load of 10^8 spores g^{-1}, the amount of exoglucanase produced by the fungus considerably exceeds that produced with 10^4 spores g^{-1}. Xylanases and cellulases purified from filtrates of the cultures were investigated to hydrolyze a bagasse pulp prepared with alkaline peroxide. Xylanase or sulphuric acid were used as pretreatments for xylan removal, increasing the cellulase performance on pulp bagasse. However, results revealed that the removal of hemicellulose is not the only main factor limiting the cellulose hydrolysis. **Conclusion:** Results indicate that the xylanase action on alkaline-pretreated sugar cane bagasse enhances the cellulolytic effect promoted by a commercial cellulase. This study thus presents an evaluation of the applicability of enzymes from *Thermoascus aurantiacus* to potentially improve the enzymatic cellulose hydrolysis.

Key words: Xylanase, cellulose, *Thermoascus aurantiacus*, purification, enzymatic hydrolysis, lignocellulose

INTRODUCTION

The study of sugarcane as a feedstock for the production of biofuel is a significant endeavor (Kuo and Lee, 2009; Lee and Breessan, 2006; Baudel *et al*., 2005; Adsul *et al*., 2005). This material contains 75-80% of polysaccharides that can be converted into fuels. Due to its recalcitrance, the bagasse needs a pretreatment prior to enzymatic hydrolysis, to increase the accessibility of the substrate to enzymatic attack. The pretreatment should preferably result in removal of lignin and hemicellulose, resulting in increased surface area and substrate porosity (Varnai *et al*., 2010).

The raw material and pretreatment will affect the enzyme composition needed. An alkaline peroxide to treat bagasse was reported by Brienzo *et al*. (2009), resulting in a pulp with 5.9% lignin, 16.1% hemicellulose and 61% cellulose. However, despite pretreatment of the bagasse, cellulase preparations appear not always contain all the required activities in optimal ratios for its degradation (Rabelo *et al*., 2009). Hence, further improvements for the polysaccharide cell wall hydrolysis needs to be achieved by improving the enzyme spectrum. Supplementation with extra β-glucosidase is usually required for complete conversion of cellulose. Because of the high levels of cellulases produced by *T. reesei* strains, these seem to be a well-

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suited starting point for obtaining improved cellulose hydrolysis via boosting of co-adjuvant enzymes. However, several thermophilic cellobiohydrolases of family 7 performed better than *T. reesei* cel 7A in the hydrolysis of substrates at 45°C (Wojtczak *et al*., 1987).

Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at temperatures of 50-70°C, particularly species such as *Thielavia terrestris, Aspergillus terreus, Sporotrichum thermophile* QM-9382 and *Thermoascus aurantiacus* (Wojtczak *et al*., 1987; Romaneli *et al*., 1975). *T. aurantiacus* is specially a good producer of β-glucosidases. In general, its β-glucosidases show high stability, half lives at 70°C of 23.5 h (Gomes *et al*., 2000), optimum temperature and pH between 65 and 80°C and 4.5 and 6, respectively (Gomes *et al*., 2000; Hong *et al*., 2007). Different *T. aurantiacus* strains show endoglucanases with acid pI (around 3.5-3.7) (Hong *et al*., 2007; Parry *et al*., 2001) that display high stability (at 70°C half live of 98 h) (Gomes *et al*., 2000), optimum temperature and pH between 65 and 80°C and 4.0 and 5.5, respectively (Parry *et al*., 2001; Kalogeris *et al*., 2003). Besides producing cellulases, *T. aurantiacus* can secret most of the hemicellulolytic enzymes, endo-xylanase being the main enzyme detected in its culture, similar to several other well known hemicellulase-producing microorganisms such as *Aspergillus niger* (Bailey and Poutanen, 1989; Kang *et al*., 2004; Coral *et al*., 2002) and *Trichoderma reesei* (Juhasz *et al*., 2005).

