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ORIGINAL PAPER

Characterization of commercial cellulases and their use in the saccharification of a sugarcane bagasse sample pretreated with dilute sulfuric acid

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Abstract This study aimed to correlate the efficiency of enzymatic hydrolysis of the cellulose contained in a sugarcane bagasse sample pretreated with dilute H_2SO_4 with the levels of independent variables such as initial content of solids and loadings of enzymes and surfactant (Tween 20), for two cellulolytic commercial preparations. The preparations, designated cellulase I and cellulase II, were characterized regarding the activities of total cellulases, endoglucanase, cellobiohydrolase, cellobiase, β-glucosidase, xylanase, and phenoloxidases (laccase, manganese and lignin peroxidases), as well as protein contents. Both extracts showed complete cellulolytic complexes and considerable activities of xylanases, without activities of phenoloxidases. For the enzymatic hydrolyses, two 2^3 central composite full factorial designs were employed to evaluate the effects caused by the initial content of solids (1.19–4.81%, w/w) and loadings of enzymes (1.9–38.1 FPU/g bagasse) and Tween 20 (0.0–0.1 g/g bagasse) on the cellulose digestibility. Within 24 h of enzymatic hydrolysis, all three independent variables influenced the conversion of cellulose by cellulase I. Using cellulase II, only enzyme and surfactant loadings showed significant effects on cellulose conversion. An additional experiment demonstrated the possibility of increasing the initial content of solids to values much higher than 4.81% (w/w) without compromising the efficiency of cellulose conversion, consequently improving the glucose concentration in the hydrolysate.

Keywords Enzymatic hydrolysis · Statistical design · Surfactant · Fermentable sugars

Introduction

Sugarcane bagasse is generated after processing and extraction of sugar, mainly sucrose, from sugarcane plant. Although most of the sugarcane bagasse is burned to produce steam power, there is still a surplus of this material whose use as a source of sugars for ethanol production would allow the industry to enhance its ethanol production without increasing the demand for more sugarcane [9].

Different strategies for the conversion of lignocellulosics into sugars have been demonstrated on laboratory and pilot scales. The general concept involves a pretreatment followed by enzymatic hydrolysis [19]. There are several pretreatment technologies using different strategies to enhance the enzymatic digestibility of lignocellulosic materials. Overall, these technologies change or remove hemicellulose and/or lignin, increasing the superficial area and improving the access of the cellulolytic enzymes to their substrate [30, 45].

Pretreatment of lignocelullosic materials with dilute sulfuric acid is one of the most effective processes for removing and solubilizing hemicelluloses, with lower effects on extraction of lignin [36]. Structural changes and redistribution of lignin are the factors that enhance the saccharification of such pretreated materials [17]. In general, the structure of the complex lignin–hemicellulose–cellulose is completely modified after the dilute sulfuric

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acid hydrolysis, allowing good accessibility of hydrolytic enzymes to cellulose even in the presence of great amounts of lignin [16].

For the hydrolysis of cellulose, three different enzymes are required: (1) exo-1,4-β-D-glucanase (EC 3.2.1.91), which hydrolyzes the cellulose chain from its ends releasing cellobiose, (2) endo-1,4-β-D-glucanase (EC 3.2.1.4), which hydrolyzes the cellulose chain randomly, and (3) 1,4-β-D-glucosidase (EC 3.2.1.21), which promotes the hydrolysis of cellobiose to glucose and may also cleave glucosidic units from oligosaccharides. These three enzymes act synergistically to hydrolyze cellulose, creating accessible sites to each other and relieving problems of inhibition by products [12, 43].

Depending on the conditions employed during the pretreatment, part of the hemicellulose remains in the pretreated material and may affect the enzymatic hydrolysis of cellulose. In such cases, the efficient hydrolysis of the pretreated material requires the use of hemicellulases to increase the accessibility of the cellulases to their substrate [19, 32].

The aim of this study was to employ 2^3 central composite full factorial designs to evaluate the effects caused by the initial content of pretreated solids and loadings of enzyme and Tween 20 on the cellulose digestibility of a sugarcane bagasse sample pretreated with diluted sulfuric acid, for two commercial enzyme preparations. The commercial preparations were also characterized regarding the activities of cellulolytic, xylanolytic, and phenoloxidative enzymes as well as protein contents.

