J Ind Microbiol Biotechnol (2011) 38:809–817
DOI 10.1007/s10295-010-0815-5

ORIGINAL PAPER

Fermentation of cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse pretreated by hydrothermal processing

Vinícius F. N. Silva · Priscila V. Arruda · Maria G. A. Felipe · Adilson R. Gonçalves · George J. M. Rocha

Received: 31 May 2010 / Accepted: 10 August 2010 / Published online: 26 August 2010

Abstract This work aims to evaluate the fermentability of cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse pretreated by hydrothermal processing using *Candida guilliermondii* FTI 20037 yeast. The inoculum was obtained from yeast culture in a medium containing glucose as a carbon source supplemented with rice bran extract, $CaCl_2{\cdot}2H_2O$ and $(NH_4)_2SO_4$ in 50 mL Erlenmeyer flasks, containing 20 mL of medium, initial 5.5 pH under agitation of an orbital shaker (200 rpm) at 30°C for 24 h. The cellulosic hydrolysates, prior to being used as a fermentation medium, were autoclaved for 15 min at 0.5 atm and supplemented with the same nutrients employed for the inoculum, except the glucose, using the same conditions for the inoculum, but with a period of 48 h. Preliminary results showed the highest consumption of glucose (97%) for all the hydrolysates, at 28 h of fermentation. The highest concentration of ethanol (20.5 g/L) was found in the procedure of sugarcane bagasse pretreated by hydrothermal processing (195°C/10 min in 20 L reactor) and delignificated with NaOH 1.0% (w/v), 100°C, 1 h in 500 mL stainless steel ampoules immersed in an oil bath.

Keywords Sugarcane bagasse · Ethanol · Fermentation · Hydrothermal processing · Enzymatic saccharification

This article is based on a presentation at the 32nd Symposium on Biotechnology for Fuels and Chemicals.

V. F. N. Silva (✉) · P. V. Arruda · M. G. A. Felipe · A. R. Gonçalves · G. J. M. Rocha
Departamento de Biotecnologia, Escola de Engenharia de Lorena, Universidade de São Paulo, Estrada Municipal do Campinho, s/n -CEP: 12.602-810, Lorena, SP, Brazil
e-mail: viniciusfns@yahoo.com.br

Introduction

Lignocellulosic raw materials are widely used as substrates for the production of ethanol or other value-added compounds. Various lignocellulosic materials, including wood, agricultural and agro-industrial residues can be used for this purpose. Sugarcane bagasse, a sugar-alcohol sector by-product, has received considerable attention because it is cheap, available in large quantities and has a high carbohydrate content [17].

The lignocellulosic biomass is a complex structure comprised mainly of three polymeric fractions: cellulose, hemicellulose and lignin. Organization of and interactions among these polymers of the cell wall (constructed for strength and resistance to biological, physical, and chemical attack) constitute a barrier to be accessed by depolymerizing enzymes, and must be partially deconstructed in the pretreatment step prior to saccharification [6].

For this purpose, a pretreatment step is usually used to reduce recalcitrance by depolymerizing and solubilizing hemicelluloses. Removal of hemicellulose from the microfibrils is thought to expose the crystalline cellulose core, which can then be hydrolysed by cellulase enzymes. In addition, pretreatment typically breaks down the macroscopic rigidity of the biomass and decreases the physical barriers to mass transport [7].

In this context, hydrothermal pretreatment has been gaining increasing attention as both an environmentally friendly solvent and an attractive reaction media for a variety of applications. In this process, at 150–230°C range temperatures, lignocellulosic materials undergo hydrolysis reactions in the presence of the hydronium ions generated by water auto-ionization, which act as catalysts. The heterocyclic ether bonds of hemicelluloses are the most susceptible to this type of reaction, leading both to

generation of oligosaccharides and to splitting of the acetyl groups from the hemicellulosic fraction of the raw materials.

In further reaction stages, the hydronium ions generated from acetic acid auto-ionization also act as catalysts in the degradation of polysaccharides. Other fractions of lignocellulosic materials different from hemicelluloses can also react in the presence of water; for example, cellulose and lignin can be partially depolymerized by similar hydrolysis reactions [4].

On the other hand, pretreatment alone is not sufficient to extensively remove lignin, as the lignin also forms a protective physical barrier against enzymatic attack. So, alkaline extraction is used for lignin removal, contributing to fast enzymatic saccharification of biomass with a high sugar yield (for both hexoses and pentoses) [14].

However, enzymatic hydrolysis has demonstrated good results for the subsequent fermentation, because, although the process is slower, no degradation components of glucose are formed [18].