More efficient hydrolysis of lignocellulose is obtained by using only thermostable enzyme components (endoglucanase, exoglucanse, β-glucosidase and xylanase) (Viikari *et al*., 2007). An aproach to develop thermophilic hydrolytic mixtures is the purification of the candidate enzymes and further evaluation of the most interesting proteins by using characterized mixtures of purified proteins to hydrolyze substrates. In this work, we examined the extracellular enzymes produced by *T. aurantiacus* in various culture conditions. The main enzymes known to be involved in lignocellulose biodegradation were purified and characterized. Mixtures of the most promising enzymes were evaluated to find the best cocktail for the hydrolysis of a pulp of sugarcane bagasse.

MATERIALS AND METHODS

Microorganism and culture conditions: *Thermoascus aurantiacus* ATCC 204492 was used in this study. Fungal inoculum was prepared from mycelia grown on 2% (w/v) Potato Dextrose Agar (PDA), incubated at 45°C during 4 days under static conditions. Solid culture media were inoculated with spore suspensions prepared by adding 10 mL of distilled water to slant cultures whose surfaces were gently scraped with a sterile wire loop. The spores were counted in a Neubauer chamber and a standardized suspension (10^4 or 10^8 spores $^{-1}$g substrate) was inoculated into Erlenmeyer flasks of 300 mL with 15 g of the different agroindustrial residues: sugarcane bagasse, sugarcane straw, wheat straw and corncob, all of them milled to pass through a 25 mesh screen. The moisture was adjusted to 81 with 2% (v/v) Vogel solution (Vogel, 1956). After stationary incubation at 45°C during the different periods, the content of each flask was washed and recovered with 100 mL of 50 mM sodium acetate buffer at pH 5.5 under agitation for 1 h at 60 rpm.

Enzymatic activities: The xylanase activity was determined in a mixture containing 800 µL of birchwood xylan (Sigma-ST. Louis, USA, 1% w/v), prepared in 50 mM sodium acetate buffer, pH 5.0, plus 100 µL of *T. aurantiacus* extract to initiate the reaction. The mixture was incubated at 50°C for 5 min. After incubation time, reducing sugars were determined by adding 1.5 mL of DNS (3, 5-dinitrosalicylic acid) reagent, boiling for 5 min, cooling and measuring the absorbance at 540 nm (Bailey *et al*., 1992). The endoglucanase activity was determined by the degradation of CMC (0.44% w/v) (Sigma-St. Louis, USA, 1% w/v, medium viscosity), prepared in 50 mM sodium acetate buffer, pH 5.0. After each incubation time, 1mL of the sample was recovered and assayed for reducing sugars. The exoglucanase activity was determined according to Wood and Bhat (1988), using a solution of Avicel 1%. The filter paper activity of the preparations was determined using the procedure recommended by the IUPAC method (Ghose, 1987). β-Glucosidase activity was determined by monitoring the hydrolysis of p-nitropheny l-β-1, 4-O-glucoside. β-xylosidase activity was determined using *p*-nitrophenyl-β-D-xylopyranoside, according to Tanaka *et al*. (1981). The enzyme activities were expressed in International Units (IU), where 1 IU is equivalent to the amount of enzyme necessary to releases 1 µmol product min^{-1}.

Enzyme purification: A volume of 50 mL of enzymes of *T. aurantiacus* produced in corn cob, with 17 days of cultivation, was loaded into a column (10.2×0.98 cm) packed with the anion-exchange resin DEAE Sepharose CL 6B (*GE Healthcare*). Previous to the loading of the extracts, the column was equilibrated with 50 mM sodium acetate buffer at pH 3.5. Loading and washing operations were carried out at a flow rate of 0.50-

0.75 mL min^{-1}. Elution afterwards was by 0.2 M buffered NaCl at the same flow rate (Khandke *et al*., 1989). The protein content was determined with a UV detector at 280 nm (Pharmacia LKB-Optical unit UV-1). Denaturing electrophoresis was performed in 12.5% polyacrylamide gels, using a discontinuous system. Proteins were stained with silver nitrate (Ornstein, 1964).

