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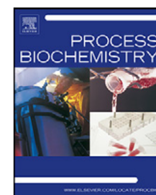

Short communication

Enzymatic hydrolysis of pretreated sugar cane bagasse using *Penicillium funiculosum* and *Trichoderma harzianum* cellulases

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ABSTRACT

In this study, we investigated the enzymatic hydrolysis of pretreated sugarcane bagasse using eight different enzymatic blends obtained from concentrated crude enzyme extracts produced by *Penicillium funiculosum* and *Trichoderma harzianum* as well as from the extracts in combination with a commercial enzymatic cocktail. The influence of different levels of biomass delignification, degree of crystallinity of lignicellulose, composition of enzymatic activities and BSA on enzymatic hydrolysis yields (HYs) was evaluated. Our X-ray diffraction studies showed that crystallinity of lignocellulose is not a key determinant of its recalcitrance toward enzymatic hydrolysis. In fact, under the experimental conditions of our study, an increase in crystallinity of lignocellulosic samples resulted in increased glucose release by enzymatic hydrolysis. Furthermore, under the same conditions, the addition of BSA had no significant effect on enzymatic hydrolysis. The most efficient enzyme blends were obtained by mixing a commercial enzymatic cocktail with *P. funiculosum* or *T. harzianum* cellulase preparations (HYs above 97%) followed by the concentrated extract of *P. funiculosum* alone (HY = 88.5%). Increased hydrolytic efficiencies appeared to correlate with having an adequate level of both β-glucosidase and xylanase activities in the blends.

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1. Introduction

The conversion of lignocellulosic biomass to glucose requires the use of cellulolytic enzymes. It is well established that hydrolytic efficiency is a result of the concerted and synergistic actions of a multicomponent enzymatic system consisting of at least three major groups of enzymes: endo-β-glucanases, exo-β-glucanases and β-glucosidases [1–7]. Various enzymatic compositions produced and secreted by filamentous fungi and other microorganisms have very different effects on biomass depolymerization. Cellulases produced by *Trichoderma reesei* and *Aspergillus niger* are the most common enzymes used in the hydrolysis of lignocellulosic biomasses. The former microorganism produces large quantities of exo- and endoglucosidases but little β-glucosidase activity, whereas the latter secretes massive quantities of β-glucosidases, though its cellulase activities are somewhat limited. To date, no natural microorganism that produces an ideal enzyme preparation for biomass hydrolysis has been discovered. Therefore, inadequate enzymatic activities must be supplemented with native or recombinant enzymes for use in particular biotechnological applications.

Additives, such as surfactants, have been used to enhance cellulose hydrolysis yields by reducing non-productive enzyme adsorption on the lignin in the substrate [8]. Proteins such as bovine serum albumin (BSA) can be competitively and irreversibly adsorbed by lignin, thus shielding the lignin and reducing enzyme adsorption. This impedes the non-productive binding of cellulases, therefore increasing the efficiency of enzymatic hydrolysis [9].

Here, we investigated the hydrolysis of pretreated bagasse using cellulases from *Penicillium funiculosum* and *Trichoderma harzianum* with the aim of obtaining blends that are superior to commercial enzymatic preparations. In addition, we analyzed the effects of cellulignin crystallinity, the concentration of alkali used in the

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bagasse pretreatment and addition of BSA on the yields of enzymatic hydrolysis of sugar cane bagasse.

2. Materials and methods

2.1. Bagasse pretreatment

Sugarcane bagasse was kindly provided by Dedini S.A. (Piracicaba, SP, Brazil). The sugarcane bagasse was pretreated with 1% H_2SO_4 solution at 121 °C for 45 min to partially remove the hemicellulosic and lignin fractions, thus allowing the enzyme access to the cellulose. After pretreatment, the cellulose-rich solid phase was separated in a hydraulic press filter by applying 2 tons of pressure over an area of 201 cm^2. The solid fraction was submitted to an additional step of partial delignification by alkaline treatment with various concentrations of NaOH (0.1, 0.5, 1.0 and 4.0% m/v) with a 1:20 solid:liquid ratio at 121 °C for 30 min as described in [10]. After thermal pressurization, the solid fraction (partially delignified cellulignin) was separated using a stainless steel sieve (0.5 mm) and exhaustively washed; the pH was then adjusted to 5.0 using 2 M HCl.

