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Strategies of detoxification and fermentation for biotechnological production of xylitol from sugarcane bagasse

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ABSTRACT

The aim of this study was to evaluate strategies of detoxification and fermentation of the hemicellulosic liquors obtained from sugarcane bagasse autohydrolysis, for the biotechnological production of xylitol. Different sequences of detoxification treatments were performed, and their effects on sugars loss and inhibitors removal were evaluated. Fermentation assays were accomplished with commercial xylose to select the best yeast (*C. guilliermondii*, *C. tropicalis*) and the fermentation conditions of the detoxified spent liquors. Detoxification through a sequence of treatments, including $Ca(OH)_2$, IR-120 resin, activated charcoal, and IRA-67 resin, practically removed all inhibitors from the hemicellulosic spent liquors. Maximal concentration of xylitol obtained was 32.0 g L^{-1} (*C. tropicalis*, xylose: 104.1 g L^{-1}, yield: 0.46 g g^{-1}, productivity: 0.27 g L^{-1} h^{-1}). The technological parameters to obtain a detoxified spent liquor rich in xylose and its bioconversion to xylitol were determined.

1. Introduction

Developing countries are in search of strategies for converting their production technologies so as to open up new markets and improve regional economies. The final disposal of lignocellulosic wastes generated during agricultural raw materials processing (sugarcane bagasse, rice husks, stalks of sunflowers, etc.), creates environmental problems associated with burning and/or accumulation. Therefore, one of these strategies is based on the use of lignocellulosic waste from agroindustrial raw materials to obtain high value products through the application of various conversion processes.

Sugarcane bagasse is the lignocellulosic waste generated in sugar mills (180–280 kg bagasse per ton of sugarcane) and constitutes an important source of renewable material, available in large quantities at low cost (Rao et al., 2012). Its composition depends on the climatic and soil conditions in which sugarcane has grown, but a typical composition is: 43–45% cellulose, 21–23% lignin, 25–32% hemicelluloses (mainly xylans) and minor amounts of extractive and ash (Area et al., 2012; Vallejos et al., 2012).

Xylans are composed of xylose units and may contain different substituents in the chain. They can be depolymerized into xylose, a primary carbon source for the bioproduction of xylitol, ethanol, and others. Xylitol is used as a sweetening agent and it is industrially produced by chemical reduction of xylose by expensive processes (De Albuquerque et al., 2014; Hou-Rui, 2012; Rao et al., 2012). Xylitol can also be produced biotechnologically by xylose conversion using specific microorganisms, being a metabolic intermediate product of xylose (Ikeuchi et al., 1999; Sasaki et al., 2012). The yeasts which have shown the highest yields of fermentation of xylose to xylitol are: *Candida guilliermondii*, *Candida tropicalis*, *Candida boidinii*, *Candida parapsilosis* and *Pichias* (Rafiqul and Sakinah, 2013).

The biotechnological process represents a lower cost alternative to the chemical one, as it is performed at atmospheric pressure and the purification of hydrolysates is less complex (Canilha et al., 2006; Rafiqul and Sakinah, 2013). Studies on culture medium prepared from lignocellulosic hydrolysates are focused on the removal of compounds that cause inhibition of microbial metabolism and decrease cell growth and product yield (Rafiqul and Sakinah, 2013). Some degradation products of sugars and lignin can adversely affect the fermentation process because they are toxic to microorganisms and inhibit their metabolism. Factors affecting xylitol production are: initial inoculum concentration, type of substrate, composition of culture medium, inhibitor compounds, temperature, pH, and oxygen transfer (Rao et al., 2004).