Materials and methods

Sugarcane bagasse sample pretreated with diluted H_2SO_4

A sugarcane bagasse sample acquired from a local mill was pretreated with dilute H_2SO_4 in 200-ml stainless steel containers, in a thermostatic oil bath. The pretreatment conditions, previously defined in our laboratories, were as follows: solids content of 17.5% (w/w), H_2SO_4 loading of 1.7% (w/w), temperature of 150°C, and residence time of 30 min. The chemical composition of the pretreated bagasse, determined according to the methodology validated by Gouveia et al. [15], was as follows: cellulose (54.8%), hemicellulose (13.3%), lignin (32.7%), and ash (1.1%); this chemical composition was used to determine the subsequent saccharification yields. As determined by using the National Renewable Energy Laboratory's laboratory analytical procedures [37], the chemical composition of this same sample was as follows: glucan (59.2%), xylan (10.2%), arabinan (0.3%), acid insoluble lignin (21.6%), acid soluble lignin (2.2%), acetyl groups (0.8%), and ash (1.3%).

Commercial cellulolytic preparations

The commercial cellulases used in this study were enzyme preparations intended to be used by biomass-processing industries, kindly donated by Genencor and Novozymes. One of them was a cellulolytic preparation produced by a genetically modified strain of *Trichoderma reesei*; the other consisted of a mix (9:1 v/v) of cellulolytic enzymes produced by *Trichoderma reesei* and β-glucosidases produced by *Aspergillus* sp.

Enzyme activities and protein contents in the commercial cellulolytic preparations

Commercial cellulolytic preparations (I and II) were characterized regarding the activities of total cellulases [13], endoglucanase [13], cellobiase [13], cellobiohydrolase [44], β-glucosidase [44], and xylanase [4]. Phenoloxidase activities, namely laccase [6], manganese peroxidase [22], and lignin peroxidase [40], were also evaluated. As with enzymatic activities, the protein contents in the enzymatic preparations were determined following a standard procedure [13]. All the activity assays for the hydrolytic enzymes were carried out in sodium citrate buffer (50 mM, pH 4.8), because this buffer was used in the enzymatic hydrolysis of the pretreated bagasse.

Enzymatic hydrolysis of the pretreated bagasse

The pretreated bagasse used in the enzymatic hydrolysis assays was exhaustively washed with distilled water and dried at room temperature. The assays were performed in 125-ml Erlenmeyer flasks. The volume of sodium citrate buffer (100 mM, pH 4.8) supplemented with sodium azide (0.02% w/v) to prevent contamination was 12.5 ml. The volumes of the enzyme preparations and of surfactant as well as the mass of pretreated bagasse added to the flasks in the assays varied according to the experimental design shown in Table 1. The final volume was always completed with distilled water to 25 ml (1 g = 1 ml). The saccharification was conducted at 45°C in an orbital shaker (Sppencer SP3060-25), under agitation of 70 rpm. Aliquots of 1 ml were taken at 24, 72, and 168 h, boiled for 5 min, and centrifuged at 12,000×*g* (Beckman Microfuge 12 Centrifuge) for 30 min. The supernatants were collected and filtered in Sep-Pak C_{18} cartridges. The concentrations of sugars were then determined by HPLC (Waters) using a BIORAD HPX-87H (300 × 7.8 mm) column, eluted at 0.6 ml/min with 0.01 N sulfuric acid. Sugars were detected at 45°C, using a RID2414 refractometric detector. The

Table 1 Real and coded values of independent variables in the 2^3 central composite full factorial design with 6 central points

Exp.	Solid (% w/w)	Enzyme (FPU/g solid)	Surfactant (g/g solid)	Solid	Enzyme	Surfactant
1	2	10	0.02	−1	−1	−1
2	4	10	0.02	1	−1	−1
3	2	30	0.02	−1	1	−1
4	4	30	0.02	1	1	−1
5	2	10	0.08	−1	−1	1
6	4	10	0.08	1	−1	1
7	2	30	0.08	−1	1	1
8	4	30	0.08	1	1	1
9	3	20	0.05	0	0	0
10	3	20	0.05	0	0	0
11	3	20	0.05	0	0	0
12	1.19	20	0.05	−1.81	0	0
13	4.81	20	0.05	1.81	0	0
14	3	1.91	0.05	0	−1.81	0
15	3	38.09	0.05	0	1.81	0
16	3	20	0.00	0	0	−1.81
17	3	20	0.10	0	0	1.81
18	3	20	0.05	0	0	0
19	3	20	0.05	0	0	0
20	3	20	0.05	0	0	0

enzymatic digestibility of cellulose was calculated according to Eq. 1:

$$D_C = \{[(G_C \times R_V) \times 0.90]/I_M\} \times 100 \quad (1)$$

where G_C is the glucose concentration in the reaction medium, R_V is the reaction volume, and I_M is the initial mass of cellulose added to the reaction.