2.2. Sugar cane bagasse and partially delignified cellulignin composition analysis

The bagasse and partially delignified cellulignin were milled and passed through a 0.5 mm sieve. The bagasse and cellulignin compositions were determined by chemical hydrolysis with H_2SO_4, which was performed in two steps according to the procedures established by the National Renewable Energy Laboratory (NREL) [11,12].

2.3. Crystallinity analysis of pretreated bagasse

Material crystallinity, defined as the weight ratio between cellulose crystals and dry matter, was determined using the X-ray diffraction method recently developed by Driemeier and Calligaris [13]. X-ray diffraction was performed in transmission fiber geometry on samples inserted inside 2-mm glass capillaries using a Rigaku UltraX-18HF rotating anode generator operated with Cu Kα radiation (λ = 1.54 Å) and a Mar345 image plate detector at a 120 mm sample-to-detector distance. Each area detector diffraction pattern was corrected for X-ray absorption and dark counts. Instrumental line broadening and detector position and tilts were calibrated with α-alumina. The absorption-corrected area detector patterns were modeled by the Rietveld method [14] using the MAUD program [15], cellulose I_β structure [16] and a harmonic description of the preferred orientation of the crystals. Crystallinity, x_{cr}, was determined using the following expression:

$$x_{cr} = \frac{Q[I'_{cr}]}{Q[I_{\text{exp}}] - (T_{\text{exp}}/T_0)Q[I_0]} \frac{1 + 1.2x_m}{1 - \varepsilon_{\text{inc}}}$$

where $Q[I]$ is a function that integrates $I(s)\ s^2\ ds$ in the standard interval 0.11–0.50 $Å^{-1}$, with $s = 2\sin\theta/\lambda$. I'_{cr} and I_{exp} are Rietveld-reconstructed isotropic intensities from diffraction and total scattering, respectively. I_0 is the scattered intensity from the empty capillary. T_{exp} and T_0 are the transmitted X-ray intensities (at $2\theta = 0$) of the sample and empty capillary, respectively. The corrective term x_m is the dry-basis moisture content determined gravimetrically after heating at 105 °C to a constant weight while ε_{inc} is the incoherent scattering factor, as calculated in [13] ($\varepsilon_{\text{inc}} = 0.102$). The reported uncertainties in x_{cr} consist of two major components: (i) variability (std. dev. ≈10%) in $Q[I_0]/T_0$ measurements and (ii) uncertainty in x_m (≈1%). Hence, the reported uncertainties do not include possible a systematic bias introduced by the crystallinity method, which was estimated to be below ± 5% [13].

2.4. Cellulase production by P. funiculosum and T. harzianum and concentration of crude enzyme extracts

The cellulases were produced by the filamentous fungi *P. funiculosum* ATCC 11797 and *T. harzianum* IOC 3844. *T. harzianum* was cultivated in Mandels' medium [17], and *P. funiculosum* was cultivated in a previously described medium [18]. Microcrystalline cellulose (Avicel®) and partially delignified cellulignin were used as carbon sources for *T. harzianum* and *P. funiculosum*, respectively. The cellulases were produced in 500 mL Erlenmeyer flasks in 200 mL of media. The flasks were maintained at a constant agitation of 200 rpm for 72 h at 30 °C for *P. funiculosum* and at 27 °C for *T. harzianum*. The solutions were then centrifuged for 30 min at 5000 × *g*, and the supernatants (crude enzymatic extracts) were concentrated in a vacuum rotary evaporator at 45 °C under 760 mmHg negative pressure. The enzymatic extracts were concentrated 30 and 25 fold for *T. harzianum* and *P. funiculosum* supernatants, respectively, prior to the hydrolysis assay of pretreated bagasse.