Substrate preparation: Sugarcane bagasse, milled to particles smaller than 25 mesh, was washed with 0.2% (w/v) Ethylenediamine Tetraacetic Acid (EDTA) for 1 h at 90°C to remove metal cations, such as iron and manganese. These ions promote decomposition of hydrogen peroxide reducing its delignification performance. Approximately 10 g of milled sample was extracted with 95% ethanol for 6 h in a Soxhlet apparatus. The material was submitted to a pretreatment using alkaline solution of hydrogen peroxide. A sample of 10 g of dewaxed bagasse was treated with alkaline peroxide (4%) and magnesium sulfate (0.5%) with the pH adjusted to 11.6 with NaOH, in a reaction volume of 200 mL incubated at 40°C. After 10 h, the insoluble residue, named bagasse pulp, was collected by filtration, washed with distilled water until the pH was neutral and then dried at 45°C (Brienzo *et al*., 2009). The cellulose content in the pulp was 70.3% and it also contained residual hemicellulose (9.3%) and lignin (5.6%).

Hemicellulose removal of substrate by acid and enzymatic hydrolysis: The bagasse pulp (5% w/v) was ressuspended in a solution of diluted sulfuric acid at two concentrations, 0.009 and 0.018%. The hydrolysis was carried out in 125 ml Erlenmeyer flasks for 45 min at 121°C. Each sample was then centrifuged and the supernatant was collected, filtered and subjected to HPLC analysis for glucose, xylose and cellobiose quantification. The bagasse pulp (5% w/v) was also treated with a purified xylanase from *T. aurantiacus* for 24 h and the sugars were analyzed by HPLC. The concentrations of monomeric sugars in the soluble fraction were determined by HPLC using a Biorad HPX87H column at 45°C, eluted at 0.6 mL min^{-1} with 0.005 mol L^{-1} sulfuric acid. Sugars were detected in a 30°C-temperature controlled RI detector. The concentrations of Xylooligomers (XOS) were determined using a HPX42A column at 60°C, eluted at 0.6 mL min^{-1} with deionized water. The XOS formed were quantified by comparing the peak area of XOS with that of standards (xylobiose, xylotriose, xylotetraose and xylopentaose), being expressed as mg mL^{-1} of hydrolysate.

Enzymatic hydrolysis: Enzymatic hydrolysis experiments were carried out for 72 h by using a) commercial enzyme preparation (Celluclast 1.5L, Novozymes, Denmark) at dosage of 6.5 FPU g^{-1} bagasse (d.w.) and b) culture filtrate of *T. aurantiacus* (18.6 UI g^{-1} substrate, as endoglucanase activity). These enzyme preparations were tested for their ability to convert the bagasse pulp to soluble sugars. The purified xylanase, exoglucanase, endoglucanase and β-glucosidase preparations of *T. aurantiacus* were also evaluated in the hydrolysis of the bagasse pulp, at the same dosages as those found in the non-purified extract. The enzymatic hydrolysis was carried out in 50 mM sodium-acetate buffer, pH 5.0, at 45°C, with 5% of consistency. The reaction was stopped by heating at 100°C for 5 min, followed by centrifugation at 7800×g for 15 min. Three replicates were run for each reaction time, which varied from 8-72 h. Hydrolysates were assayed for glucose, celobiose and xylose contents, using the same HPLC procedure described before. The Cellulose Conversion (CEC) was estimated considering glucose and cellobiose as the hydrolysis products.