Experimental design and statistical analysis

In order to correlate the digestibility of pretreated bagasse with the initial content of solids (1.19–4.81%, w/w) and the loadings of enzymes (1.91–38.09 FPU/g of pretreated bagasse) and surfactant (0.0–0.1 g/g of pretreated bagasse), two 2^3 full experimental designs with six central points were employed [7]. The real values of the independent variables evaluated in the experiments are shown in Table 1. For statistical analysis, these values were codified in levels according to Eq. 2.

$$CV = (RV - V0)/(\Delta RV/2) \quad (2)$$

where CV is the codified value of the independent variable; RV is the real value of the independent variable; V0 is the real value of the independent variable on central level, and ΔRV is the difference between maximum real value and minimum real value of the independent variable [7].

Conditions that maximize the digestion of the pretreated sugarcane bagasse were defined by using the methodologies of statistical design of experiments and analysis of response surface plots. Mathematical models were developed through the adjustment of quadratic models (Eq. 3) to the experimental data by the least squares technique, where Y_i represents the dependent variable, b_0, b_i, b_{ii}, and b_{ij} represent the regression coefficients, and x_i and x_j represent the independent variables. The regression coefficients kept in the models were those coefficients which were significant at 5% significance level ($p < 0.05$). The response surfaces described by the models were plotted with the software Statistica 5.0.

$$Y_i = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (3)$$

Effect of increased contents of solids on polysaccharides conversion

The initial contents of solids evaluated in this additional experiment ranged from 4 to 12%. The enzyme loading (cellulase II) was 10 FPU/g pretreated bagasse; the surfactant loading, 0.05 g/g pretreated bagasse. The assay was carried out under the same conditions as those described in Sect. "Enzymatic hydrolysis of the pretreated bagasse", except that the 1-ml samples were taken at 24, 48, and 72 h. The experiments were performed in duplicates and the efficiencies of polysaccharides conversion were compared among them using analysis of variance. The enzymatic digestibility of polysaccharides was calculated according to Eq. 4:

$$D_P = \{[(G_C \times R_V) \times 0.90] + [(X_C \times R_V) \times 0.88]/I_M\} \times 100 \quad (4)$$

where G_C is the glucose concentration in the reaction medium, R_V is the reaction volume, X_C is the xylose concentration in the reaction medium, and I_M is the initial mass of polysaccharides added to the reaction.

Results and discussion

Characterization of the commercial cellulolytic preparations

The commercial preparations, designated cellulase I and cellulase II, were characterized regarding the activities of hydrolytic and phenoloxidative enzymes, and protein contents (Table 2). Both preparations presented complete cellulolytic complexes and expressive activities of xylanases, thus being recommended for the saccharification of xylan-rich pretreated lignocellulosic materials.

Table 2 Characterization of enzyme activities and protein contents of enzyme preparations I and II

	Cellulase I	Cellulase II
Total cellulase (FPU/ml)	54.0 ± 0.6[a]	93.5 ± 9.2[a]
Endoglucanase (CMCU/ml)	2,643.0	925.0
Cellobiohydrolase (IU/ml)	38.4	98.0
Cellobiase (CBU/ml)	93.0	115.8
β-Glucosidase (IU/ml)	395.0	139.0
Xylanase (IU/ml)	1,280.6	1,249.0
Manganese peroxidase (IU/ml)	–	–
Laccase (IU/ml)	–	–
Lignin peroxidase (IU/ml)	–	–
Protein (mg/ml)	105.2 ± 6.6[a]	152.5 ± 10.0[a]

– Not detected

[a] Standard deviation was calculated from triplicates

Differences between cellulases I and II regarding the activities of hydrolytic enzymes were noticed. The total cellulase activity in preparation II was 93.5 ± 9.2 FPU/ml, while 54.0 ± 0.6 FPU/ml was detected in preparation I. Kovacs et al. [23] detected a total cellulase activity of 56.2 FPU/ml in Celluclast 1.5L. Compared with the previously mentioned results, Martins et al. [28] obtained a higher cellulolytic activity (110.2 FPU/ml) in Celluclast 1.5L FG. On the other hand, these same authors detected lower values of endoglucanase, β-glucosidase, and cellobiase activities than we found in preparations I and II. Cellulase I showed 2,643.0 CMCU/ml of endoglucanase, 38.4 IU/ml of cellobiohydrolase, 93.0 CBU/ml of cellobiase, and 395.0 IU/ml of β-glucosidase, while cellulase II exhibited 925.0 CMCU/ml, 98.0 IU/ml, 115.8 CBU/ml, and 139.0 IU/ml, respectively.

In both preparations we found β-glucosidase activities higher than cellobiase activities. However, the difference between β-glucosidase and cellobiase activities detected in cellulase I was much higher than that observed in cellulase II. The difference between β-glucosidase and cellobiase activities can be explained by the existence of three types of β-glucosidases: (i) cellobiases sensu stricto, which cleave exclusively cellobiose and have no action on aryl-β-glucosides; (ii) β-glucosidases possessing broad specificity in relation to the non-saccharide groups of the molecule; and (iii) aryl-β-glucosidases, which cleave only β-glucosides in which the non-saccharide group is an aryl alcohol (e.g., phenol, *p*-nitrophenol, saligenin, and 6-bromo-2-naphthol) [35]. However, for the enzymatic hydrolysis of cellulose, cellobiase activity is the most important since cellobiose is the natural substrate.