2.5. Protein and enzymatic assays

The protein concentration of the cellulase preparations was measured using the Bradford Protein Assay (Bio-Rad) using bovine serum albumin as a standard (5–30 μg/mL) [19]. The activities of FPase, Avicelase, CMCase, β-glucosidase and xylanase were measured using filter paper, Avicel®, carboxymethylcellulose, cellobiose and xylan as substrates, respectively [20]. The carboxymethyl cellulose sodium salt (medium viscosity), cellobiose and xylan were purchased from Sigma–Aldrich. Grade 1 Whatman filter paper and Avicel® were purchased from Whatman and Fluka, respectively. Test tubes containing substrate and crude enzyme extract were incubated under continuous agitation at 50 °C for 2 h and1 h for measurements of avicelase and filter paper activities, respectively. A 15 min incubation time was used for CMCase, β-glucosidase and xylanase activities. All enzymatic activities were determined under the same conditions (pH and temperature) used for cellulignin hydrolysis.

Cellulase, β-glucosidase and xylanase activities were expressed in international activity units (U), which is defined as the amount of enzymatic extract that releases 1 μmol of sugars (glucose equivalent) per minute.

2.6. Enzymatic hydrolysis of cellulignin and the effect of BSA on hydrolysis yields

Cellulignin (25 g/L) that had been partially delignified with 4% NaOH was used in the enzymatic hydrolysis assays. Hydrolysis was carried out in 100 mL Erlenmeyer flasks containing 50 mL of a cellulignin suspension in 50 mM citrate buffer (pH 5.0) under constant agitation.

The following commercial enzymatic blends and their combinations with the indicated enzymatic concentrations of *P. funiculosum* and *T. harzianum* were studied: Multifect CX 10 L (Genencor), based on enzyme load (FPU) per gram of cellulignin; M25 (100% commercial Multifect at 25 FPU/g); M12.5 (100% Commercial Multifect at 12.5 FPU/g); P (100% *P. funiculosum* supernatant at 25 FPU/g); T (100% *T. harzianum* at 25 FPU/g); MP (12.5 FPU/g of Multifect + 12.5 FPU/g of concentrated *P. funiculosum* extract); MT (12.5 FPU/g of Multifect + 12.5 FPU/g of concentrated *T. harzianum* extract); PT (12.5 FPU/g of *P. funiculosum* + 12.5 FPU/g of *T. harzianum* extracts); MTP (8.3 FPU/g of Multifect + 8.3 FPU/g of *P. funiculosum* + 8.3 FPU/g of *T. harzianum* extracts).

The enzymatic blends were added to the cellulignin suspension at 25 FPU/g of FPase activity except for the M12.5 blend, which consisted of pure commercial Multifect®, which was added at an activity of 12.5 FPU/g. The flasks containing bagasse and enzyme were maintained under constant agitation at 200 rpm at 50 °C for 18 h. During this procedure, samples were periodically taken for a kinetic profile. The samples were centrifuged at 10,000 × *g* for 10 min, and the sugars released in the supernatant were quantified using HPLC.

It is generally accepted that cellulases adsorb irreversibly to the lignin in lignocellulosic materials, thus decreasing the hydrolysis efficiency [21,22]. This effect can be partially prevented by the addition of BSA [9]. To study the influence of BSA on the hydrolysis yields, a 1.5 g/L solution of BSA was added to the cellulignins with different degrees of delignification 2 h prior to the addition of the enzyme mixtures (enzymatic load of 18 FPU/g). In parallel, control hydrolysis experiments were performed in the absence of BSA.

The hydrolysis yield was determined using the following equation:

$$HY = \frac{released\ glucose\ (g/L)}{25 \times 0{,}68 \times 1.11} \times 100$$

where HY is the hydrolysis yield; 25 is the cellulignin concentration (g/L); 0.68 is the cellulose content in the cellulignin (g/g), and 1.11 is a correction factor that accounts for the addition of water molecules to the anhydroglucose residues in cellulose.

The experiments were repeated three times. The results were evaluated using Analysis of Variance (ANOVA) at a 5% significance level; Tukey's test was applied to the averages.