The present work evaluated the fermentability of cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse pretreated by hydrothermal processing. Sugarcane bagasse was pretreated only by a hydrothermal process, and then submitted to enzymatic hydrolysis. Pretreated samples of sugarcane bagasse were delignificated by an alkaline process and also submitted to enzymatic hydrolysis. Preliminary experiments using cellulosic hydrolysates obtained from pretreated sugarcane bagasse and from pretreated and delignificated sugarcane bagasse were evaluated in relation to fermentability by *Candida guilliermondii* FTI 20037, a yeast which is extensively studied in the research about the use of lignocellulosic materials and which produces ethanol as a byproduct.

Materials and methods

Raw material pretreatment and delignification

The sugarcane bagasse used in this study was provided by the Cia. Açucareira Vale do Rosário of Orlândia/SP—Brazil. As soon as obtained, the material was washed with water until neutral in pH and dried at 50 ± 5°C to attain 10% moisture content (untreated material).

The untreated material was submitted to hydrothermal processing pretreatment in a 20L reactor (model REGMED AU/E-20, with a mixture system for complete rotation (360°), under these conditions: 185°C/10 min, 190°C/10 min and 195°C/10 min at 1:10 w:v solid:liquid ratio with 6 rpm agitation. The pretreated sugarcane bagasse was washed with 60 L of water, until neutral in pH, for removal of residual quantities of hemicellulosic hydrolysate, and then it was measured to determine mass yield.

Afterwards, some of the lignocellulosic material was delignificated with NaOH 1.0% (w/v), 100°C, 1 h in 500 mL stainless steel ampoules immersed in an oil bath. At the end of the reaction, the residual solid material (cellulose pulp) was separated by filtration in 100% polyester cloth, washed with water to remove the residual alkali, and dried at 50 ± 5°C to attain 10% moisture content.

Enzymes

The enzymes used were commercial cellulase concentrates (Celluclast 1.5L), produced by *Trichoderma reesei*, supplemented with Novozym 188 (β-glucosidase), produced by *Aspergillus niger* (kindly supplied by Novozymes Latin America Ltda).

Filter paper activity of the commercial cellulase concentrate was measured according to Mandels; Andreotti and Roche [10], and expressed in filter paper units (FPU). The formed reducing sugar was estimated by dinitrosalicylic acid [11]. One unit of FPU is defined as the amount of enzyme required to liberate 1 μmol of glucose from Whatman no.1 filter paper per minute at 50°C. Activity of the β-glucosidase was measured according to Mongkolthanaruk and Dharmsthiti [12].

Enzymatic hydrolysis

The cellulase (15 FPU/g substrate) and β-glucosidase (10 IU/g substrate) were added to 0.05 mol L^{-1} sodium citrate buffer (pH 4.8) supplemented with 0.02% (w/v) sodium azide to inhibit microbial contamination, and then mixed with the substrate at 1:10 (w/v) solid:liquid ratio. The experiments were carried out in 125 mL Erlenmeyer flasks containing 30 mL total reaction volume. The flasks were sealed with a plastic film and incubated in a rotary shaker at 100 rpm, 45°C during 72 h.

This protocol was chosen for evaluation of enzymatic saccharification of sugarcane bagasse in raw, pretreated and delignificated forms. After 72 h reaction, the hydrolysates were heated for 5 min in a boiling water bath to precipitate the protein and prevent further hydrolysis. Glucose, cellobiose and xylose concentrations in the hydrolysates were quantitatively determined by HPLC.

The cellulose conversion ratio was calculated according to the expression:

$$CC = \frac{m_{glucose} \times f_h}{m_{initial} \times y_i} \times 100$$

Where: CC: enzymatic cellulose conversion; $m_{glucose}$: glucose mass in the hydrolysate (w); $m_{initial}$: dry mass of lignocellulosic material, before enzymatic hydrolysis step (w); y_i: cellulose content in the lignocellulosic material and f_h: hydrolysis factor of cellulose (correspondent to 0.9).

Chemical characterization of lignocellulosic materials

Chemical composition (cellulose, hemicelluloses and lignin) of the original starting material (untreated), pretreated (cellulignin) and cellulose pulp, was determined according to Gouveia et al. [5]. Carbohydrates and organic acids were determined by HPLC in a Shimadzu LC-10AD chromatograph equipped with a Shimadzu RID-6A refractive index detector and a Aminex HPX-87H (300 × 7.8 mm, Bio-Rad Laboratories Ltd) column.

The samples were diluted with deionized water, filtered through Sep-Pak C_{18} filters (Millipore) and thus injected in the chromatograph under these conditions: 45°C column temperature, 0.005 mol L^{-1} sulfuric acid as mobile phase at 0.6 mL.min^{-1} flow rate.

