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The influence of pretreatment methods on saccharification of sugarcane bagasse by an enzyme extract from *Chrysoporthe cubensis* and commercial cocktails: A comparative study

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HIGHLIGHTS

- *Chrysoporthe cubensis* produces an enzyme mixture with high specific activities.
- The alkali-pretreated bagasse resulted in the best sugars release by all mixtures.
- The *C. cubensis* mixture was the best one for sugarcane bagasse saccharification.
- *C. cubensis* has a great potential as enzyme producer for biomass hydrolysis.

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ABSTRACT

Biomass enzymatic hydrolysis depends on the pretreatment methods employed, the composition of initial feedstock and the enzyme cocktail used to release sugars for subsequent fermentation into ethanol. In this study, sugarcane bagasse was pretreated with 1% H_2SO_4 and 1% NaOH and the biomass saccharification was performed with 8% solids loading using 10 FPase units/g of bagasse of the enzymatic extract from *Chrysoporthe cubensis* and three commercial cocktails for a comparative study. Overall, the best glucose and xylose release was obtained from alkaline pretreated sugarcane bagasse. The *C. cubensis* extract promoted higher release of glucose (5.32 g/L) and xylose (9.00 g/L) than the commercial mixtures. Moreover, the *C. cubensis* extract presented high specific enzyme activities when compared to commercial cocktails mainly concerning to endoglucanase (331.84 U/mg of protein), β-glucosidase (29.48 U/mg of protein), β-xylosidase (2.95 U/mg of protein), pectinase (127.46 U/mg of protein) and laccase (2.49 U/mg of protein).

1. Introduction

Lignocellulosic biomass is the most abundant organic material in the world and it has the potential to be a very promising alternative source of fuels and chemicals. Enzymatic hydrolysis of biomass for its conversion into liquid fuels requires the action of cellulases and hemicellulases, since lignocellulose consists of a network of cellulose and hemicellulose bound by lignin (Suhardi et al., 2013). For cellulose degradation, three enzymes typically act in synergy: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.176) and β-glucosidase (EC 3.2.1.21) (Horn et al., 2012). Concerning hemicellulose hydrolysis, a more complex set of enzymes is necessary. In the case of xylan hydrolysis, the major hemicellulose polymer, the action of endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and some accessory enzymes are required to increase sugars yields in the hydrolysis step. Examples of these accessory enzymes are α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase

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(EC3.1.1.72) and feruloyl esterase (3.1.173) (de Vries et al., 2000; van den Brink and de Vries, 2011; Van Dyk and Pletschke, 2012).

To achieve complete conversion of lignocellulosic biomass to ethanol, there are still some hurdles to overcome. The major bottlenecks of this process remain the pretreatment methods, which have to be efficient and economical, and the high costs of enzymes, which have to be sufficiently robust to produce high yields of fermentable sugars (Jonsson et al., 2013).

Comparing various pretreatment methods for enzymatic saccharification enables the analysis of their efficiency on a particular biomass (Harrison et al., 2013). The essential factors of any pretreatment are to improve the availability of monomeric sugars, to prevent their degradation, to avoid inhibitor formation and to be low cost (Sun and Cheng, 2002).

Dilute acid pretreatments are normally used to degrade the hemicellulosic fraction and increase biomass porosity, improving the enzymatic hydrolysis of cellulose. This pretreatment is very commonly used because of its low cost and also due to the fact that the used acids are commonly available. The disadvantage of acid pretreatments is the formation of furan and short chain aliphatic acid derivatives, which are considered strong inhibitors in microbial fermentation (Hendriks and Zeeman, 2009; Kumar et al., 2009; Liu and Song, 2009). The most commonly used pretreatment methods for biomass hydrolysis are acid-based, mainly because most fungal enzymes, which are essential for enzymatic saccharification, have optimal pH values in the range of 4.0–5.0 (Dashtban et al., 2009).