2.7. Quantification of sugars using High performance liquid chromatography (HPLC)

Released sugars were quantified using a Waters 2414 High Performance Liquid Chromatographic system on an Aminex HPX87P column (Bio-Rad) at 60 °C with a differential refractive index detector (Waters). The mobile phase was Milli-Q water at a flow rate of 0.6 mL/min. Glucose and cellobiose were used as standards.

3. Results and discussion

3.1. Sugar cane bagasse and cellulignin composition

Lignin represents one of the main obstacles to using lignocellulosic materials in biotechnological applications based on biomass cellulose [23]. The purpose of pretreatment is to remove part of the lignin and hemicellulose, reduce cellulose crystallinity and enhance porosity [6,24]. Table 1 presents the experimentally determined composition of sugar cane bagasse and partially delignified cellulignin obtained in our study. The sum of all components (95.5 ± 4.3% and 97.0 ± 3.4% for sugar cane bagasse and cellulignin, respectively) was close to 100%, taking into account experimental error and extractives that were partially unaccounted for.

Table 1
Composition of sugar cane bagasse and partially delignified cellulignin after acid pre-treatment followed by pre-treatment with 4.0% of NaOH.

Components	Sugar cane bagasse (% w/w)	Partially delignified cellulignin (% w/w)
Cellulose	34.1 ± 1.2	68.0 ± 1.3
Hemicellulose	29.6 ± 1.4	12.2 ± 0.9
Lignin	19.4 ± 0.4	9.3 ± 0.6
Ash	7.9 ± 1.1	3.5 ± 0.4
Moisture	4.4 ± 0.1	4.0 ± 0.2
Total	95.5 ± 4.3	97.0 ± 3.4

The results show that the pretreatments (acid and alkaline) were efficient in reducing the hemicellulose (29.6–12.2%) and lignin (19.4–9.3%) fractions, with a concomitant increase in the cellulose content of cellulignin from 34.1% to 68.0%.

3.2. Enzymatic hydrolysis of bagasse pretreated with different degrees of delignification and the effect of BSA on hydrolysis yields

In this study, we attempted to identify the minimum concentration of sodium hydroxide that could be used in the pretreatment procedure while maintaining hydrolysis efficiency. The influence of the concentration of sodium hydroxide (NaOH) on cellulignin hydrolysis is shown in Fig. 1. The rates of hydrolysis of cellulignin pretreated with 1% or 4% NaOH were comparable whereas pretreatment of samples with 0.5% sodium hydroxide slightly reduced the efficiency of hydrolysis. The enzymatic hydrolysis of cellulignin upon treatment with 0.1% NaOH was not efficient (Fig. 1).

The decrease in the hydrolysis efficiency of cellulignin pretreated with the lowest NaOH concentration is likely due to the physical barrier generated by residual lignin, which impeded the enzyme from accessing the cellulose microfibrils.

In a separate set of experiments, BSA was added to the cellulignin prior to adding the enzymatic blend. Surprisingly, our results indicate no statistically significant difference in hydrolytic efficiency of the cellulase blend in BSA-pretreated and BSA-untreated cellulignin (Fig. 1). This contradicts results reported by Yang and Wyman [9] who observed a significant increase in the efficiency of the enzymatic hydrolysis of dilute acid-pretreated corn stover upon addition of BSA. The same enhancement, however, was not observed for Avicel®, which does not have exposed lignin surface areas that would allow BSA adsorption [9]. We argue that the absence of any influence of BSA on the yields of enzymatic hydrolyses may indicate that NaOH pretreatment was efficient in at least partially removing the lignin wrapping, thus preventing non-productive adsorption of the enzymes.