Considerable xylanase activities were detected in both preparations, I and II: 1,280.6 and 1,249.0 IU/ml, respectively. Martins et al. [28] detected a similar xylanase activity in Celluclast 1.5L FG (1,232.8 IU/ml). It should be remembered here that the supplementation of cellulolytic complexes with xylanases is an alternative to increasing the enzymatic conversion of cellulose in certain pretreated lignocellulosic materials; xylanases act in the hydrolysis of xylan-rich hemicelluloses remaining in the solid material after the pretreatment, increasing the affordability of cellulolytic enzymes to hydrolyze cellulose [32].

The protein contents observed in the cellulolytic preparations I and II also differed considerably. Cellulase II showed a higher protein content in comparison to cellulase I (152.5 ± 10.0 mg/ml and 105.2 ± 6.6 mg/ml, respectively). Mussato et al. [31], for example, observed a much lower protein content (27.0 mg/ml) in an enzyme preparation widely used for saccharification of lignocellulosic materials.

Phenoloxidase activities (manganese peroxidase, laccase, and lignin peroxidase) could not be detected in any of the preparations. In general, *Trichoderma* sp. is not a potential or large producer of phenoloxidases [34, 38]. However, some researchers reported the ability of some strains to produce oxidative enzymes, especially laccases [3, 14, 18]. There are also papers reporting the production of laccase [25], manganese peroxidase [21], and lignin peroxidase [1] by *Aspergillus* sp. strains. This gender has also been studied in the expression of heterologous genes, especially from basidiomycetes, for the production of phenoloxidases [10, 39].

Saccharification of the sugarcane bagasse sample pretreated with dilute H_2SO_4

The sugarcane bagasse sample pretreated with dilute H_2SO_4 was subjected to enzymatic hydrolysis using the commercial preparations I and II. In order to correlate the initial content of solids and the loadings of enzymes and surfactant with the cellulose conversion at 24, 72, and 168 h of hydrolysis, we performed two complete 2^3 central composite experimental designs. Digestion values of cellulose by cellulases I and II are shown in Table 3.

As can be observed in Table 3, cellulose conversion into glucose varied according to hydrolysis conditions and cellulase preparation used. When cellulase I was employed, the cellulose conversion ranged between 27.5 and 47.7% within 24 h of hydrolysis. Meanwhile, when cellulase II was used, the yield of saccharification at the same time of hydrolysis varied from 15.9 to 48.8%.

Lower values of cellulose saccharification, for both cellulolytic preparations, were obtained under the condition that employed the lowest amount of enzyme (1.91 FPU/g pretreated bagasse). These results demonstrate the importance and influence of enzymes loading on cellulose saccharification from lignocellulose. Kaar and Holtzapple [20]

Table 3 Enzymatic digestibility of cellulose from pretreated sugarcane bagasse at 24, 72, and 168 h of hydrolysis, by cellulolytic preparations I and II

Exp.	Cellulose conversion (%)					
	Cellulase I			Cellulase II		
	24 h	72 h	168 h	24 h	72 h	168 h
1	29.3	45.2	47.3	30.2	39.9	46.1
2	34.5	42.8	58.0	28.8	36.8	44.4
3	30.2	48.7	58.7	37.8	43.9	46.5
4	42.5	49.2	62.0	40.8	46.2	55.1
5	38.9	44.9	55.2	38.1	45.8	55.4
6	36.7	41.1	58.9	36.8	46.6	57.6
7	36.6	54.2	62.4	48.8	53.1	63.6
8	47.7	54.7	61.9	48.1	54.4	62.9
9	43.5	52.7	60.7	38.6	47.0	55.4
10	35.6	47.4	60.6	36.7	45.8	53.8
11	30.0	50.0	50.6	41.1	41.5	56.4
12	33.2	47.9	61.6	41.6	50.7	65.0
13	44.6	51.2	63.0	37.0	48.0	57.1
14	27.5	37.8	47.1	15.9	23.9	29.1
15	44.7	50.5	62.9	40.4	48.0	56.1
16	30.8	38.0	41.5	34.5	41.6	48.8
17	45.0	53.9	62.7	40.1	47.1	57.1
18	41.3	49.2	55.1	36.2	45.3	55.0
19	42.9	50.7	53.8	38.1	46.2	54.6
20	44.1	54.0	61.1	35.4	46.8	53.5

noticed remarkable differences in corn stover saccharification depending on the loading of enzymes (experimental range between 1 and 15 FPU/g). Their report also demonstrated the necessity of exploring the effect of enzyme loading on the digestion of lignocellulosic materials since there is a limit above which further increases in the enzymes loading do not result in higher saccharification rates and yields. For example, Kaar and Holtzapple did not note significant differences in the cellulose saccharification yield when they varied the loading of cellulases from 10 to 15 FPU/g of pretreated corn stover. Similarly, Zheng et al. [48] did not report differences in saccharification of *Leymus triticoides* pretreated with diluted sulfuric acid when they employed 60, 100, or 150 FPU/g material.