Fermentability of the cellulosic hydrolysates

The fermentation tests of cellulosic hydrolysates used *Candida guilliermondii* 20037 yeast maintained in malt extract agar at 4°C. The inoculum was obtained from yeast culture in a synthetic medium containing glucose (30 g/L) as a carbon source supplemented with rice bran extract, $CaCl_2{\cdot}2H_2O$ and $(NH_4)_2SO_4$ in 50 mL Erlenmeyer flasks, containing 20 mL of medium, initial pH 5.5 under agitation of an orbital shaker (200 rpm) at 30°C for 24 h.

The cellulosic hydrolysates, prior to being used as a fermentation medium, were autoclaved for 15 min at 0.5 atm and, later, supplemented with the same nutrients employed for the inoculum, except the glucose, using the same conditions for the inoculum, but with a period of 48 h. The initial cell concentration in each Erlenmeyer flask was 1.0 g/L. The control experiment was performed containing only glucose as carbon source under the same conditions specified for the hydrolysates.

Hydrolysate analyses were made by high performance liquid chromatographic (HPLC), using the methodology described in topic *Chemical characterization of lignocellulosic materials*, regarding the composition of glucose, xylose and ethanol, at 0, 28 and 48 h.

Cell viability was assessed using slide microscopy analysis of fresh material prepared and stained with methylene blue. For seed culture, the final density of cells was 5.0×10^7 cells/mL. Table 1 shows the conditions for obtaining hydrolysates as well as the abbreviations used for each one.

Results and discussion

Raw material pretreatment and delignification

The composition of the raw material used in this work is shown in Table 2. The sugarcane bagasse showed a high carbohydrate content (about 42.8% of cellulose). This table also shows the proportional increase of the cellulose and

Table 1 Cellulosic hydrolysates obtained from sugarcane bagasse obtained from the hydrothermal process used to fermentability test by *Candida guilliermondii* yeast

Hydrolysates	Conditions for obtaining hydrolysates
RPTB 1 A	Sugarcane bagasse pretreated at 195°C/10 min, not delignificated
RPTB 1 B	Sugarcane bagasse pretreated at 195°C/10 min and delignificated
RPTB 2 A	Sugarcane bagasse pretreated at 190°C/10 min, not delignificated
RPTB 2 B	Sugarcane bagasse pretreated at 190°C/10 min and delignificated
RPTB 3 A	Sugarcane bagasse pretreated at 185°C/10 min, not delignificated
RPTB 3 B	Sugarcane bagasse pretreated at 185°C/10 min and delignificated
Control	Synthetic medium (pure sugar)

Table 2 Chemical composition and mass balance of sugarcane bagasse before (raw) and after pretreatments

Components	Pretreatment conditions of sugarcane bagasse						
	Raw sugarcane bagasse	185°C/10 min		190°C/10 min		195°C/10 min	
Yield	–	–	55.5%[a]	–	51.7%[b]	–	49.6%[c]
Cellulose	42.8 ± 0.3	58.8 ± 0.7	32.5 ± 0.7	60.8 ± 0.9	31.4 ± 0.9	63.4 ± 1.1	31.4 ± 1.1
Hemicelullose	25.9 ± 0.3	15.1 ± 0.4	8.4 ± 0.4	8.9 ± 0.4	4.6 ± 0.4	5.9 ± 0.1	2.9 ± 0.1
Lignin	22.1 ± 0.2	24.8 ± 0.1	13.8 ± 0.1	24.9 ± 0.7	12.9 ± 0.7	28.5 ± 1.2	14.1 ± 1.2
Ashes	1.4 ± 0.1	1.3 ± 0.0	0.7 ± 0.0	5.4 ± 0.1	2.8 ± 0.1	2.1 ± 0.1	1.0 ± 0.1
Extractives	6.1 ± 0.1	–	–	–	–	–	–
Total	98.3 ± 1.0	100.0 ± 1.2	–	100.0 ± 2.1	–	99.9 ± 2.5	–

[a,b,c] Values of chemical characterization of sugarcane bagasse, adjusted by yield from the pretreatment steps

Table 3 Solubilization of components of sugarcane bagasse after hydrothermal pretreatment processing

Solubilized components after pretreatment	Pretreatment conditions of sugarcane bagasse		
	185°C/10 min (%)	190°C/10 min (%)	195°C/10 min (%)
Cellulose	23.8	26.6	26.5
Hemicellulose	67.6	82.2	88.7
Lignin	37.7	41.8	36.0

Table 4 Chemical composition and mass balance of sugarcane bagasse delignificated, after hydrothermal pretreatment process