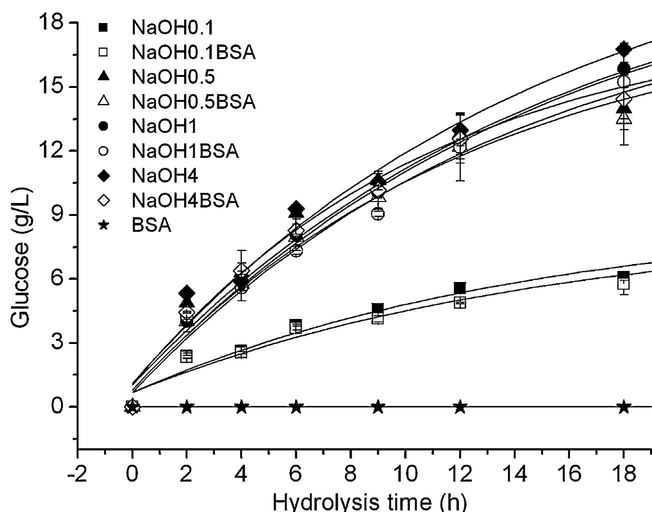

Fig. 1. Effects of varying degrees of delignification (different NaOH concentrations in the pretreatment procedure) and the presence of BSA on the enzymatic hydrolysis of cellulignin. Solids concentration: 25 g/L; enzymatic load of the mixture of Multifect®, *T. harzianum* and *P. funiculosum* extracts (MTP) was set to 18 FPU/g in equal proportions.

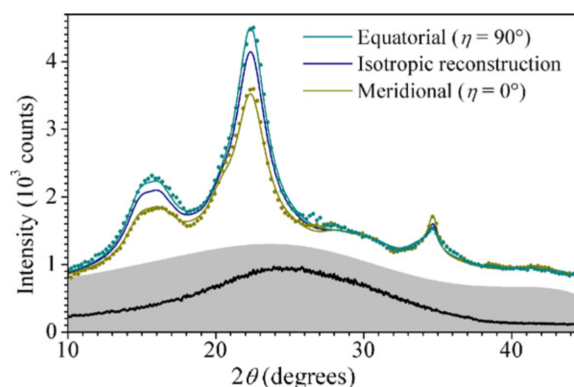

Fig. 2. Experimental (dots) and modeled (thin lines) diffractograms from bagasse pretreated with 4% NaOH. The isotropic reconstructed diffractogram (blue line), model-resolved diffuse background (grey area) and non-sample contribution (thick black line) are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Crystallinity of lignocellulose samples

To gain further insights into the physical parameters of cellulignin samples, we investigated their crystallinity using an X-ray diffraction method based on Rietveld-reconstructed isotropic intensities [13]. Fig. 2 shows meridional ($\eta = 0°$) and equatorial ($\eta = 90°$) diffractograms from the material pretreated with 4% NaOH. The two diffractograms represent the variability observed within the 63 slices taken from each area detector diffraction image ($-155° \leq \eta \leq 155$, with slices of 5°; Section 2). The differences between the two diffractograms arise from differences in the preferred orientation of the cellulose crystals. The anisotropy differences, as well as other major features of the diffractograms, are adequately reproduced by the Rietveld model. Isotropic reconstructions were generated by eliminating the crystals' preferred orientation in the model and used to calculate the degree of crystallinity. Fig. 2 also shows a model-resolved diffuse scattering background as well as the non-sample contribution (predominantly from the capillary).

The degree of crystallinity of the materials pretreated with NaOH (0.1%, 0.5%, 1% and 4%) is reported in Table 2. An increase in cellulignin crystallinity was observed with increased NaOH concentration during the alkali pretreatment steps. Because the degree of crystallinity is a weight ratio, the crystallinity increase can be attributed to the preferential removal of amorphous matter such as lignin, and to a lesser extent, hemicellulose and non-crystalline cellulose.

Quantification of the crystalline content in cellulose is important and provides an estimate of the recalcitrance of the biomass to enzymatic attack [25]. Enzymatic cellulose hydrolysis is typically 3–30 times faster for amorphous cellulose than for highly crystalline cellulose [6]. Therefore, it could be concluded that the

Table 2
Degrees of crystallinity of bagasse samples after acid pre-treatment followed by treatment with variable NaOH concentrations (calculated on a dry-weight basis).