Alkaline pretreatments differ from acid pretreatments in that they are able to remove lignin. The hydrolysis of ester linkages between hemicellulose residues and lignin promotes an increase of porosity in the biomass, and as a result cellulose and hemicellulose become more accessible to enzyme action (Cardona et al., 2010; Sun and Cheng, 2002). As this pre-treatment results in a large fraction of both cellulose and hemicellulose to remain intact, it has the potential for hydrolysis of a much larger fraction of the pretreated biomass, releasing glucose from cellulose and additional pentose sugars from hemicellulose. In addition, this occurs in an environment free of strong acids and fermentation inhibitors (Visser et al., 2013). Under these conditions, the degradation of sugars is minimal (Sharma et al., 2013). Although hydroxides are not expensive, the main downside of this process is that it consumes a lot of water for washing the sodium (or calcium) salts that incorporate into the biomass. These are difficult to remove, and in addition some enzyme inhibitors can be generated during lignin depolymerization (Chaturvedi and Verma, 2013).

The high costs and/or low efficiencies of the enzymes used for biomass hydrolysis are one of the main obstacles of lignocellulosic ethanol production. Enzymes must be stable, efficient, highly active and low cost. The majority of the cellulases applied in industries are produced by fungi of the genus *Trichoderma*. However, the amount of β-glucosidase secreted by *Trichoderma* species is very low, which can compromise complete cellulose hydrolysis due to the cellobiose accumulation (Jiang et al., 2011). *Penicillium* and *Aspergillus* are also good producers of cellulases, with higher levels of β-glucosidases, but they present lower FPase activity – total cellulase activity (Falkoski et al., 2013).

Plant pathogenic fungi produce extracellular enzymes to degrade plant cell walls, and there is a close relationship between hydrolase secretion capacity and the virulence of these microorganisms (Kikot et al., 2009). *Chrysoporthe cubensis* no exception to this and is able to produce high titers of cellulases and hemicellulases, mainly β-glucosidase, xylanase and some interesting accessory enzymes. The efficiency of *C. cubensis* enzymatic extract in the saccharification of alkali-pretreated sugarcane bagasse is around 60% for glucan and 90% for xylan degradation, respectively (Falkoski et al., 2013; Visser et al., 2013).

Few studies evaluate the effect of different enzymatic cocktails in the hydrolysis efficiency of acid- or alkali-pretreated biomass. In this study, saccharification of acid- and alkali-pretreated sugarcane bagasse was compared using different enzymatic mixtures. The aim of this work was to investigate the influence of these two pretreatment methods in view of enhancing the digestibility of the sugarcane bagasse and also to compare the effect of *C. cubensis* enzyme extract and three known commercial enzymes on the digestibility of pretreated material (acid- or alkaline-pretreated sugarcane bagasse).

2. Methods

2.1. Materials

Substrates including *p*-nitrophenyl-β-D-glucopyranoside (pNPGlc), *p*-nitrophenyl-β-D-xylopyranoside (pNPXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPMan), *p*-nitrophenyl-β-D-galactopyranoside (pNPGal), *p*-nitrophenyl-α-D-arabinofuranoside (pNPAra), *p*-nitrophenyl-β-D-cellobioside (pNPCel), carboxymethylcellulose (CMC), xylan from birchwood, locust bean gum, polygalacturonic acid, 2,2′-azino-bis(3-ethylben zothiazoline-6-sulfonic acid (ABTS) and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). The chemical reagents NaOH, H_2SO_4 and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). The commercial enzymatic mixtures Multifect® CL, Multifect® XL and Accellerase® 1500 were purchased from Dupont/Genencor International Inc. (Rochester, NY, USA). Sugarcane bagasse was kindly donated by Jatiboca Sugar and Ethanol Plant, Urucânia, MG, Brazil. Wheat bran was obtained on the local market. All others reagents used in this study were of analytical grade.

2.2. Strain and culture conditions

The Forest Pathology Laboratory (LPF), Federal University of Viçosa, MG, Brazil, kindly offered the fungus *C. cubensis* LPF-1 from its mycological collection. This fungus was maintained on PDA plates at 28 °C and periodically subcultured. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and incubated in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized using a Polytron® device and immediately used to inoculate the solid culture media. For enzyme production via solid state fermentation (SSF), 250 mL Erlenmeyer flasks contained 12.5 g of wheat bran and 18.75 mL of the culture media (final moisture of 60%) consisting of, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2.0. Furthermore, $MnCl_2$ (0.1 mg/L), H_3BO_3 (0.075 mg/L), Na_2MoO_4 (0.02 mg/L), $FeCl_3$ (1.0 mg/L) and $ZnSO_4$ (3.5 mg/L) were also added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 5 mL (containing 1.5×10^7 spores/mL) of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and enzyme extraction was performed after 7 days of fermentation. Enzymes secreted during SSF were extracted

solubilized in sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), with agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15,000*g* for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis.