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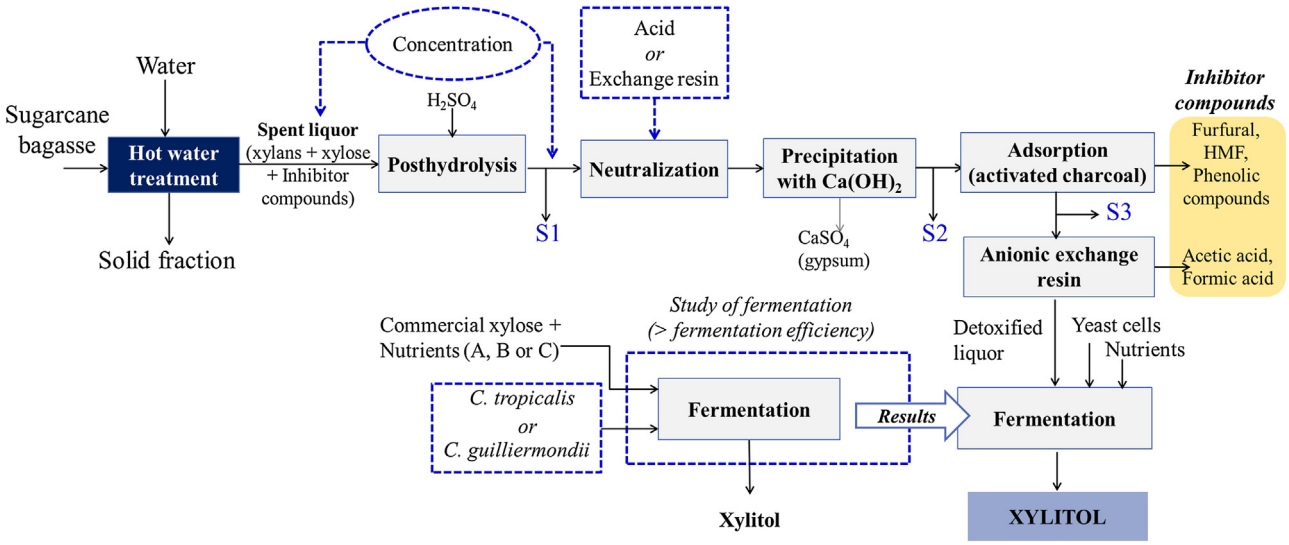

Fig. 1. Complete methodology used to obtain xylose and to convert it to xylitol (dotted line: alternatives).

Hot water treatment, named also autohydrolysis, is effective for hemicelluloses solubilization. This technology is environment friendly as it does not use any chemicals other than water (Garrote et al., 1999). In this process (aqueous medium, 150–250 °C, pH between 3 and 4), the hemicelluloses are hydrolyzed to oligo and monosaccharides (Caparrós et al., 2007; Vallejos et al., 2015). These conditions also promote the formation of low molecular weight aliphatic acids, furfural, and hydroxymethylfurfural (Jönsson and Martín, 2016). Lignin undergoes reactions of degradation and repolymerization, generating partially water soluble phenolic derivatives and insoluble condensation products, respectively (Rafiqul and Sakinah, 2013). Despite their low concentration, these compounds may act as inhibitors of microorganisms in the fermentation of hydrolysates from lignocellulosic materials (Larsson et al., 2000).

The concentration of inhibitors and sugars in spent liquors depends on raw materials and processing conditions. Detoxification may be accomplished by several methods, namely: (i) vacuum evaporation, (ii) ion exchange resins, (iii) activated charcoal, (iv) enzymes or microorganisms, (v) extraction with ether or ethyl acetate, and (vi) alkaline neutralization and precipitation. Detoxification methods cannot be compared with each other when different spent liquors and microorganisms are used, since liquors may have different amount and type of inhibitors and microorganisms present different tolerances to them (Jönsson et al., 2013). The identification and quantification of each compound is difficult because of the numerous and various aromatic compounds that can be found in different lignocellulosic hydrolysates.

Xylose sources and conversion treatments are keys to allow a cost-effective production of xylitol. Some strategies were explored to produce xylitol in an economical and environmentally friendly way (Clauser et al., 2015; Franceschin et al., 2011; Hernández et al., 2014). Because of the dissimilar prices of xylitol and ethanol, the co-production of xylitol with ethanol may improve the viability of lignocellulosic biorefineries, making feasible their installation in small-scale (Rao et al., 2012; Rueda et al., 2016). Nevertheless, there are few reports about an integral study of the processes involved in xylose extraction from sugarcane bagasse, its purification, and its conversion to xylitol (Rao et al., 2006; Silva et al., 2005).

The aim of this study was to evaluate strategies of detoxification and fermentation of the hemicellulosic spent liquors from sugarcane bagasse autohydrolysis for the biotechnological production of xylitol.