In the present study, the conversion of the cellulose contained in the pretreated bagasse did not differ substantially among the different preparations tested, in all the hydrolysis times. The results showed that the majority of the conversion (more than 66.0%) was obtained within 24 h of hydrolysis. For example, in 168 h of hydrolysis with the cellulolytic preparation I, we observed an average of 57.3% of cellulose conversion; an average of 38.0% was obtained within 24 h of hydrolysis. With preparation II, we noticed an average of 53.7% of cellulose conversion after 168 h of hydrolysis; a conversion of 37.3% was observed in the first 24 h of hydrolysis. We decided to statistically analyze the results obtained at 24 h of enzymatic hydrolysis, for both cellulolytic preparations. In this way, we correlated the cellulose digestibility with the levels of the independent variables evaluated in the factorial design.

Table 4 Variance analysis of the model proposed for predicting cellulose conversion by cellulolytic preparation I within 24 h hydrolysis

Source	DF	SS	MS	*F* value	*p*
Model	3	481.30	160.43	9.12	0.0009
A	1	151.84	151.84	8.64	0.0096
B	1	163.0	163.0	9.27	0.0077
C	1	166.45	166.45	9.47	0.0072
Residual	16	281.35	17.58		
Lack of fit	11	124.06	11.27	0.36	0.9274
Pure error	5	157.28	31.45		
Total	19	762.64			

DF degree of freedom, *SS* sum of squares, *MS* mean square, *A* solid, *B* enzyme, *C* surfactant. $R^2 = 0.63$

Table 5 Variance analysis of the model proposed for predicting cellulose conversion by cellulolytic preparation II within 24 h hydrolysis

Source	DF	SS	MS	*F* value	*p*
Model	3	766.84	255.61	28.57	<0.0001
B	1	509.54	509.54	56.96	<0.0001
C	1	134.92	134.92	15.08	0.0013
B^2	1	122.38	122.38	13.68	0.0019
Residual	16	143.13	8.95		
Lack of fit	11	122.16	11.11	2.65	0.1462
Pure error	5	20.97	4.19		
Total	19	909.97			

DF degree of freedom, *SS* sum of squares, *MS* mean square, *B* enzyme, *C* surfactant, B^2 quadratic term. $R^2 = 0.84$

The variance analyses of the models proposed to explain the cellulose conversion within 24 h of hydrolysis by cellulases I and II are shown in Tables 4 and 5, respectively. Using cellulase I, we observed that all three factors evaluated (initial content of solids and loadings of enzymes and surfactant) were significant. However, with cellulase II, the initial content of solids was not a significant factor influencing cellulose saccharification within 24 h of hydrolysis. Cara et al. [8], evaluating the enzymatic hydrolysis of olive tree biomass pretreated by steam explosion, reported that solids loadings varying from 2 to 10% did not influence the cellulose conversion in the first 48 h of hydrolysis. According to these authors, increasing the initial substrate

concentration during the enzymatic hydrolysis would lead to a solution with higher concentration of glucose, enhancing the subsequent ethanol production. Nevertheless, they also recommended being careful when increasing the solids loading during the enzymatic digestion since excessive concentrations may hinder homogenization of the material and lead to a high concentration of glucose in the reaction mixture; then, a possible inhibition of cellulolytic enzymes may occur.

The addition of surfactants during the enzymatic hydrolysis of lignocellulosic materials has been shown to be beneficial in the conversion of cellulose, due to the reduction in the unproductive adsorption of enzymes by lignin [5, 26] and to the increase in the stability of such proteins against denaturation [20]. Eriksson et al. [11], for example, reported an increase of 63% in cellulose conversion from steam-exploded spruce wood by supplementing the reaction mixture with 5 g/l Tween 20. Even reducing the enzyme loading by 50%, they managed to achieve similar results in terms of cellulose conversion by adding 2.5 g/l Tween 20 to the reaction mixture. Kaar and Holtzapple [20] demonstrated that the benefit of Tween is loading dependent, not concentration dependent; the best results being achieved with 0.15 g Tween/g solids. Alkasrawi et al. [2], on the other hand, suggested adding Tween to the reaction mixture up to a final concentration of 2.5 g/l. In our study, the addition of Tween 20 to the reaction mixture improved the saccharification of the bagasse cellulose, with both cellulolytic preparations; the higher the loading of surfactant, the more efficient was the cellulose saccharification. In a recent economic analysis made in the context of ethanol production from organosolv-pretreated softwood, the use of surfactants during the enzymatic hydrolysis of cellulose was selected as a good strategy to reduce processing costs owing to the advantage of recycling the cellulases [41]. Yang and Wyman [46] also showed an increase in the enzymatic hydrolysis of sulfuric acid-pretreated corn stover, from 82.3 to 91.7%, by addition of bovine albumin to the reaction mixture. These authors attributed the increase in the percentage of saccharification to the adsorption of bovine albumin to lignin, which reduced the unproductive adsorption of cellulases.