2.3. Protein analysis

Protein concentration in the enzymatic extract from *C. cubensis* and in the commercial enzymatic mixtures was determined by the Coomassie Blue binding method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.4. Enzymatic assays

2.4.1. Total reducing sugars

FPase and endoglucanase activities were determined using Whatman No. 1 filter paper (1 × 6 cm, 50 mg) and 1.25% (w/v) CMC as substrates, respectively, according to previously described standard conditions (Ghose, 1987). The total reducing sugars released during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as the standard. Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1% w/v), locust bean gum (0.4% w/v) and polygalacturonic acid (0.25% w/v) as substrates, respectively, combined with the DNS method. The enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. The enzymatic reactions were initiated by the addition of 100 μL of the appropriately diluted enzyme solution to 400 μL of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 30 min and the total reducing sugar content released was determined via the DNS method using xylose, mannose and galacturonic acid as standards.

One unit of enzymatic activity (U) was defined as the amount of enzyme that released 1 μmol of the corresponding product (glucose equivalent, xylose, mannose, galacturonic acid) per minute, under the assay conditions used.

2.4.2. ρNP assays

β-Glucosidase, β-xylosidase, β-mannosidase, α-galactosidase, α-arabinofuranosidase and cellobiohydrolase activities were measured using ρPNGlc, ρNPXyl, ρNPMan, ρNPGal, ρNPAra and ρNPCel as substrates, respectively. The enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. The reaction mixtures contained 100 μL of the appropriately diluted enzyme solution, 125 μL of the synthetic substrate solution (4 mM at final concentration) and 275 μL of buffer. The reaction mixtures were incubated for 30 min and stopped by addition of 0.5 mL of a sodium carbonate solution (0.5 M). Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve.

One unit of enzymatic activity (U) was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute, under the assay conditions used.

2.4.3. Laccase activity

Laccase activity was determined by monitoring the oxidation of the substrate ABTS. The enzymatic assay was carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. It was performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. The reaction mixtures contained 100 μL of the appropriately diluted enzyme solution, 350 μL of the buffer and 50 μL of 10 mM ABTS. This mixture was incubated for 10 min and, at the end of the incubation period, absorbance was immediately measured at 420 nm. Laccase activity was calculated by the Lambert-Beer principle, using a molar extinction coefficient of $3.6 \times 10^4\ M^{-1}cm^{-1}$.

2.5. Biomass pretreatments

Sugarcane bagasse was washed and dried in an oven at 70 °C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline or acid pretreatments prior to being employed in saccharification experiments. Sodium hydroxide and sulfuric acid, both at concentrations of 1.0%, were used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v). The pretreatments were performed in an autoclave at 120 °C for 60 min. Pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at −20 °C.

2.6. Chemical composition of the bagasse samples

Approximately 3 g of milled samples were extracted with 95% ethanol for 6 h in a Soxhlet apparatus. Extracted samples were hydrolyzed with 72% (w/w) sulfuric acid at 30 °C for 1 h (300 mg of sample and 3 mL of sulfuric acid) as described by (Ferraz et al., 2000). Acid was diluted by addition of 79 mL of water and the mixture was heated to 121 °C for 1 h. The resulting material was cooled and filtered through a number 3 porous glass filter. Solids were dried to a constant weight at 105 °C, from which the insoluble lignin content was determined. Soluble lignin in the filtrate was determined by UV spectroscopy at 205 nm. An absorptivity value of 105 L/g cm was used to calculate the amount of acid-soluble lignin present in the hydrolysate. Concentrations of monomeric sugars in the soluble fraction were determined by HPLC using a BIO-RAD HPX-87H column at 45 °C eluted at 0.6 mL/min with 5 mM sulfuric acid. Sugars were detected with a temperature-controlled RI detector.