RESULTS

Production of cellulases and hemicellulases: Different carbon sources were used to inducer the production of cellulolytic and hemicellulolytic enzymes. The productions were performed in solid state fermentations. The main enzymatic activities produced by *T. aurantiacus* in these conditions are listed in Table 1. The cellullose/hemicellulose ratio was similar among the substrates; however lignin content was different and, apparently, played a critical factor in enzymes production. Xylanase activities were at their maximum levels on the sugarcane straw or bagasse media after 9 days (Fig. 1). In wheat straw and corn cob media, the peaks of xylanase occurred at 37 days. For the hydrolysis of substrates containing a mixture of hemicellulosic and cellulosic sugars, a culture filtrate containing thermostable activities of both hemicellulases and celullases may be interesting to carry out such hydrolysis at elevated temperatures for prolonged periods, in order to produce a hydrolysate rich in pentose and hexose sugars.

The profiles of endoglucanase and β-glucosidase productions apparently coincided with that of xylanase, indicating that the production of cellulases and xylanase are linked when the fungus is grown on agro-industrial residues (Fig. 1), similarly to what was reported previously (Royer and Nakas, 1990). There were major differences among the different media in terms of exoglucanase activity.

Table 1: Production of *T. aurantiacus* enzymes on solid state cultivation

Composition (g/100 g of original substrate)				Enzymatic activities (IU g^{-1})				
	Glucan	Hemicellulose	Lignin	xylanase	endoglucanase	exoglucanase	β-glucosidase	β-xylosidase
Wheat straw	41.0	26.0	24.0	1315.9	45.4	1.6	1.7	0.4
Sugarcane straw	40.6	28.7	20.5	1679.8	91.8	26.1	29.9	2.2
Sugarcane bagasse	43.7	27.4	24.4	978.1	108.9	11.1	7.2	0.8
corncob	42.4	25.2	19.6	672.6	37	46	2.6	5.2