The proposed models to quantify the effects of the independent variables on the enzymatic digestion of cellulose by preparations I and II within 24 h of hydrolysis were simplified by setting the initial content of solids to its maximum level (4.81% w/w), giving rise to Eqs. 5 and 6, respectively. The proposed model for cellulose conversion with preparation I is significant and shows no lack of fit, but presents low prediction capacity (Table 4). The proposed model for cellulose conversion with preparation II, on the other hand, besides being significant and not presenting lack of fit, shows a more accurate prediction capacity (Table 5).

$$ICc_{24h} = 43.82 + 3.35 \times B + 3.38 \times C \quad (5)$$

$$IICc_{24h} = 39.11 + 5.92 \times B + 3.04 \times C - 2.55 \times B^2 \quad (6)$$

where ICc_{24h} and $IICc_{24h}$ represent cellulose conversion into glucose within 24 h employing commercial cellulolytic preparations I and II, respectively, as a function of the coded values of loadings of enzymes (B) and surfactant (C).

Response surface plots were elaborated from Eqs. 5 (cellulase I) and 6 (cellulase II), being presented, respectively, in Figs. 1 and 2. As can be observed in these figures, when cellulolytic preparation I is employed for the digestion of the pretreated bagasse, it seems to be possible to enhance the cellulose conversion indefinitely by increasing the loadings of both enzymes and surfactant since there is no curvature in the response surface plot (Fig. 1). However, when preparation II is used, a curvature in the area of study is clearly observed for the loading of enzymes (Fig. 2), i.e., from a certain point the increase in amount of enzymes added to the mixture does not cause a corresponding increase in the conversion of cellulose. This latter behavior is similar to those observed by Kaar and Holtzapple [20] and Zheng et al. [48], as discussed previously, but differs from the behavior displayed by commercial preparation I. The graphs also show that the cellulose digestion can be increased, apparently indefinitely, by increasing the concentration of surfactant in the reaction mixture.

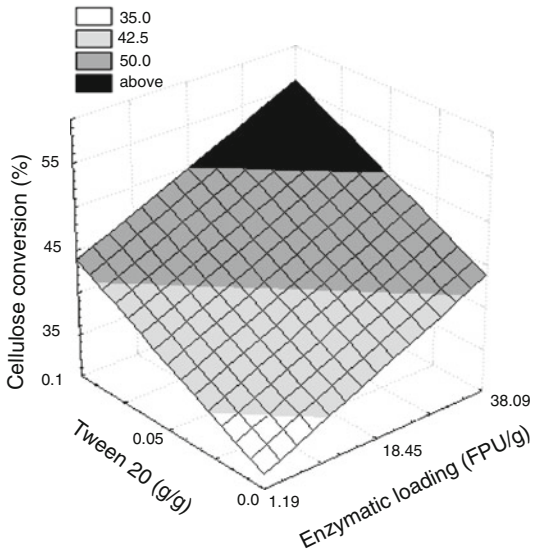

Fig. 1 Response surface showing the effects of the loadings of enzymes and surfactant on the conversion of cellulose within 24 h, by cellulolytic preparation I

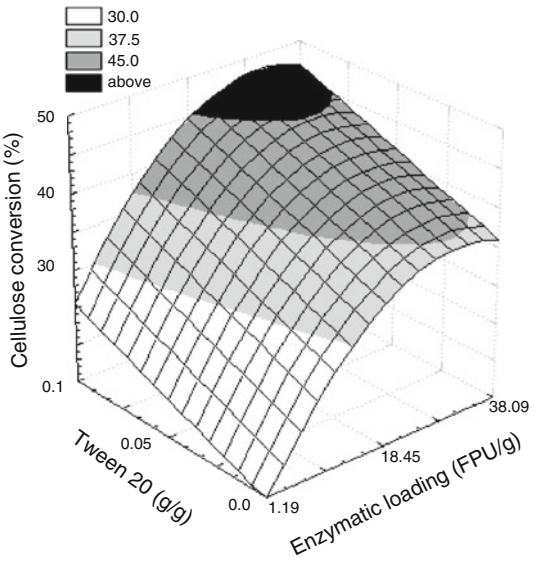

Fig. 2 Response surface showing the effects of the loadings of enzymes and surfactant on the conversion of cellulose within 24 h, by cellulolytic preparation II