2. Materials and methods

2.1. Raw material

Sugarcane bagasse was collected in a local sugar mill (San Javier Sugar Mill, Misiones, Argentina). Bagasse pith was removed in two stages. In first stage, bagasse was wet-depithed to break its structure in a Bauer disc refiner (plate gap of 0.01 in), after which the bagasse pith was removed by screening, in a second stage, using a plate with 2 mm wide slits (Wenmber). Finally, depithed bagasse was centrifuged and preserved in a refrigerator. The bagasse was characterized in a previous work (Vallejos et al., 2015). The complete methodology used in this work for obtaining xylose and for converting it to xylitol is schematized in Fig. 1x.

2.2. Autohydrolysis of sugarcane bagasse and post-hydrolysis detoxification of spent liquors

The sequential study of autohydrolysis, post-hydrolysis and detoxification treatments is described below and shown in Fig. 2.

2.2.1. Autohydrolysis of sugarcane bagasse

Sugarcane bagasse was treated with hot water under isothermal conditions at 180 °C and 20 min. The hot water pretreatment conditions were selected on the base of results obtained in a previous study about the kinetic study of the extraction of hemicellulosic carbohydrates from sugarcane bagasse by this kind of treatment. A detailed description of the treatment can be found in Vallejos et al., 2015. The chemical composition of sugarcane bagasse was: 43.1% glucans, 23.8% xylans, 1.7% arabinans, 1.7% acetyl groups, 21.3% lignin, 4.8% extractives, and 1.5% ash.

Hot water pretreatments were performed with two different liquid-solid ratios (LSR) to obtain dilute and concentrated liquors. Dilute liquor was obtained using LSR of 14:1 in a MK digester (7 L) with a recirculation system using 350 g OD of bagasse, whilst concentrated liquor was obtained using LSR of 4:1 in a multipurpose reactor (4 L) heated by direct steam without stirring, using 300 g OD of bagasse. The extraction of xylans (wt.%) in the pretreatments and the chemical composition of the liquors were determined.

2.2.2. Post-hydrolysis of spent liquors

Hot water pretreatment produces partial solubilization of xylans mainly as *xylo*-oligomers, therefore post-hydrolysis of spent

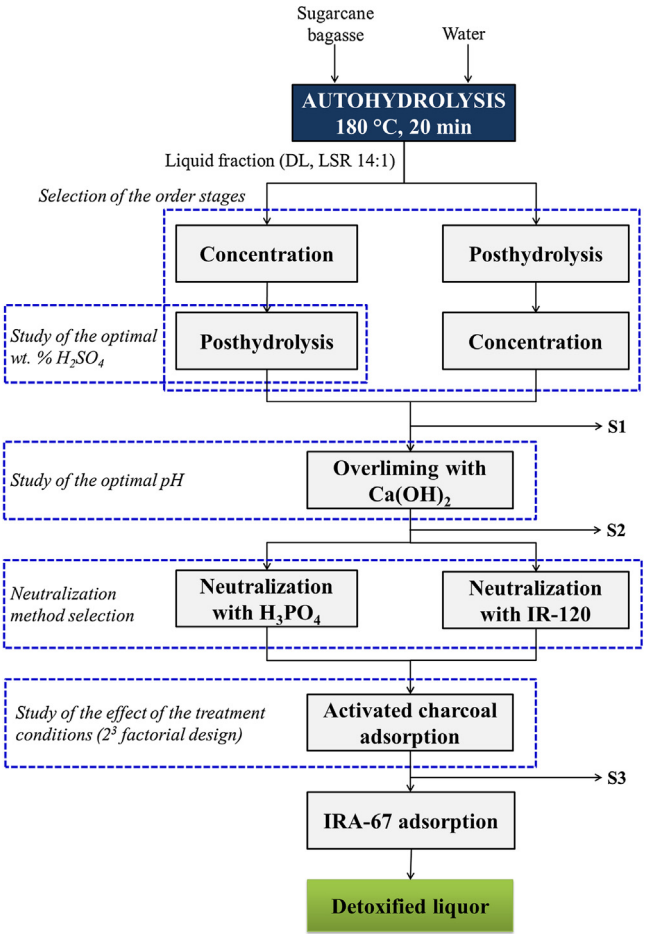

Fig. 2. Detail of sequential treatments of autohydrolysis, post-hydrolysis and detoxification of spent liquors (S1–S3: samples taken for fermentation experiments).