2.7. Sugarcane bagasse saccharification

The crude enzymatic extract from *C. cubensis* and the commercial cocktails (Multifect® CL, Multifect® XL and Accellerase® 1500) were applied in a biomass saccharification experiment. The *C. cubensis* enzymatic extract was concentrated 5-fold before the experiment using an Amicon Ultrafiltration system (Millipore Co. Billerica, MA, USA) with a membrane filter (Cut-off Mr 10,000 Da). Enzymatic saccharification of alkali- and acid-pretreated sugarcane bagasse was performed in 125 mL Erlenmeyer flasks with 50 mL working volume, at an initial solid concentration of 8% dry matter (w/v) in 50 mM sodium acetate buffer at pH 5.0. Enzyme loading was specified as 10 FPase units per gram of biomass with the addition of sodium azide (10 mM) and tetracycline (40 $\mu g mL^{-1}$) to the reaction mixture to inhibit microbial contamination. The reaction was carried out in an orbital shaker at 250 rpm and 50 °C for 72 h. Samples (1.0 mL) were taken from the reaction mixture at different time intervals for process monitoring. These samples were immediately heated to 100 °C to denature the enzymes, cooled and then centrifuged for 5 min at 15,000*g*.

2.8. Analysis of hydrolysis products

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) using a Shimadzu series 10A chromatograph as described by (Falkoski et al., 2013). The HPLC was equipped with an Aminex HPX-87P column (300 × 7.8 mm) and refractive index detector. The column was eluted with water at a flow rate of 0.6 mL/min and it operated at 80 °C.

3. Results and discussion

3.1. Activities of the enzymatic extracts

In order to establish a comparison between the enzymatic profile of the pathogenic fungus *C. cubensis* and the commercial cocktails (Multifect® CL, Multifect® XL and Accellerase® 1500), the activities of cellulases, hemicellulases, pectinase and laccase were determined (Table 1).

The commercial mixtures consist of highly concentrated industrial enzyme preparations and contain different additives to maintain all proteins in a very stable solution. In contrast, the extract from *C. cubensis* was prepared at the laboratory level in bench top fermenters. For this reason, and also to level the amount of enzymatic volumes, the extract from *C. cubensis* was concentrated 5 times.

The measured activities were then indexed to the protein content and expressed in specific activity. The *C. cubensis* extract contains higher endoglucanase activity, 1.5, 6.0 and 2.0-fold compared to Multifect® CL, Multifect® XL and Accellerase® 1500 commercial cocktails, respectively. This is significant since endoglucanase is believed to be the first enzyme to act on the cellulose structure, releasing substrates for other cellulases (Quay et al., 2011). The *C. cubensis* cocktail also presents high β-glucosidase activity, 3.0 and 24.0-fold compared to Multifect® CL and Multifect® XL, respectively. β-Glucosidase is necessary to release glucose and avoid the accumulation of cellobiose. The *C. cubensis* extract contains higher β-xylosidase activity, 2.5-fold compared to Multifect® XL and 15-fold compared to Multifect® CL and Accellerase® 1500, respectively. β-Glucosidase and β-xylosidase constitute the activities that are often insufficiently present in commercial cocktails, limiting their use for cellulose and hemicellulose hydrolysis (Borges et al., 2014). These two enzymes from *C. cubensis* could therefore be used to supplement commercial cocktails to avoid product inhibition that commonly retards or stops the action of enzymes during biomass hydrolysis.

In addition, the *C. cubensis* extract contains higher hemicellulolytic and pectinolytic activities than the other commercial mixtures. These accessory enzymes support the complete hydrolysis of biomass and they contribute to better action of the enzymatic mixture, since these enzymes are fundamental for the synergistic effect. The hydrolysis of biomass not only depends on the presence and isolated action of cellulases, but efficient degradation is a function of a balanced proportion of different enzymes that act in synergy to breakdown the complex structure of the lignocellulose (Van Dyk and Pletschke, 2012). When enzymes act in synergy, the total effect is greater than the sum of the effects of the individual components (Kostylev and Wilson, 2011).

Furthermore, the *C. cubensis* extract contains significant laccase activity, 2.49 U/mg protein, while in the other enzyme mixtures only traces of this activity could be measured. Laccase can assist in degradation of lignin, which is an obstacle to the biomass hydrolysis process. Extracts rich in laccase positively contribute for higher saccharification and fermentation yields since these enzymes can attack phenolic compounds derived from lignin degradation during the pretreatment which act as inhibitors (Kudanga and Le Roes-Hill, 2014).

3.2. Biomass pretreatment

After acid or alkaline pretreatment, the sugarcane bagasse was filtered and the solid fraction was dried for moisture content determination and compositional analysis (Table 2).