Similar to what was observed within 24 h of digestion, considerable variations in the cellulose conversion at 72 and 168 h of hydrolysis were observed when employing cellulolytic preparation II, with values ranging from 23.9 to 54.5% and from 29.1 to 63.6%, respectively. The variations in the cellulose conversions by preparation I were not very different and ranged from 37.8 to 54.7% within 72 h, and from 41.5 to 63.0% within 168 h of hydrolysis. For all the experimental conditions and times tested we did not detect the presence of cellobiose in the reaction mixture, which confirmed that both commercial preparations presented sufficiently high activities of cellobiase. Such a property is interesting since cellobiose is known to strongly inhibit the activity of cellulases [47].

Last but not least, xylose was observed in the reaction mixture during the saccharification of the pretreated bagasse in all the conditions and reaction times, for both commercial preparations (data not shown). The solubilization of xylose is related to the action of xylanases, present in the enzymatic preparations, on the residual hemicellulose contained in the pretreated bagasse. The highest hemicellulose conversions were obtained after 168 h of digestion, with 43.7 and 38.9% for the cellulases I and II, respectively. The xylose generated during the enzymatic digestion of xylan-rich lignocellulosic materials can be an additional source of fermentable sugars to increase the production of ethanol. It is noteworthy that different strains of *Saccharomyces cerevisiae* were already engineered to ferment xylose to ethanol [29]. Concomitantly, the search for yeast strains able to ferment different types of sugars into ethanol has been intense in the recent years. As an example, a strain of *Kluyveromyces* sp., recently isolated from soil samples collected from a landfill of bagasse in a sugar mill, was shown to be able to produce ethanol from carbon sources as diverse as glucose, xylose, mannose, galactose, lactose, sucrose, and cellobiose [24]. Besides ethanol, the xylose sugar could also be used for the production of specialties like xylitol, a sweetener effective in preventing dental caries [27] and in combating acute otitis [42], among others.

Validation of the proposed models

Additional experiments were carried out to validate the models proposed to quantify the enzymatic saccharification of the pretreated bagasse within 24 h of hydrolysis as a function of the independent variables, namely initial content of solids and loadings of enzymes and surfactant. For each one of the two commercial preparations, three experiments were performed in duplicates as shown in Table 6. The lowest cellulose conversions, 9.3 and 7.6% (cellulases I and II, respectively), were obtained when the experiments were carried out using the lowest levels of all the independent variables. Significantly higher cellulose

Table 6 Prediction intervals, experimental conversions, and real levels of the independent variables used to validate the proposed models

	Independent variable			Cellulose conversion (%)[a]	Prediction interval (%)*
	Solid (% w/w)	Enzyme (FPU/g solid)	Surfactant (g/g solid)		
Cellulase I	1.19	1.91	0	9.3 ± 0.6	8.3–31.6
	3	20	0.05	34.2 ± 0.0	28.9–47.1
	4.81	38.09	0.1	44.5 ± 0.2	44.3–67.7
Cellulase II	1.19	1.91	0	7.6 ± 0.2	5.9–23.2
	3	20	0.05	33.1 ± 0.2	32.5–45.7
	4.81	38.09	0.1	43.4 ± 3.8	38.4–55.6

* $p < 0.05$

[a] Standard deviation was calculated from duplicates

conversions (44.5 and 43.4%, respectively) were achieved when the variables were used at their highest levels. The results achieved in these additional experiments validated the proposed models since the efficiencies of digestion were consistent with the prediction intervals, as shown in Table 6.

Effect of increased contents of solids on polysaccharides conversion

An additional experiment was carried out to evaluate the effect of increasing the initial content of solids to values higher than those previously evaluated in the statistical designs. In this experiment, initial solids contents varying from 4 to 12% were tested. The efficiency of polysaccharides digestion and the concentration of fermentable sugars (glucose and xylose) were considered as the response variables. Cellulolytic preparation II was used in the experiment because the model proposed to explain the results achieved with this cellulase showed a better prediction capacity, with an R^2 value of 0.84.