Components	Delignification of sugarcane bagasse pretreated—Conditions: NaOH 1.0% (w/v), 100°C for 1 h, from the pretreatments at:					
	185°C/10 min		190°C/10 min		195°C/10 min	
Yield	–	74.4%[a]	–	68.6%[b]	–	59.7%[c]
Celullose	68.5 ± 0.1	28.3 ± 0.1	73.1 ± 0.6	25.9 ± 0.6	79.2 ± 0.6	23.5 ± 0.6
Hemicelullose	9.9 ± 0.2	4.1 ± 0.2	7.1 ± 0.1	2.5 ± 0.1	3.7 ± 0.2	1.1 ± 0.2
Lignin	18.8 ± 1.3	7.8 ± 1.3	17.3 ± 0.9	6.1 ± 0.9	14.2 ± 0.3	4.2 ± 0.3
Ashes	2.0 ± 0.2	0.8 ± 0.2	2.6 ± 0.0	0.9 ± 0.0	3.6 ± 0.4	1.1 ± 0.4
Total	99.2 ± 1.8	–	100.1 ± 1.6	–	100.7 ± 1.5	–

[a,b,c] Values of chemical characterization of sugarcane bagasse delignificated, adjusted by yield from steps of pretreatment and delignification

lignin content in the pretreated sugarcane bagasse (185°, 190° and 195°C for 10 min) in relation to the raw sugarcane bagasse, due to solubilization of the hemicellulosic fractions.

Note that there is a yield reaction (Table 2), for both pretreatment and delignification steps, implying a reduction of the amount of each component of the biomass during its processing.

Analysis of solubilized fractions after the hydrothermal pretreatment process (Table 3, below) showed that the highest severity of pretreatment process (195°C/10 min) provided the highest solubilization of the hemicellulose, reaching 88.7%.

According to studies of hydrolysis of lignocellulosic materials [3], in hydrothermal pretreatment processes conducted in elevated temperatures, the heating time of the reactor may be relatively long compared to the reaction time in the target temperature, providing a pronounced solubilization of the hemicellulosic fraction during the heating phase.

In Table 3, a little increase in solubility of some cellulosics fractions was observed , achieving approximately 26.6% between 190 and 195°C/10 min, and also solubilization of the lignin fraction from sugarcane bagasse, reaching 41.8% in 190°C/10 min.

Several agricultural residues have presented different behaviors in relation to increase of the susceptibility of cellulosic fraction in relation to hydrolysis, reaching 50% of cellulosic degradation in experiments conducted at temperatures in the range of application for the hydrothermal pretreatment process, ranging from approximately 150–230°C, according with previous reports [4].

Table 5 Total solubilization of the components of sugarcane bagasse after hydrothermal pretreatment process and alkaline delignification steps

Biomass components	185°C/10 min (%)	190°C/10 min (%)	195°C/10 min (%)
Cellulose	33.9	39.4	45.2
Hemicellulose	84.2	90.3	95.8
Lignin	64.9	72.2	80.9

However, factors such as solid–liquid ratio and the reaction time employed are important in controlling degradation of the cellulosic fraction, avoiding the waste of yield in the process of cellulose enzymatic conversion to glucose for ethanol production.

After the hydrothermal pretreatment process, the resulting material from this step had a better susceptibility to alkaline delignification, as shown in Tables 4 and 5.

The delignification process with NaOH 1.0% (w/v), at 100°C during 1 h provided a solubilization of large amounts of lignin (~81%) when the condition of the hydrothermal pretreatment process at 195°C/10 min was employed. However, a significant amount of the cellulosic fraction was solubilized, achieving ~45% for the same conditions of pretreatment of sugarcane bagasse. In a global process of ethanol production, the solubilization of a large amount of cellulosic fraction is not desirable , as it can lead to a loss of yield in this process.

The hydrothermal pretreatment process produced high solubilization of hemicellulosic fraction and solubilization of lignin fragments with low molecular weight, providing a reduction in the recalcitrance of lignocellulosic material, as

Table 6 Effect of hydrothermal pretreatment and alkaline delignification on enzymatic saccharification of sugarcane bagasse

Material	Pretreatment condition	Glucose concentration in hydrolysate (g/L)	Cellulose conversion (%)
Raw sugarcane bagasse	–	8.3 ± 0.2	6.0 ± 0.3
Sugarcane bagasse pretreated	185°C/10 min	42.5 ± 0.4	40.8 ± 1.8
	190°C/10 min	57.6 ± 0.6	56.9 ± 0.7
	195°C/10 min	66.8 ± 1.3	69.2 ± 2.6
Sugarcane bagasse pretreated and delignificated	185°C/10 min	71.6 ± 0.8	78.5 ± 0.6
	190°C/10 min	74.5 ± 0.5	82.3 ± 0.6
	195°C/10 min	86.0 ± 0.4	89.2 ± 2.2

also reported by another study [15]. This increased the available surface cellulosic area, promoting extensive degradation of this polymer in the process of sugarcane bagasse alkaline delignification by the pretreatment with more severity.