The acid pretreatment was responsible for 28.3% of biomass loss while 27.8% of sugarcane bagasse was lost after the alkaline pretreatment (Table 3). The percentage of biomass loss is associated with the severity of the specific pretreatment method and it affects the final yield increasing costs of the finished product (Chaturvedi and Verma, 2013).

The compositional analysis of the raw and the pretreated sugarcane bagasse samples shows that after the acid pretreatment the cellulose content decreased slightly (90% recovery) unlike in the alkaline pretreatment. The acid pretreatment can degrade some portions of the cellulose structure, contributing to reduce the crystallinity and the polymerization degree of this polysaccharide. The hemicellulose content in the alkali-pretreated sugarcane bagasse decreased only slightly (81% recovery), while a significant part of this fraction was removed in the acid pretreatment (27% recovery). The lignin content was reduced after the alkaline pretreatment (44% recovery), but the acid pretreatment did not result in a significant change in the lignin amount (93% recovery). These effects of acid and alkaline pretreatments, especially on hemicellulose and

Table 1
Comparative analysis of cellulases, hemicellulases, pectinase and laccase activities present in the crude extract from *Chrysoporthe cubensis* and the commercial cocktails Multifect® CL, Multifect® XL and Accellerase®1500.

Units of enzymatic activity/mg of protein				
Enzyme	*C. cubensis* extract	Multifect® CL	Multifect® XL	Accellerase® 1500
FPase	2.66 ± 0.015	23.50 ± 0.019	0.83 ± 0.018	10.95 ± 0.020
Endoglucanase	331.84 ± 0.065	208.32 ± 0.112	53.76 ± 0.024	178.37 ± 0.019
β-Glucosidase	29.48 ± 0.031	10.02 ± 0.020	1.21 ± 0.018	33.42 ± 0.086
Mannosidase	0.85 ± 0.017	2.11 ± 0.041	0.83 ± 0.05	6.63 ± 0.036
Xylanase	183.04 ± 0.019	313.17 ± 0.002	3135.02 ± 0.061	97.03 ± 0.009
β-Xylosidase	2.95 ± 0.034	0.20 ± 0.04	1.13 ± 0.001	0.20 ± 0.041
β-Mannosidase	1.69 ± 0.057	n.d.	0.002 ± 0	n.d.
Mannanase	14.12 ± 0.033	2.33 ± 0.056	3.17 ± 0.098	3.18 ± 0.21
Pectinase	127.46 ± 0.011	10.40 ± 0.012	20.54 ± 0.036	7.76 ± 0.004
α-Galactosidase	6.25 ± 0.051	0.03 ± 0.016	0.18 ± 0.005	n.d.
α-Arabinofuranosidase	8.70 ± 0.134	0.38 ± 0.002	0.20 ± 0.028	n.d.
Laccase	2.49 ± 0.077	n.d.	n.d.	n.d.

n.d., not detected.

Table 2
Compositional analysis of the raw and the pretreated sugarcane bagasse. The amounts of cellulose, hemicellulose, lignin and ash are based on dry weight.

Sample	Composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Total
SCB	34.01 ± 0.86	22.71 ± 0.45	30.07 ± 1.67	4.81	91.59
SCB after acid pretreatment	42.88 ± 0.74	8.69 ± 0.08	38.91 ± 0.69	5.58	96.07
SCB after alkaline pretreatment	47.99 ± 1.89	25.47 ± 1.05	18.52 ± 0.10	7.17	99.15

SCB, sugarcane bagasse.

lignin degradation, respectively, are in agreement with a previous study performed by Harrison et al. (Harrison et al., 2013).

The material provided by alkaline pretreatment contained approximately 20% more hemicellulose than the acid pretreated sugarcane bagasse and therefore could yield more xylose after biomass saccharification. However, glucose release would be expected to be very similar for both pretreatments since the amount of cellulose was 42.88% and 47.99% for acid and alkali-pretreated sugarcane bagasse, respectively. Nevertheless, the removal of lignin by the alkaline pretreatment, which is crucial for effective enzymatic hydrolysis of the biomass, could be responsible for a larger release of glucose. Lignin acts as a shield limiting the hydrolysis rate of the digestible portions in the plant cell wall and it can also absorb proteins in solution (Yang and Wyman, 2004). Almost 70% of all enzymes added for hydrolysis can become unproductive due to the nonspecific adsorption by lignin (Berlin et al., 2005). Therefore, the higher amount of lignin in the acid pretreated sugarcane bagasse contributes to nonspecific adsorption of cellulases and hemicellulases, leading to a reduced release of monosaccharides after biomass saccharification.