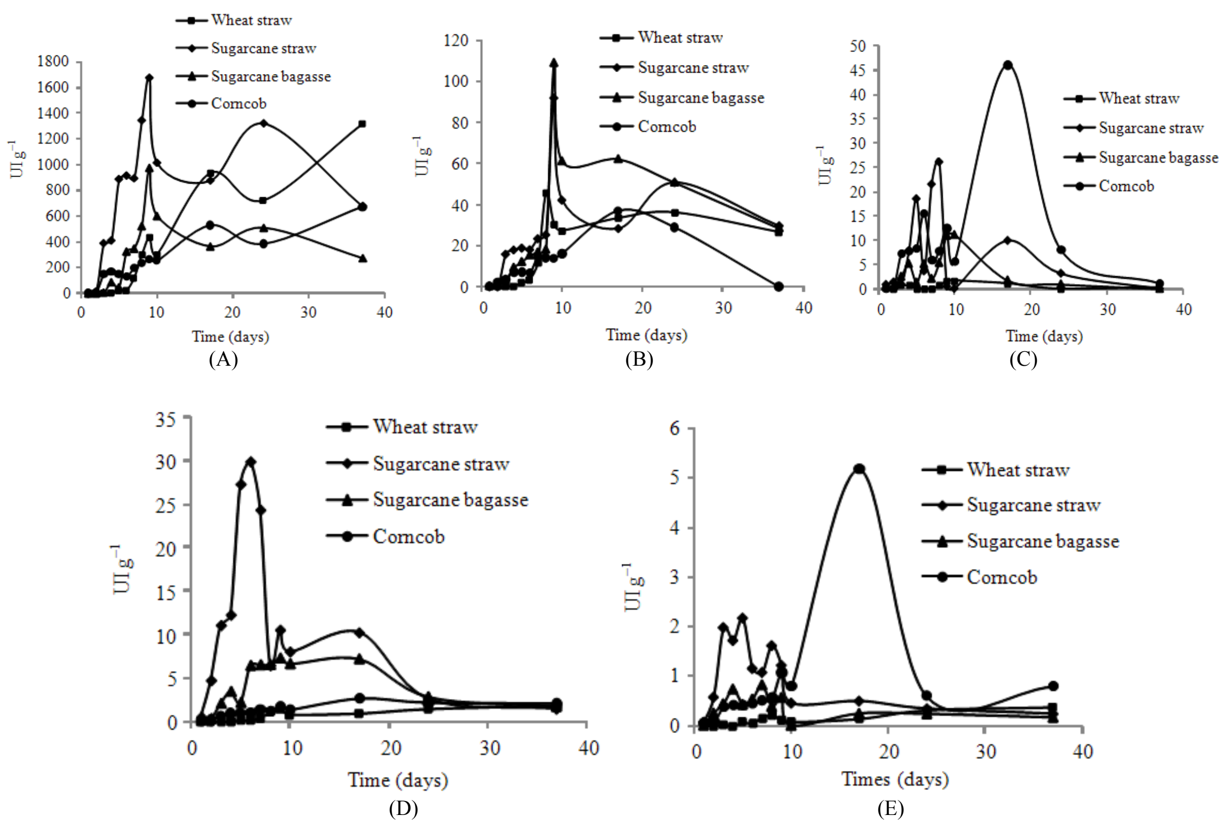

Fig. 1: Influence of culture medium on enzymatic activities in four different media. (A) Xylanase; (B) Endoglucanase; (C) Exoglucanase; (D) β-glucosidase; (E) β-xylosidase

The production of exoglucanase was generally much lower than that of endoglucanase; detectable exoglucanase production occurred only in corn cob medium. The best production was obtained on day 17 of cultivation, which increased markedly enzyme production.

The influence of two spore concentrations on enzyme production was studied using the sugarcane bagasse medium. *T. aurantiacus* produced different amounts of cellulases and hemicellulases activities depending on inoculum size. Table 2 summarizes the results obtained with the two inoculum sizes. Even if the peaks of activities using a reduced inoculum of 10^4 spores g^{-1} or a big inoculum of 10^8 spores g^{-1} were coincident, the use of a high inoculum spore concentration led to higher cellulases activities. At low inoculum size, xylanase was dominant.

Finally, comparing the commercially available and the in-house produced enzymes, it was seen that the activity of xylanase in the commercial extract was lower than that achieved in the extracted produced in-house with the corn cob medium. In spite of this, total cellulase activity (FPA) was not detected in the filtrate of the *T. aurantiacus* cultivation, since the amount of sugar released was less than 2 mg mL^{-1} glucose (Ghose, 1987). The enzymes produced in-house with corn cob and the comercial cellulase (Celluclast 1.5 L) were both characterized with respect to the various activities (Table 3).

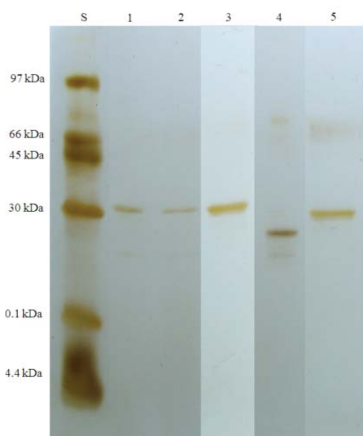

Fig. 2: Protein profile of purified hydrolytic enzymes of *T. aurantiacus*. Denaturing gel electrophoresis of Bis/acrylamide (SDS-PAGE) stained with 12.5% silver nitrate. Values of *M*R in kDa S: protein standard. 1 and 2: xylanases; 3: endoglucanase; 4 and 5: β-glucosidase

Table 2: Enzymes from *T. aurantiacus* obtained with different inoculum sizes in the sugarcane bagasse medium

	Enzymatic Activities (IU g^{-1})	
Enzymes	10^4 spores mL^{-1}	10^8 spores mL^{-1}
Xylanase	1315.9	978.1
Endoglucanase	45.4	108.9
Exoglucanase	1.6	11.1
β-glucosidase	1.7	7.2
β-xylosidase	0.4	0.8