Enzymatic hydrolysis of sugarcane bagasse

The Table 6 shows that, after the hydrothermal pretreatment step, the maximum conversion of cellulose obtained (for the pretreatment condition: 195°C/10 min, followed by delignification with NaOH 1.0% (w/v) at 100°C/1 h) was 89.2%, with a glucose concentration of 86.0 g/L in the hydrolysate.

These values were higher than those obtained by enzymatic saccharification of raw sugarcane bagasse, which achieved 6.0% of cellulose conversion and with a glucose concentration of 8.3 g/L in the hydrolysate. It can also be verified in this table that these values were higher than those obtained by enzymatic saccharification of sugarcane bagasse that was only pretreated, which achieved 69.2% of cellulosic conversion with a glucose concentration of 66.8 g/L in the hydrolysate.

Table 3 verified that the increase of pretreatment severity (from 185°C/10 min to 195°C/10 min) enhanced the solubilization of the hemicellulosic fraction and provided a change in the cellulosic structure (in relation to their physical–chemical characteristic mainly) which made it more accessible to the action of cellulolytic enzymes.

This factor directly reflected in the increase of the conversion of cellulose into glucose, as shown by the increase in the glucose concentration in the enzymatic hydrolysate (Table 6). Besides, the sugarcane bagasse coming from the hydrothermal pretreatment step at 195°C/10 min, when delignificated with NaOH 1.0% (w/v) at 100°C for 1 h, showed a better enzymatic conversion of cellulose than the other conditions of pretreatment and delignification employed.

This material probably had a lignin structure that was easier to remove, a small amount of hemicellulose in the fiber and a cellulosic fraction with a lower degree of polymerization and more available to the action of cellulolytic complexes. This indicates that there is a significant improvement in the enzymatic conversion of the cellulose after the pretreatment and delignification steps, a fact previously reported by other authors [13, 16].

Testing of fermentability of cellulosic hydrolysates

The graphs in Fig. 1a and b show the behavior of *Candida guilliermondii* yeast during the cultivation in cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse after different conditions of hydrothermal pretreatment and control experiments, respectively.

Analyzing the results of Fig. 1a and Table 7, we found a similarity in glucose consumption (higher than 98.5%), regardless of the pretreatment conditions employed, except for the condition RPTB 3B (94.9%). We also observed that the yeast consumes glucose rather than xylose during the first 28 h. This fact was already previously verified using the same yeast [19]. Xylose was slowly assimilated in all the pretreatment conditions used; its maximum consumption (92.4%) occurred in the condition RPTB 3A, in which we observed the maximum glucose consumption (100%). Concerning ethanol production, the maximum concentration (20.5 g/L) occurred in 28 h using the enzymatic hydrolysate of cellulosic fraction of bagasse pretreated at 195°C/10 min and delignificated (RPTB 1B).

This condition coincided with one in which the more soluble fractions of hemicellulose and lignin were obtained (Table 3). It was also shown by Fig. 1 that, except for the hydrolysate from the condition RPTB 3B, the ethanol was assimilated by the yeast, as verified previously [1]. The behavior of the yeast during cultivation in a medium containing only glucose as carbon source (Fig. 1b) showed a slow glucose consumption, different from that observed in hydrolysates. Possibly the hydrolysates contained some compounds that favored the consumption of glucose and consequently ethanol formation.