3.3. Saccharification of pretreated sugarcane bagasse by commercial enzyme mixtures

Three commercial enzymatic cocktails were applied for saccharification of pretreated sugarcane bagasse with the same enzyme loading of 10 FPase units/g of dried pretreated bagasse. Overall, the saccharification assays resulted in higher release of sugars from the alkaline pretreated sugarcane bagasse than the acid pretreated biomass (Fig. 1). Multifect® CL and Accellerase® 1500, commercial enzymatic mixtures containing high cellulase activities, were able to promote the release of 5.30 g/L and 3.19 g/L of glucose from the alkaline pretreated sugarcane bagasse, respectively, but only 1.29 g/L and 0.37 g/L of xylose, respectively (Fig 1A and C). In contrast, hydrolysis of the same bagasse with Multifect® XL, a commercial enzymatic mixture containing higher xylanase activity, released 8.83 g/L of xylose and 1.90 g/L of glucose (Fig. 1B).

The hydrolysis of acid pretreated sugarcane bagasse using Multifect® CL and Multifect® XL extracts promoted a lower release of glucose and xylose (Fig. 1D and E). However, Accellerase® 1500 achieved a higher release of xylose after saccharification of the acid pretreated sugarcane bagasse and this was the only situation that the acid pretreatment showed to be more efficient (Fig 1F).

Table 3
Pretreatment yields: comparison between acid and alkaline pretreatment.

	Pretreatments	
	Acid	Alkaline
Initial dry weight (g)	100.0	100.0
Final weight (g)	377.25	451.02
Humidity (%)	81	84
Final dry weight (g)	71.7	72.16

Multifect® CL was the only cocktail resulting in significant levels of cellobiose (4.07 g/L) and xylobiose (3.68 g/L) after saccharification of alkali-pretreated sugarcane bagasse, suggesting that the amount of β-glucosidase and β-xylosidase in this cocktail is not sufficient to convert all oligosaccharides to glucose and xylose (Fig 1A).

3.4. Saccharification of pretreated sugarcane bagasse by C. cubensis enzymatic extract

Concerning utilization of the *C. cubensis* extract, the saccharification assays also resulted in higher release of sugars from the alkaline pretreated sugarcane bagasse than the acid pretreated biomass (Fig. 2). Beyond that, the *C. cubensis* extract was more efficient to promote the release of glucose and xylose from alkaline pretreated bagasse compared to the commercial cocktails. These results suggest that, under these conditions, the *C. cubensis* extract presents an adequate balance between the different enzymatic activities involved in biomass degradation.

Falkoski et al. (2013) compared the action of enzymes from *C. cubensis* and the Multifect® CL on the saccharification of alkali-pretreated sugarcane bagasse with 2% solids loading, and found that the non-commercial mixture was more effective for biomass hydrolysis than the commercial cocktail. Here, this study was expanded to include two other commercial enzyme mixtures and also to use higher solids loading, since the higher solids concentration in the hydrolysis step will positively influence the fermentation and distillation yields. The *C. cubensis* extract maintained a superior hydrolysis capacity compared to the commercial cocktails in both low and high solids loading.

The *C. cubensis* extract presents numerous characteristics that could explain this better performance. The large amount of β-glucosidase prevents accumulation of cellobiose during the hydrolysis step, and therefore there is no end-product inhibition of endoglucanases and cellobiohydrolases, which results in higher saccharification yields (Jiang et al., 2011). Furthermore, the *C. cubensis* extract also contains a high concentration of endoglucanase which may contribute to a better action of the other cellulases due to the rapid formation of their substrates. Another explanation could be related to the fact that the *C. cubensis* extract has more accessory enzymes when compared to the commercial mixtures. Although these enzymes are not able to produce glucose directly, they play an important role in stimulating cellulose hydrolysis by facilitating the access of cellulolytic enzymes to the cellulose fraction, and thus increasing the monosaccharide concentrations for posterior fermentation (Hu et al., 2011; Várnai et al., 2011). Moreover, the action of these enzymes can release pentoses and hexoses which may also be fermented to ethanol or other higher-value products. Finally, the *C. cubensis* extract has a higher laccase activity when compared to the commercial mixtures. Laccases assist in removing the residual lignin of the lignocellulosic biomass as well as in oxidation of phenolic compounds which inhibit the cellulase enzymes, facilitating the access of cellulases to the