liquors is necessary to convert these *xylo*-oligomers to xylose, which can be directly metabolized by yeast. Two strategies of post-hydrolysis were tried, using sulfuric acid as catalyst:

2.2.2.1. Strategy 1. Dilute liquor was concentrated in a rotary evaporator (Senco Technology Co., Ltd.) at 55 °C under vacuum (concentration factor: 3.2) and then post-hydrolysed. Different concentrations of H_2SO_4 were studied to limit the formation of inhibitor compounds generated from the dehydration of the pentoses and hexoses (furfural and HMF), and to achieve maximal xylose concentration. The post-hydrolysis was carried out by autoclaving the liquor in 10 mL vials using different H_2SO_4 concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 wt.%) at 121 °C for 60 min.

2.2.2.2. Strategy 2. Dilute liquor was post-hydrolyzed using optimal H_2SO_4 concentration from Strategy 1 and then concentrated. In these conditions, concentration of spent liquor avails the steam-stripping of furfural and HMF generated in the autohydrolysis and post-hydrolysis stages. The methods and conditions of the detoxification stage were based on the results of Strategy 1.

2.2.3. Detoxification of spent liquors

In the first strategy, sulfuric acid used in the post-hydrolysis was removed by overliming with $Ca(OH)_2$ at ambient temperature. This alkaline treatment also reduces the concentrations of furfural, HMF, and phenolic compounds. Assays of the treatment with $Ca(OH)_2$ were performed at various pH (6, 8 and 10) to assess the effect of overliming on inhibitors removal and xylose loss. To achieve different pH, anhydrous $Ca(OH)_2$ was added to 50 mL of each liquor, mixing vigorously. Samples were taken at each pH and the composition of liquors was determined by HPLC. The liquor was subsequently centrifuged at 2500 rpm for 10 min to precipitate solids (gypsum).

Two methods were applied to adjust spent liquor to pH 5. The first method involved the addition of phosphoric acid to pH 5 followed by liquor centrifugation to precipitate mainly $Ca_3(PO_4)_2$. The second method was based on the total removal of calcium ions by stirring the liquor with Amberlite IRA-120 cationic resin (in hydrogen form), which was recovered by centrifugation.

A 2^3 factorial design was used to determine the effect of temperature, activated charcoal concentration, and contact time, on the removal of phenolic and other compounds (Table 1). The real values of the variables were codified, so that the low and high levels of all coded variables were −1 and +1, respectively.

Table 1
Levels of the variables in the 2^3 factorial design for the activated charcoal treatment.

	Coded levels	
Variable	−1	+1
Activated charcoal concentration (%, g/100 mL), X_1	1	3
Temperature (°C), X_2	30	60
Contact time (min), X_3	10	60

Liquor samples (50 mL each) in 100 mL Erlenmeyer flasks were treated with activated charcoal in a thermostatic bath with orbital shaking (Numak) at 120 rpm. The characteristics of the activated charcoal (CLARIMEX, DICA, Argentina) were: powdered acid type, pH 2–4; Number of iodine (I_2 mg g^{-1} CA): 850; particle size: 80% minimum passing 400 mesh. The statistical analysis of the experimental design was performed by STATGRAPHICS Centurion software.

Acids in spent liquors (mainly acetic acid) were removed by weak-base anion-exchange Amberlite IRA-67 resin (Adrem Corporación Industrial S.A., Argentina). Preliminary assays were performed with acetic acid solution (1 g L^{-1}) to adjust ionic exchange treatment conditions. Spent liquor was stirred with Amberlite IRA-67 resin (capacity: 1.6 eq. L^{-1}) until a constant pH. The resin was recovered by centrifugation.

2.3. Inoculum preparation and fermentation

The strains of *Candida guilliermondii* and *Candida tropicalis* yeast were obtained from the Mycology Department of the National Institute of Infectious Diseases, ANLIS "Dr. Carlos G. Malbrán". The yeast cultures were maintained on 2% water agar at 4 °C.