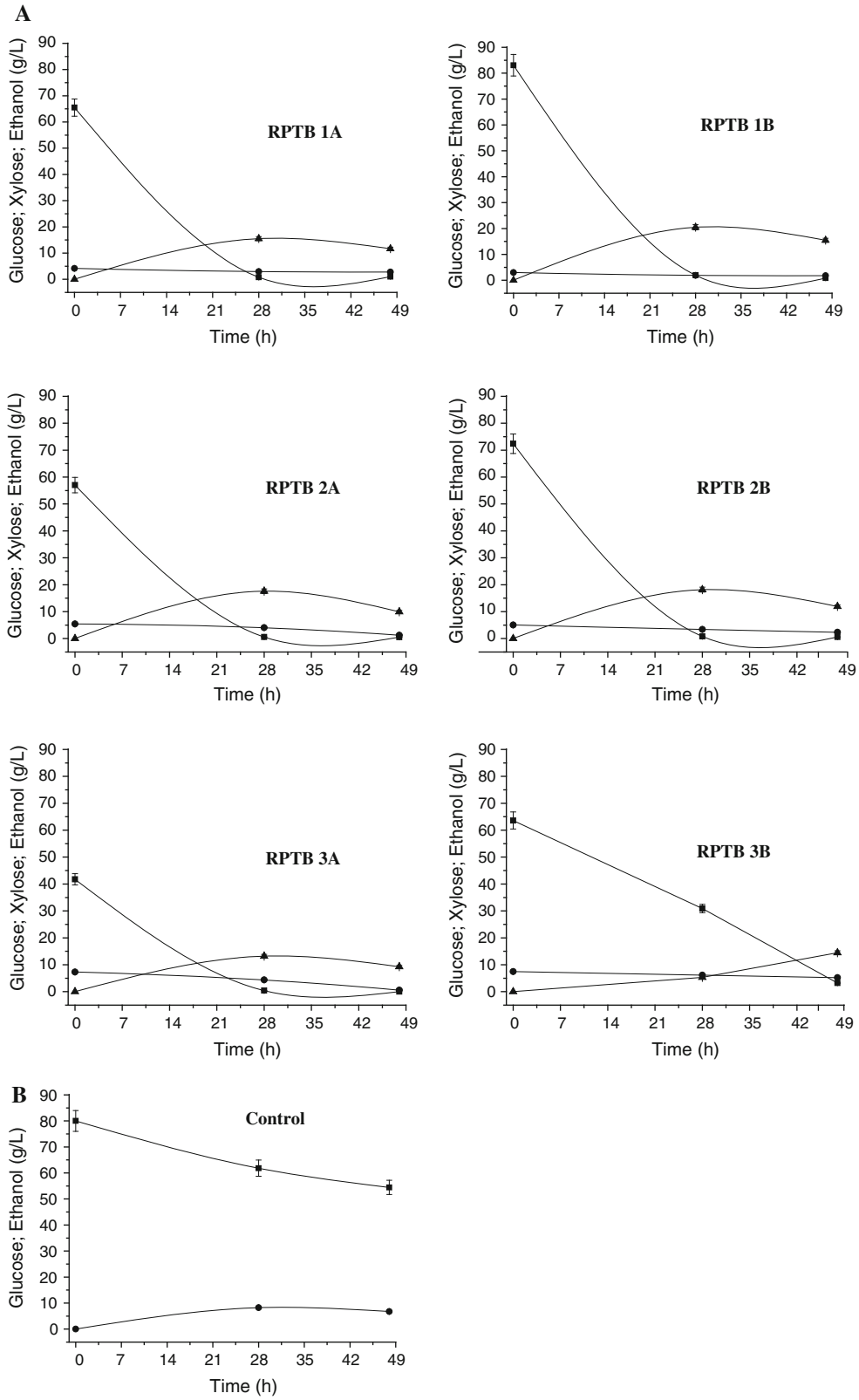

Fig. 1 Xylose *filled circle* and glucose *filled square* consumption and ethanol production (*filled triangle*) by *Candida guilliermondii* yeast during cultivation in **a** cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse from different conditions of hydrothermal pretreatment. (RPTB 1—195°C/10 min; RPTB 2—190°C/10 min; RPTB 3—185°C/10 min; A—not delignificated and B—delignificated) and **b** Control

Table 7 Glucose and xylose consumption (%) and ethanol formation by *Candida guilliermondii* yeast

Enzymatics hydrolysates of	Glucose consumption (%)		Xylose consumption (%)		Concentration of ethanol (g/L)	
	28 h	48 h	28 h	48 h	28 h	48 h
RPTB 1A	98.8	98.5	29.9	33.6	15.5	11.7
RPTB 1B	97.7	98.9	37.2	40.9	20.5	15.5
RPTB 2A	98.9	99.1	25.4	77.4	17.6	9.9
RPTB 2B	98.9	99.2	33.3	53.6	18.2	11.9
RPTB 3A	99.1	100	40.1	92.4	13.2	9.3
RPTB 3B	51.4	94.9	17.6	30.5	5.3	14.5
Control	24.6	33.6	–	–	8.3	6.8

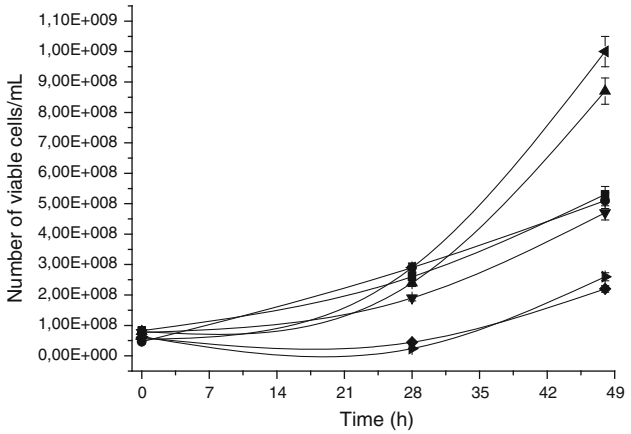

Fig. 2 Number of viable cells during cultivation in cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse from hydrothermal pretreatment conditions: RPTB 1A *filled square*, RPTB 1B *filled circle*, RPTB 2A *filled triangle*, RPTB 2B *filled inverted triangle*, RPTB 3A *filled left pointing triangle*, RPTB 3B *filled right pointing triangle* and Control *filled diamond*

A similar profile for glucose and xylose consumption and ethanol production was also observed in other studies [2, 8, 9, 20], but employing the yeast *Saccharomyces cerevisiae*, commonly used in processes for ethanol production. According to Krishnan et al. 2010 [8], the use of this yeast provided ethanol concentrations higher (between 34 and 36 g/L) than those observed in this work. Once, in their research, the employed enzymatic hydrolysates were obtained from sugarcane bagasse pretreated with ammonia (AFEX).