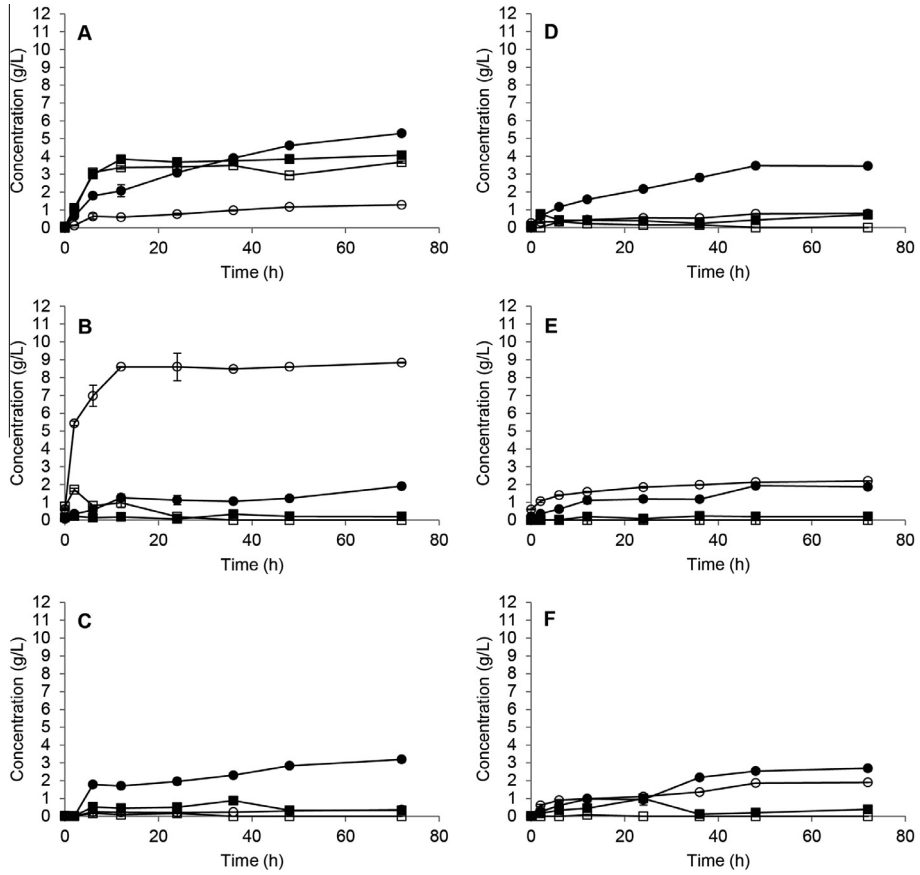

Fig. 1. Saccharification of sugarcane bagasse after 72 hours at 50 °C using commercial enzyme mixtures. Alkali-pretreated sugarcane bagasse hydrolysis using: (A) Multifect® CL, (B) Multifect® XL and (C) Accellerase® 1500. Acid-pretreated sugarcane bagasse hydrolysis using: (D) Multifect® CL, (E) Multifect® XL and (F) Accellerase® 1500. Sugars released: (■) cellobiose, (□) xylobiose, (●) glucose and (○) xylose.

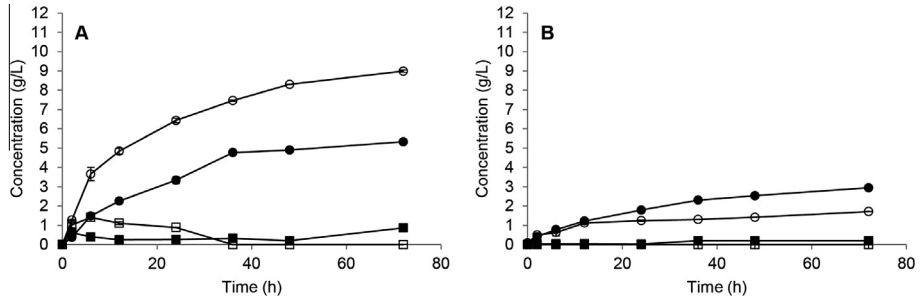

Fig. 2. Saccharification of sugarcane bagasse after 72 hours at 50 °C using the *C. cubensis* extract. (A) Alkali-pretreated sugarcane bagasse hydrolysis. (B) Acid- pretreated sugarcane bagasse hydrolysis. Sugars released: (■) cellobiose, (□) xylobiose, (●) glucose and (○) xylose.

cellulose fraction (Chen et al., 2012; Gutiérrez et al., 2012; Rico et al., 2014; Ximenes et al., 2010).