Table 3: Enzymatic activities (IU g^{-1} substrate) of Celluclast and corn cob culture filtrates of *Thermoascus aurantiacus* used to treat pulp bagasse

Enzymes	Celluclast 1.5 L (Novozymes)	*T. aurantiacus* filtrate
FPA	6.51	0.00
β-glucosidase	0.54	0.40
Exoglucanase	4.79	0.26
Endoglucanase	18.60	18.60
Xylanase	20.61	123.34
β-xylosidase	0.59	0.08

Xylanase and cellulase purification: The 17 day-old culture grown on corn cob was mixed with the aforementioned buffer and extracted to obtain a culture filtrate. Besides xylanases, endoglucanases and β-glucosidases, the enzymatic extract produced on corn cob contained a high exoglucanase activity and, for this reason, it was chosen for purification of enzymes. These four enzymes are involved in cell wall polysaccharide degradation.

Xylanase and cellulases secreted by *T. aurantiacus* were partially purified from culture fluid in a one-step chromatographic procedure on DEAE-Sephadex A-50 at pH 3.5, which resulted in the isolation of a xylanase activity. Application of a sodium chloride gradient (0-0.2 M) led to the elution of cellulase enzymes. It is seen, from Fig. 2, that all four enzymes were identified on SDS-PAGE. Exoglucanase (line 4) or endoglucanase (line 5) were contaminated with low activities of β-glucosidase. Similar results were reported for the same fungus, both in solid cultivation in wheat straw (Mamma *et al*., 2009) and in submerged cultivation using paper as substrate (Tong *et al*., 1980) or corn cobs (Parry *et al*., 2002).

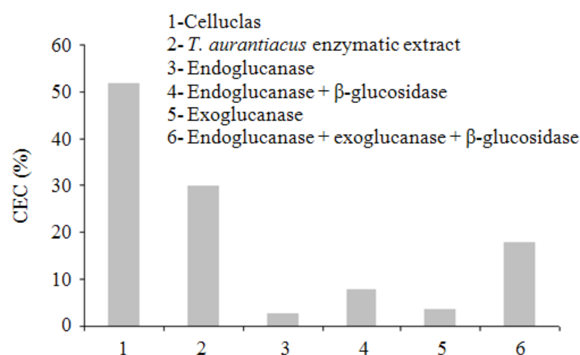

Fig. 3: Extent of cellulose conversion of 24 h enzymatic hydrolysis of alkali-pulp bagasse (5%). The dosage was 6.5 FPU Celluclast per gram pulp (18.6 IU endoglucanase/g), 2-Dosage of *T.aurantiacus* extract was the same as the endoglucanase activity in Celluclast, 3- 18.6 U g^{-1} *T. aurantiacus* endoglucanase, 4-18.6 U g^{-1} endoglucanase plus 0.4 U g^{-1} β-glucosidase, 5-0.26 U g^{-1} exoglucanase, 6-18.6 U g^{-1} endoglucanase plus 0.4 U g^{-1} β-glucosidase and 0.26 U g^{-1} exoglucanase

Hydrolysis of the bagasse pulp by the different extracts: Commercial cellulase and culture filtrate of *T. aurantiacus* were tested for their ability to convert the bagasse pulp into soluble sugars. A comparison of the glucose and cellobiose release and of the cellulose conversion was made during prolonged saccharifications of the bagasse pulp at pH 5, 50°C. Treatment with Celluclast at a dosage of 6.5 FPU g^{-1} substrate gave high yield of glucose, corresponding to 50% cellulose conversion (Fig. 3). This cellulase preparation had an activity of endoglucanase equal to 18.6 IU g^{-1} substrate (Table 3).

To assess the potential cellulolytic effect of *T. aurantiacus*, the same dosage of endoglucanase was used for pulp saccharification. However, the cellulose conversion was very low compared to the yield obtained with the Celluclast extract (Fig. 3).