Regarding the number of viable cells, there was an increase in the number of cells in all hydrolysates used, with the increased time of fermentation (Fig. 2).

We also observed an increase in the number of cells in 48 h of fermentation for conditions RPTB 2A and RPTB 3A; these conditions were those that had lower ethanol concentrations in the medium and a preference of biomass production over ethanol production (Tables 7 and 8).

The Fig. 3 shows a diagram of the whole mass balance at the optimum conditions of pretreatment of sugarcane bagasse.

Conclusions

The ethanol production by *Candida guilliermondii* in cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse was verified independently of the employed pretreatment conditions. For all evaluated conditions, delignification contributed to improving the ethanol production by this yeast, except for the condition of sugarcane bagasse pretreated at 185°C/10 min and delignificated (RPTB 3B). Cell growth was observed in all the employed hydrolysates, achieving its highest value in condition RPTB 3A at a fermentation time of 48 h.

Table 8 Cell counts in a Neubauer chamber at times 0, 28 and 48 h

Enzymatic hydrolysates of	Cell counts in Neubauer chamber (cell/mL)		
	0 h	28 h	48 h
RPTB 1A	0.83×10^8	5.3×10^8	2.6×10^8
RPTB 1B	0.46×10^8	2.9×10^8	5.1×10^8
RPTB 2A	0.80×10^8	2.4×10^8	8.7×10^8
RPTB 2B	0.77×10^8	1.9×10^8	4.7×10^8
RPTB 3A	0.55×10^8	2.9×10^8	10.0×10^8
RPTB 3B	0.63×10^8	0.24×10^8	2.6×10^8
Control	0.61×10^8	0.45×10^8	2.2×10^8