The results show that when the enzyme loading was adjusted based on FPase activity (10 FPase units/g of biomass) for the alkaline pretreated sugarcane bagasse hydrolysis (Fig. 1A–B and 2A), the *C. cubensis* extract was able to promote similar release of glucose (5.32 g/L) compared to Multifect® CL (5.30 g/L), which is cocktail rich in cellulases, and similar release of xylose (9.00 g/L) compared to and Multifect® XL (8.83 g/L), which is a rich xylanase mixture.

Concerning acid pretreatment (Fig. 2B), the released amount of sugars by the *C. cubensis* enzyme extract was 2.94 g/L of glucose and 1.71 g/L of xylose. The dilute acid pretreatment eliminates or reduces the need for hemicellulases (Saha et al., 2005) and *C. cubensis* extract is very rich in hemicellulases and accessory enzymes. However, *C. cubensis* enzymes presented a better performance for the saccharification of acid pretreated sugarcane bagasse than the commercial cocktails, being only behind Multifect® CL for glucose release and Multifect® XL for xylose release (Fig. 1D–E).

Small levels of cellobiose, 0.87 g/L and 0.20 g/L, remained after the action of the *C. cubensis* extract on alkali and acid-pretreated sugarcane bagasse, respectively, while xylobiose was not detected after the hydrolysis of pretreated bagasse (Fig. 2).

The *C. cubensis* extract was able to convert 12.5% of glucan and 44% of xylan after saccharification of alkali-pretreated sugarcane bagasse. For the hydrolysis of acid pretreated sugarcane bagasse, *C. cubensis* enzymes promoted 7.7% and 25% of glucan and xylan conversions, respectively. The saccharification yields were lower when compared to previous studies (Falkoski et al., 2013; Visser

et al., 2013). This may be explained by three factors: 1- the sugarcane bagasse used in this study was obtained from industry and it contains more lignin than the sugarcane bagasse used in the previous studies. Lignin is one of the major obstacles for high saccharification yields because it can promote non-specific linkages of enzymes that harm their action; 2 - the saccharification experiments of this work utilized 8% biomass loading instead of 2%. The utilization of high substrate concentrations generates excessively viscous mixtures that complicate homogenization and the action of enzymes; 3 - the enzymatic cocktails were used alone, i.e., there was no testing of enzyme blends.

Therefore, for this study, the *C. cubensis* enzymatic extract was the best enzyme source for glucose and xylose release, compared to the commercial mixtures, when sugarcane bagasse was pretreated with sodium hydroxide. This enzyme mixture could possibly be a good supplementation for the commonly used *Trichoderma reesei* cellulase mixtures. It is important to emphasize that the *C. cubensis* extract presents lower costs since it is obtained from a simple carbon source and no technical improvement was performed to ameliorate the protein secretions. In fact, the objective of this work was not to establish the best conditions for sugarcane bagasse saccharification, but instead to test and compare two pretreatment methods for different enzymatic cocktails. Indeed, the best enzymatic mixture is the one which presents the most appropriate set of enzymes for the pretreatment applied to the biomass to generate higher sugar yields with higher solids loading.

4. Conclusions

The different pretreatment methods can be only compared for a specific biomass and a specific enzymatic cocktail and the several available enzymatic mixtures can be compared with respect to efficiency and solids loading used in the saccharification step. For the sugarcane bagasse studied in this work, the alkaline pretreatment promoted the best saccharification yields (glucose and xylose release) when *C. cubensis* was used as the enzyme source. Furthermore, *C. cubensis* was able to produce high specific enzyme activities when compared to the commercial cocktails. The extract from *C. cubensis* showed great potential for application in biomass hydrolysis processes. For further studies, the comprehension of the *C. cubensis* proteome arising through its genome sequencing would permit a better explanation for the excellence of its enzymatic extract.

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