Conditions for fermentation of detoxified spent liquors, including yeast selection (*C. guilliermondii*, *C. tropicalis*), inoculum preparation, and fermentation (nutrients, concentration of yeast cells, and aeration), were obtained from initial experiences with commercial xylose (Biopack, Argentina).

Three different compositions of culture medium were studied for inoculum preparation and fermentation (all added to 30 g L^{-1} of xylose):

(A) $(NH_4)_2SO_4$ (3.0 g L^{-1}), $CaCl_2.2H_2O$ (0.1 g L^{-1}), rice bran extract (10% v/v);
(B) yeast extract (7.5 g L^{-1}), peptone (4.5 g L^{-1});
(C) yeast extract (10 g L^{-1}), peptone (20 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), KH_2PO_4, (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), $(NH_4)_2SO_4$ (2.0 g L^{-1}).

Yeast cells from 40% Sabouraud dextrose agar slant were transferred to the inoculum medium containing commercial xylose

(30.0 g L^{-1}) and incubated on a rotary shaker (200 rpm) at 30 °C for 48 h. Cells were collected by centrifugation at 2200 rpm for 20 min.

The collected cells were resuspended in distilled water and were placed in Erlenmeyer flasks containing commercial xylose in sterilized medium (around of 30.0 g L^{-1}). Preliminary fermentation assays were performed at 200 rpm at 30 °C, with initial pH adjusted to 5 with 1 N H_2SO_4 solution. The air-to-medium ratio in Erlenmeyer flask was ≤0.4 to confer semi-aerobic fermentation conditions. Erlenmeyer flasks were stoppered with cotton plugs.

A statistical study of the stages involved in the fermentation was performed to determine the variability of methods and the standard deviation of the studied fermentation parameters. The average standard deviations of yeast cells, xylose consumption, and xylitol production were determined from replicas of fermentation of commercial xylose at different yeast cell concentrations.

2.4. Xylitol production from spent liquors

Concentrated liquors with different degrees of detoxification were supplemented with nutrients and then sterilized, and the inoculum cells were resuspended in these mediums. Fermentation assays were carried out in a rotary shaker (200 rpm) at 30 °C with initial pH adjusted to 5. Samples were taken every 24 h for 120 h. Parallel experiments employing commercial xylose were also performed as control.

Resulting liquors were centrifuged, and xylose and xylitol concentrations were determined in the supernatant by liquid chromatography (HPLC).

Conversion of xylose to xylitol (g xylitol/100 g of consumed xylose) and volumetric productivity (g of produced xylitol L^{-1} h^{-1}) were determined from the concentrations of these products.

2.5. Analytical methods

The used bagasse was characterized in a previous work (Vallejos et al., 2015). Spent liquors were characterized by determinations of sugars (glucose, xylose, and arabinose), oligomers (glucans, xylans, arabinans), formic acid, acetic acid, and degradation products (furfural, 5-hydroxymethilfurfural (HMF)), according to Technical Report NREL/TP-510-42623 (January 2008) "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples", National Renewable Energy Laboratory. The quantification of these compounds and of xylitol were carried out by HPLC liquid chromatography (Waters HPLC System) using an Aminex-HPX–87 H87H column (BIO-RAD) with the following chromatographic conditions: H_2SO_4 4 mM as eluent, 0.6 mL min^{-1}, 35 °C, with refractive index and diode array detectors. The oligomers in the liquid fractions were hydrolyzed to monosaccharides. The weights of cellobiose and glucose were converted to glucans equivalents, the xylose to xylans, the arabinose to arabinans, and the acetic acid to acetyl groups, by multiplying them by the hydrolysis factors: 0.95, 0.90, 0.88 and 0.717, respectively. The pH of the samples was determined with a HANNA pHmeter.

Total phenols content was determined by the Folin–Ciocalteau method (Blainski et al., 2013), based on the ability of phenolic compounds to react with the Folin–Ciocalteau oxidizing reagent (FCR). Phenolic compounds react with 2 N FCR to form a colored complex (blue) that can be quantified by visible-light spectrophotometry (760 nm). Maximal absorbance of chromophores mainly depends on the concentration of phenolic compounds. The total phenolic content was expressed as grams of vanillin equivalents by liter (g vanillin L^{-1}) by means of a calibration curve obtained with a standard of vanillin. Vanillin was selected as reference compound based on the following criteria: (i) it was found in the highest proportion among all the analyzed phenolic compounds, and (ii) preliminary tests with xylose (60 g L^{-1}) showed negligible interference in the Folin–Ciocalteau method.