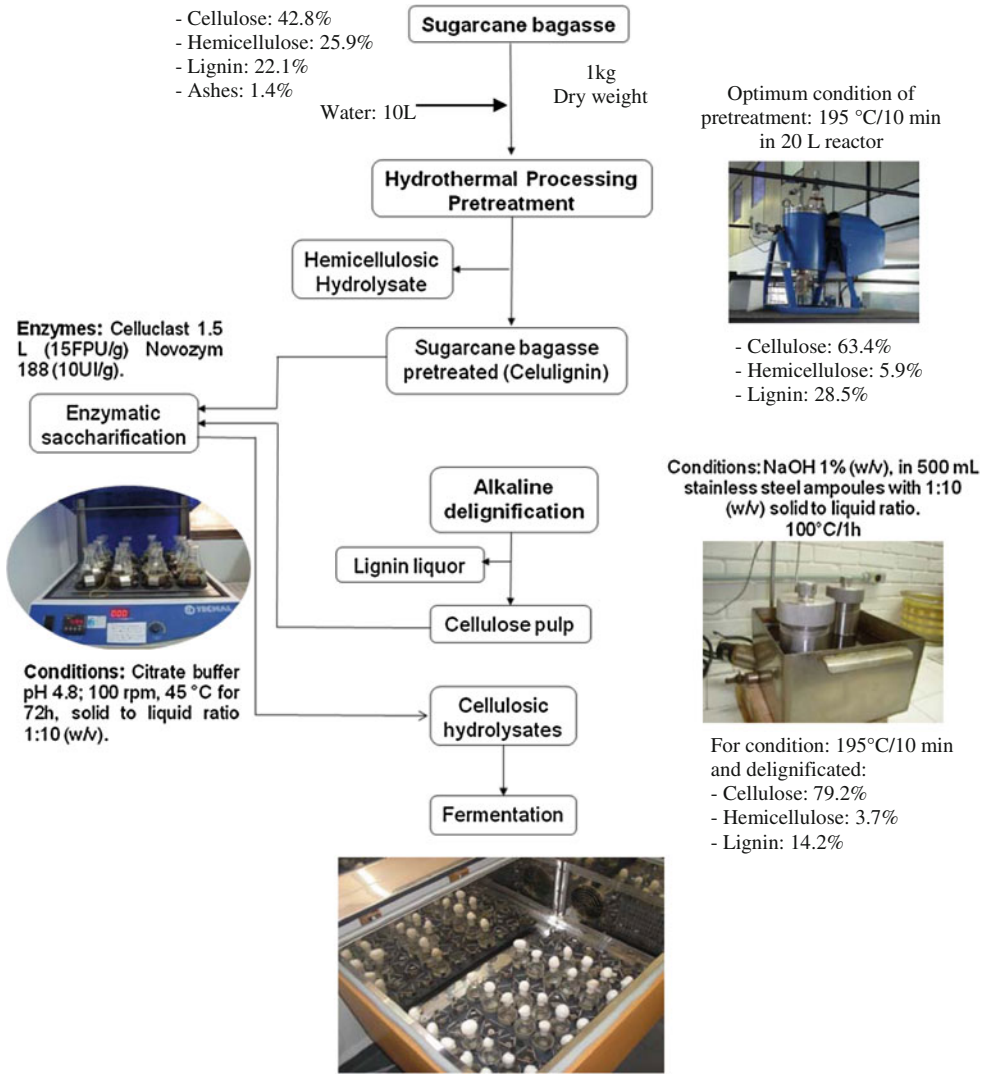

Fig. 3 Mass balance for delignificated and hydrothermal process pretreated sugarcane bagasse during enzymatic saccharification and fermentation

Acknowledgments This work was financially supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors are grateful to Novozymes Latin America Ltda for the supply of the enzymes used in this study.

References

1. Arruda PV, Felipe MGA (2009) Role of glycerol addition on xylose-to-xylitol bioconversion by *Candida guilliermondii*. Curr Microbiol 58:274–278. doi:10.1007/s00284-008-9321-7
2. Bals B, Rogers C, Jin M, Balan V, Dale B (2010) Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. Biotechnol Biofuels 1–11. doi:10.1186/1754-6834-3-1
3. Carrasco F, Roy C (1992) Kinetic study of dilute-acid prehydrolysis of xylan-containing biomass. Wood Sci Technol 26:189–208
4. Garrote G, Domínguez H, Parajó JC (1999) Hydrothermal processing of lignocellulosic materials. Eur J Wood Wood Prod 57:191–202
5. Gouveia ER, Nascimento RT, Souto-Maior AM, Rocha GJM (2009) Validação de metodologia para a caracterização química de bagaço de cana-de-açúcar. Quim Nova 32:1500–1503
6. Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100:10–18. doi:10.1016/j.biortech.2008.05.027
7. Himmel ME, Ding S, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants

and enzymes for biofuels production. Science 315:804–807. doi:10.1126/science.1137016
8. Krishnan C, Sousa LC, Jin M, Chang L, Dale BE, Balan V (2010) Alkali-based AFEX pretreatment for the conversion of sugarcane bagasse and cane leaf residues to ethanol. Biotechnol Bioeng. doi:10.1002/bit.22824
9. Lau MW, Dale BE (2009) Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A (LNH-ST). Proc Natl Acad Sci USA 106:1368–1373. doi: 10.1073/pnas.0812364106
10. Mandels M, Andreotti R, Roche C (1976) Biotechnol Bioeng Symp 6: 21–33
11. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428. doi: 10.1021/ac60147a030
12. Mongkolthanaruk W, Dharmsthiti S (2002) Biodegradation of lipid-rich wastewater by a mixed bacterial consortium. Int Biodeterior Biodegrad 50:101–105
13. Mussatto SI, Fernandes M, Milagres AMF, Roberto IC (2008) Effect of hemicelluloses and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme Microb Technol 43:124–129. doi:10.1016/j.enzmictec.2007.11.006
14. Öhgren K, Bura R, Saddler J, Zacchi G (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour Technol 98:2503–2510. doi: 10.1016/j.biortech.2006.09.003
15. Petersen MO, Larsen J, Thomsen MH (2009) Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. Biomass Bioenergy 33:834–840. doi:10.1016/j.biombioe.2009.01.004
16. Ramos LP, Nazhad MM, Saddler JN (1993) Effect of enzymatic hydrolysis on the morphology and fine structure of pretreated cellulosic residues. Enzyme Microb Technol 15:821–831. doi: 10.1016/0141-0229(93)90093-H
17. Reddy N, Yang Y (2005) Biofibers from agricultural byproducts for industrial applications. Trends Biotechnol 23:22–27. doi: 10.1016/j.tibtech.2004.11.002
18. Sánchez ÓJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feed stocks. Bioresour Technol 99:5270–5295. doi:10.1016/j.biortech.2007.11.013
19. Sene L, Felipe MGA, Vitolo M, Silva SS, Mancilha IM (1998) Adaptation and reutilization of *Candida guilliermondii* cells for xylitol production in bagasse hydrolysate. J Basic Microbiol 38:61–69
20. Zhang C, Lau MW, Balan V, Dale BE, Yuan YJ (2009) Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl Microbiol Biotechnol 84:667–676. doi:10.1007/s00253-009-2001-0