	Pre-treated bagasse (% NaOH)			
	0.1%	0.5%	1%	4%
Degree of crystallinity (%)	62.9 ± 2.2	66.6 ± 3.0	70.5 ± 2.9	68.6 ± 3.4

Table 3
Cellulase activities of the blends used in the hydrolysis assays of the cellulignin. The cellulase blends from *P. funiculosum* and *T. harzianum* were concentrated as described in Section 2.

Enzymatic blend	Activities (U/mL)					Protein (mg/mL)
	FPase	Avicelase	CMCase	β-Glucosidase	Xylanase	
Penicillium funiculosum	5.8	7.2	125.0	26.6	90.0	2.6
Trichoderma harzianum	8.4	4.2	121.4	31.4	320.0	6.4
Multifect®	174.2	99.2	6500.0	195.3	2107.0	56.8

amorphous part of the cellulose would be depolymerized first, which increases the crystallinity of the cellulose during enzymatic hydrolysis [26,27]. However, several research groups have found that crystallinity does not increase during enzymatic hydrolysis [28–31]. The uncertainty of methodologies for measuring crystallinity contributed to the differences observed in the results and conclusions [6]. Here, we applied a novel procedure to evaluate crystallinity, which was based on the Rietveld models (Section 2; [13]).

The results support the conclusion that the crystallinity of the samples increased with increased delignification following NaOH pretreatment, at least up to 1% of NaOH. There was a small decrease in crystallinity when the concentration of NaOH used in pretreatment reached 4% (from 70.5 ± 2.9% to 68.6 ± 3.4%), but uncertainties in the measurements did not permit us to conclude whether it was an artifact or an actual trend. Furthermore, the size of the cellulose crystals estimated from the diffraction data consistently increased with an increase in crystallinity (data not shown). The results suggest that delignification of the bagasse led to a steady increase in the proportion and size of the cellulose crystals within the samples. These trends were observed under the pretreatment conditions and were likely related to the removal of lignin/hemicellulose. The process appears to be accompanied by the condensation of cellulose microcrystals within the increasingly delignified cellulignin. Moreover, the results reveal that the increase in crystallinity under these conditions does not impede the enzymatic hydrolysis of the bagasse samples. On the contrary, the increase in crystallinity positively correlated with an increase in glucose release from the samples (Fig. 1).

Notably, the cellulignin pretreatment process also increased the accessibility of the cellulose microfibrils to the enzymes [6,23]. The enhanced accessibility of cellulose to enzymatic hydrolysis resulted in consistently increased glucose yields under a constant enzyme load. However, experimental studies of cellulose accessible areas in the samples would be required for a detailed characterization of this phenomenon.

3.4. Hydrolysis of cellulignin using cellulases from P. funiculosum, T. harzianum and a commercial enzymatic preparation

To evaluate the effect of *P. funiculosum* and *T. harzianum* cellulasic blends on the Multifect® commercial preparation, systematic hydrolysis procedures were carried out with each blend and with different combinations. Enzymatic activities of the tested blends and their combinations were all normalized to 25 FPU/g. For comparison, hydrolysis using the commercial enzyme at half the enzymatic load (12.5 FPU/g) was also performed. Table 3 shows the enzymatic activity and protein content of the enzyme preparations from each source.

Enzymatic hydrolysis kinetics of *P. funiculosum* and *T. harzianum* cellulasic enzymatic blends and the Multifect® commercial enzymatic blend are shown in Fig. 3a. The Multifect® commercial blend released a maximum of 12.89 g/L of sugars after 18 h of hydrolysis when added at an enzymatic load of 25 FPU/g. When the enzymatic load was 12.5 FPU/g, 9.08 g/L of sugars were released. The performances of the two blends were 68.3% and 48.1% of the theoretical hydrolysis yield, respectively. As confirmed by Tukey's test, the values increased when half of the enzymatic load was substituted by the enzymatic blend obtained from *P. funiculosum* (MP) and/or *T. harzianum* (MT), raising the released sugars to 18.9 g/L (MP) and 18.42 g/L (MT). These concentrations represent yields of 100.2% and 97.6% of the maximum theoretical value, respectively. The enzymatic blend of *P. funiculosum* (P) alone and the mixture of the three enzymatic preparations (MTP) released 16.7 g/L and 16.6 g/L of glucose, respectively, at the end of 18 h and corresponded to a hydrolysis yield of 88.5% and 88.0%. The enzymatic blend of *T. harzianum* (T) showed no statistical difference compared to the Multifect® commercial blend, releasing 12.24 g/L of glucose after