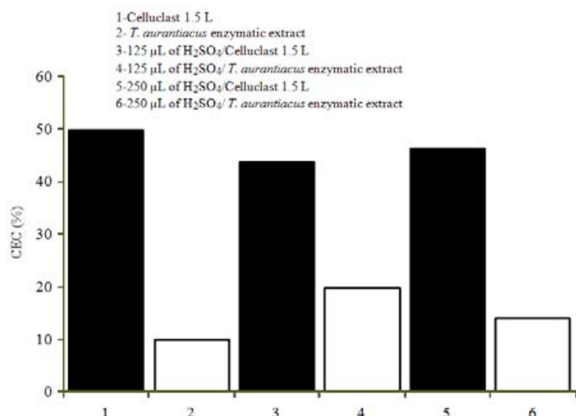

Fig. 4: Effect of hemicellulose removal by dilute acid on enzymatic hydrolysis of sugarcane bagasse cellulosic pulp

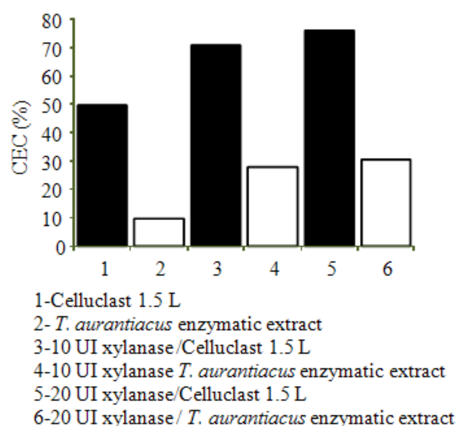

Fig. 5: Effect of hemicellulose removal by purified xylanase of *T. aurantiacus* on enzymatic hydrolysis of sugarcane bagasse cellulosic pulp

The enzymatic hydrolysis of the pulp with the culture filtrate of *T. aurantiacus* reached 30% conversion, whereas treatment with commercial cellulase achieved 50% of enzymatic conversion of cellulose (CEC). Although the *T. aurantiacus* extract does not require externally added β-glucosidase and provide endoglucanase to hydrolyze amorphous cellulose, additional activities are needed to hydrolyze crystalline cellulose. The ability of this filtrate to replace the commercial cellulase extract is still low. The culture filtrate of *T. aurantiacus* presents the entire complex of enzymes necessary for cellulose hydrolysis. However, the coordinate action of these enzymes did not result on an efficient cellulose conversion. To define the principal enzyme of the *T. aurantiacus* extract acting on the conversion of the bagasse pulp cellulose, we performed the hydrolysis adding each enzyme separately and associated. Treatment with endoglucanase alone, at a dosage of 18.6 U g^{-1}, gave very low yield of glucose release, with a cellulose conversion of 3% within 24 h of treatment. The combination of endo-glucanase and β-glucosidase did not improve the yield of glucose release very much, showing that no significant amount of cellobiose had been was produced by the sole endoglucanase. The cellulose conversion was, however, significantly increased by addition of the exoglucanase + endoglucanase + β-glucosidase, compared to the other conversions obtained with purified enzymes. Notably, the *T. aurantiacus* whole enzymatic extract gave a cellulose conversion that was almost twice superior to that observed with the mixture of purified enzymes, exoglucanase + endoglucanase + β-glucosidase. Probably, the presence of the cellulase enhancing factor in the enzymatic extracts of *T. aurantiacus* (Cel61A) has improved the hydrolysis of the sugarcane bagasse pulp, as already observed previously (Viikari *et al*., 2007).