According to the results, the efficiency of polysaccharides conversion within 24 h of hydrolysis ranged from 24.5 to 27.0% (Fig. 3a). No statistically significant differences ($p < 0.05$) were observed among the different contents of solids. Similarly to what was observed in the previous experiments, the majority of cellulose saccharification took place in the first 24 h of hydrolysis. At 48 h of hydrolysis, the cellulose conversion achieved with the highest initial content of solids (12%) was the lowest (26.0%). Considering that the bagasse was washed thoroughly with distilled water after the pretreatment, the decrease in cellulose conversion could not be explained by the presence of inhibiting compounds generated during such pretreatment [33]. Such a decrease in the efficiency of conversion should be attributed to a poorer homogenization of the reaction mixture as well as to the end-product inhibition of the enzymes [8]. Regarding the percentages of cellulose saccharification achieved at 72 h of hydrolysis, there were no significant differences in the cellulose conversions when using initial contents of solids of 4, 6, 8, or 10%. However, the average cellulose conversion observed during the reaction with 12% solids was the lowest, 30.0%. Zheng et al. [48], for example, observed a 16% drop in the enzymatic digestibility of pretreated *Leymus triticoides* when the solid content was increased from 4 to 12%.

As expected, the concentration of fermentable sugars (glucose and xylose) obtained in the enzymatic hydrolysate was maximized with the increase in the initial content of solids in the reaction mixture (Fig. 3b). The highest concentrations of fermentable sugars were obtained after 72 h of hydrolysis, 10 and 27 g/l when using 4 and 12% solids, respectively. From the technological point of view, the use of higher contents of solids is very interesting since it leads to a hydrolysate containing an elevated concentration of fermentable sugars, which will certainly reduce processing costs.

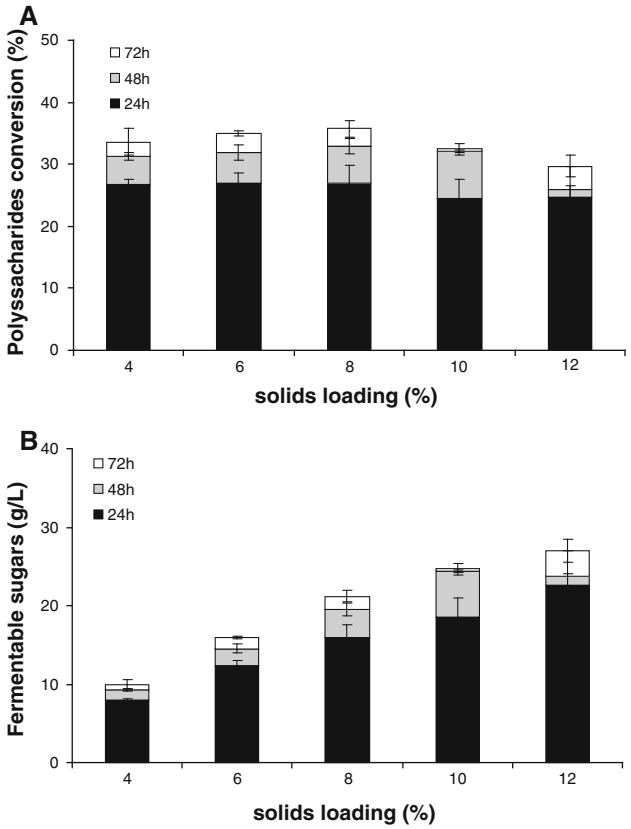

Fig. 3 Influence of solids loading on the efficiency of polysaccharides saccharification (**a**) and the concentration of fermentable sugars (**b**)

Conclusion

Both commercial cellulolytic preparations I and II, produced by leading companies, presented complete cellulolytic complexes and significant activities of xylanases, showing ability to promote saccharification of the polysaccharides contained in the sulfuric acid-pretreated sugarcane bagasse sample. Further optimization in the conditioning of the solids, prior to the enzymatic digestion, would certainly lead to better saccharification efficiencies, with both enzyme preparations. Considering that cellulase II presented higher protein content and led to a worse cellulose saccharification under the lowest enzyme loading, cellulase I was apparently more efficient than its counterpart. Such difference of behavior, under the experimental conditions employed, was neutralized by increasing the

loading of enzymes added to the material in the beginning of the reaction. All three independent variables, namely the initial content of solids and the loadings of enzymes and surfactant, significantly influenced the saccharification of the pretreated bagasse, for both preparations. Xylose was shown to be present in all the enzymatic hydrolysates and, although in low concentrations, could be used to increase a future ethanol production or, otherwise, to produce higher added value compounds. Finally, we would like to draw attention to the fact that the chemical composition of lignocellulosic materials and the constitution of complex enzyme preparations are highly variable. As the pretreatment conditions with dilute sulfuric acid leading to optimal results in terms of xylose recovery and cellulose digestibility are expected to depend of the composition of both the raw material and the enzyme preparation, a small-scale, composition-sensitive experimental approach would certainly help in defining the most adequate conditions for pretreating and saccharifying each particular sample of raw material before proceeding to larger-scale reactors. We hereby report that the statistical methodologies of screening design and response surface analysis could be successfully used to improve the enzymatic saccharification of a sugarcane bagasse sample, pretreated with dilute sulfuric acid under fixed conditions, on a small scale and in a short time.

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