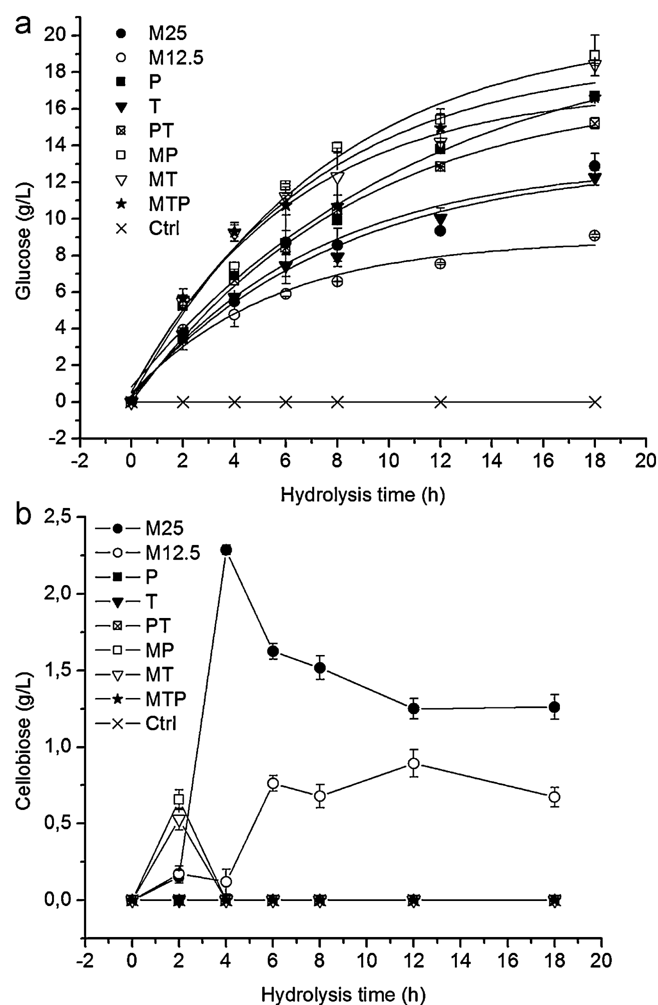

Fig. 3. (a) Glucose and (b) cellobiose released during 18 h of hydrolysis of partially delignified cellulignin, containing 68% of cellulose, at concentration of 25 g/L. M25 (Multifect® at 25 FPU/g); M12.5 (Multifect® at 12.5 FPU/g); P (*Penicillium funiculosum* enzymes at 25 FPU/g); T (*T. harzianum* enzymes at 25 FPU/g); MP (Multifect® at 12.5 FPU/g + *P. funiculosum* enzymes at 12.5 FPU/g); MT (Multifect® at 12.5 FPU/g + *T. harzianum* enzyme at 12.5 FPU/g); PT (*P. funiculosum* enzymes at 12.5 FPU/g + *T. harzianum* enzymes at 12.5 FPU/g); MTP (Multifect® at 8.3 FPU/g + *P. funiculosum* enzyme at 8.3 FPU/g + *T. harzianum* enzymes at 8.3 FPU/g).

the same time of hydrolysis (hydrolysis yield of 64.7%). However, when the 50% *P. funiculosum* and 50% *T. harzianum* blends (PT) were used simultaneously, there was an increase in the released sugars up to 15.21 g/L, corresponding to a hydrolysis yield of 80.6%. The negative control experiments (the addition of buffer instead of enzymatic blend) showed no sugar release.

In general, the hydrolysis yields achieved in this study were significantly higher than the yields reported in the literature [31–35].