Since the removal of hemicellulose from pulp bagasse can lead to an increase in the yield of cellulose hydrolysis (Varnai *et al*., 2010), we evaluated the pre-treatment of the pulp with acid or purified xylanase. Pretreatment of pulp with dilute acid was able to remove 46.6 and 59.7% hemicellulose, using 0.009 and 0.018% w/w sulphuric acid, respectively. When the pretreatment of pulp bagasse was performed with xylanase, few changes occurred in terms of polysaccharides composition. The production of XOS was not significant with the two enzyme doses evaluated and the amount of released xylose was almost independent of the enzyme load. The percentage of hemicellulose removal was 0.54% for 10 IU xylanase per gram of pulp and 0.69% for 20 IU g^{-1}. Enzymatic treatment of the pulp which was previously hydrolyzed with acid did not enhance cellulose hydrolysis (Fig. 4), although almost fifty percent of hemicellulose had been removed. When xylanase of *T. aurantiacus* was used for pre-hydrolysis of the pulp, Celluclast gave a relatively high cellulose conversion, i.e., 70% conversion within the 72 h reaction (Fig. 5). Despite the fact that the pre-hydrolysis of pulp with acid was more efficient in the removal of hemicellulose, the treatment with xylanase produced a substrate more easily hydrolysable by cellulases.

DISCUSSION

T. aurantiacus produces a complete cellulolytic system and the level of expression of such enzymes is dependent on initial biomass concentration dose and substrate composition. Xylanase was always produced in higher titres, confirming that this fungus is more hemicellulolytic than cellulolytic (Da Silva *et al*., 2005). Although the titres of the extracellular enzymes produced by *T. aurantiacus* are lower than those found in commercial preparations, *T. aurantiacus* enzymes have the advantage to be highly thermostable. In this regard, they appear particularly useful, since they can be used readily at temperatures at which they function best. However, their use as a complete system is more appropriated than in purified form. There are co-adjuvant proteins, that we did not evaluate in the commercial extract or in the culture filtrate, that seem to be important for cellulose hydrolysis. Besides, the presence of xylanases in the in-house prepared enzymatic extract also favors the cellulose hydrolysis. The pretreatment of pulp bagasse with hemicellulases prior to cellulases dramatically increased the effectiveness of the cellulose hydrolysis. Since such a pretreatment almost did not remove the residual hemicelulose of the pulp of bagasse, the beneficial effect observed may have resulted from the creation of new sites for attack by cellulases or from the prevention of non-productive adsorption of cellulases on lignin (Eriksson *et al*., 2002). The substrate enzymatically pretreated attained 75% digestibility, although practically no hemicellulose and lignin removals happened. On the other hand, removal of hemicellulose by acid hydrolysis did not reach the same results. This observation supports the idea that, during acid hydrolysis, hemicellulose removal increases the proportion of lignin in the substrate, which becomes progressively more recalcitrant and also more prone to non-productive enzyme adsorption (Selig *et al*., 2007). These two observations regarding the hydrolysis of hemicellulose with mineral acid or xylanase suggest that lignin-carbohydrate linkages might be disrupted during xylanase pretreatment into forms that favor greater accessibility of cellulase to cellulose. Most likely, the xylanase added to the pretreated bagasse adsorbed onto both lignin and hemicellulose and, consequently, reduced nonproductive binding of the cellulases.

CONCLUSION

It was concluded that the substrate used for the enzyme production was not effective in differentiating the enzymatic profile of *T. aurantiacus*, which proved to be more hemicellulolytic than cellulolytic, regardless of the substrate. The increased initial inoculum did not alter the time for maximum enzyme production; however, cellulases, especially exoglucanase, had its activity increased by almost 10 times. *T. aurantiacus* xylanase showed no hydrolytic effect on the bagasse pulp, but its addition was particularly effective when added before the cellulases, consistent with it being irreversibly bound to lignin. Consequently, treatment with xylanase has important implications in potentially reducing the need for high enzyme doses for complete cellulose digestion. Pre-treatment with dilute sulfuric acid was able to remove up to 50% of the hemicellulose present in the pulp of bagasse, but did not result in an increase in cellulose conversion, suggesting that, in addition to removal of hemicellulose, other differences in the structure of the pulp such as surface alterations also influence the saccharification by cellulases.

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