To gain a better understanding of the data from the hydrolysis of bagasse cellulignin, we determined the enzymatic activities of *T. harzianum* and *P. funiculosum* extracts and the Multifect preparation. Table 3 indicates that *P. funiculosum* is an efficient producer of cellulases and β-glucosidases, providing a fair balance between the cellulase activities in the enzyme preparation. This finding was consistent with previous studies [33,36–46].

The FPase and β-glucosidase activity ratio in the crude extract of *P. funiculosum* and *T. harzianum* were close to 1:4.5 and 1:3.7, respectively, whereas the Multifect® ratio was 1:1. The high ratio of β-glucosidase activity to FPase is important in simultaneous saccharification and fermentation (SSF) processes to avoid accumulation of cellobiohydrolases inhibitors (cellobiose) in the reaction media. The positive effect of the addition of enzyme blends from *P. funiculosum* and *T. harzianum* over the cellobiose concentration can be observed in Fig. 3b. Cellobiose accumulation in the hydrolyzate was only detected when the commercial enzyme preparation (Multifect®) was used, both at 12.5 FPU/g and at 25 FPU/g, resulting in cellobiose concentrations of 0.89 g/L and 2.28 g/L, respectively. However, in the media hydrolyzed by the mixture of commercial enzyme and *P. funiculosum* (MP) or *T. harzianum* (MT), there was an initial accumulation of cellobiose followed by conversion into glucose after 2 h of reaction, presumably as a result of the action of the β-glucosidases of *P. funiculosum* and *T. harzianum*. The hydrolysis performed with enzymes from *P. funiculosum* and *T. harzianum* alone did not result in an accumulation of cellobiose. Moreover, the *T. harzianum* extract had an approximately three-fold higher concentration of xylanase: FPase activities ratio compared to Multifect© (Table 3), which might further contribute to its efficiency. Xylooligomers have recently been identified as strong inhibitors of the enzymatic hydrolysis of cellulose, capable of significantly inhibiting biomass saccharification even at concentrations as low as 1.67 mg/mL [47]. Xylose proved to be a much weaker inhibitor, and therefore, it was less of an impediment to the saccharification process. This finding highlights the importance of xylanase activity in the enzymatic hydrolysis of biomass. Furthermore, the presence of hemicellulose hindered the access of enzymes to cellulose and reduced cellulose depolymerization. Higher xylanase activity in the *T. harzianum* extract might explain the higher efficiency of blends containing this preparation compared to *P. funiculosum* and *T. harzianum* extracts, which had lower xylanase activity. Other components of the enzymatic mixtures produced by *T. harzianum* and *P. funiculosum*, such as glycoside hydrolases (GH) from the GH family 61 [48] and swollenins [49], might also contribute to the observed enhanced enzymatic hydrolysis yields.

4. Conclusions

Based on the results of the present study, the highest sugar yields from enzymatic hydrolysis of pretreated sugar cane bagasse are obtained with an enzymatic blend prepared from the commercial enzyme preparation (Multifect®) enhanced with the same load (in terms of FPase activity) of either *P. funiculosum* or *T. harzianum* enzyme extracts. Additionally, the *P. funiculosum* enzymatic blend alone shows better hydrolytic performance on sugar cane bagasse cellulignin compared to the commercial Multifect® preparation. Adequate levels of β-glucosidase and xylanase activities appear to be important for enhancing the efficiency of cellulignin hydrolysis by the enzymatic blends. Additionally, the high crystallinity of the bagasse lignocellulose (∼70%) has no detrimental effect on the yield of hydrolysis. The experiments evaluating sugar cane bagasse alkaline pretreatment indicate that the amount of sodium hydroxide used for delignification can be reduced several fold (down to 1% or even 0.5%) without significant loss in the enzymatic hydrolysis yields, which might be of interest for reducing costs. The studies also show that the crystallinity of the bagasse increases during delignification. Finally, under the conditions used in this study, the addition of BSA has no significant effect on the enzymatic hydrolysis of sugar cane bagasse cellulignin.

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