Yeast cell concentrations (g L^{-1}) in the inoculum and fermentation mediums were determined by turbidimetry using a HANNA turbidimeter. A calibration curve for the turbidity of the suspension (NTU) in terms of grams of dried cells per liter of fermentation medium at 105 °C was elaborated. Samples extracted from the fermentation medium at different times were centrifuged and the obtained cells were re-suspended in known volumes of water to determine the cell concentrations from turbidity values using the calibration curve.

3. Results and discussion

3.1. Autohydrolysis and spent liquor post-hydrolysis strategies

A scheme summarizing the complete methodology of xylitol production from sugarcane bagasse hemicelluloses at laboratory scale is shown in Fig. 1. The sugarcane bagasse was treated by hot water at two liquid-solid ratios (14:1 and 4:1). The composition of the liquors obtained at different liquid-solid ratios is shown in Table 2. Xylose and xylans were the main compounds in spent liquors, identified and quantified by HPLC (74 and 70 wt.% of compounds for diluted and concentrated liquors, respectively). Low LSR is efficient in water usage and thus in capital and operating costs. Vallejos et al. reported the removal of almost 57% of the original amount of xylans in the raw material (equivalent to 48.29 g L^{-1} of xylans) by treating bagasse at 170 °C for 60 min, with LSR of 3 g g^{-1} (Vallejos et al., 2012). However, reduction in liquid-solid ratio from 10:1 to 4:1 in the hot water pretreatment decreased the extraction of xylans in 46%.

HMF and furfural were steam-stripped during liquor concentration, whereas acetic acid and formic acid were partially removed because of the drop in boiling point under reduced pressure (about 41% and 19%, respectively). Xylose and xylans concentrations in the concentrated spent liquor were 10.3 and 45.7 g L^{-1} respectively (concentration factor, CF: 3.2).

Table 2
Composition of spent liquors (g L^{-1}) from hot water treatment of sugarcane bagasse.

Compound concentration (g L^{-1})	Dilute liquor (DL) LSR of 14:1	Concentrated liquor (CL) LSR of 4:1
Glucose	0.07	0.25
Xylose	3.27	1.34
Arabinose	0.86	1.86
Formic acid	0.26	0.27
Acetic acid	1.39	1.19
Glucans	2.58	6.63
Xylans	14.6	24.0
Arabinans	0.31	0.99
HMF	0.02	0.02
Furfural	0.70	0.05

Table 3
Composition of spent liquors (g L^{-1}) depending on H_2SO_4 concentration (post-hydrolysis) and on the pH of $Ca(OH)_2$ treatment.

Compound	H_2SO_4					pH (post-hydrolysis with 1% H_2SO_4)			
	0.5%	1.0%	2.0%	3.0%	4.0%	1.3	6	8	10
Cellobiose	0.45	1.37	0.83	0.35	0.20	1.20	1.20	1.21	1.22
Glucose	1.63	4.90	8.60	9.09	8.91	4.93	4.85	4.86	4.87
Xylose	55.9	59.3	57.7	55.5	53.4	53.0	51.9	53.1	53.0
Arabinose	3.49	3.68	3.98	4.00	3.94	3.28	3.38	3.22	3.19
Formic acid	0.80	0.69	0.71	0.76	0.79	0.43	0.34	0.24	0.22
Acetic acid	8.07	8.43	8.34	8.24	8.18	7.70	7.46	7.55	7.65
HMF	0.10	0.21	0.09	0.11	0.07	0.11	0.08	0.09	0.09
Furfural	0.51	1.11	1.95	2.81	3.45	0.98	0.74	0.71	0.64
PT	n.d.	n.d.	n.d.	n.d.	n.d.	5.58	5.38	5.09	4.58

PT: total phenolic compounds expressed as g equivalent of vanillin per liter, n.d.: not determined.

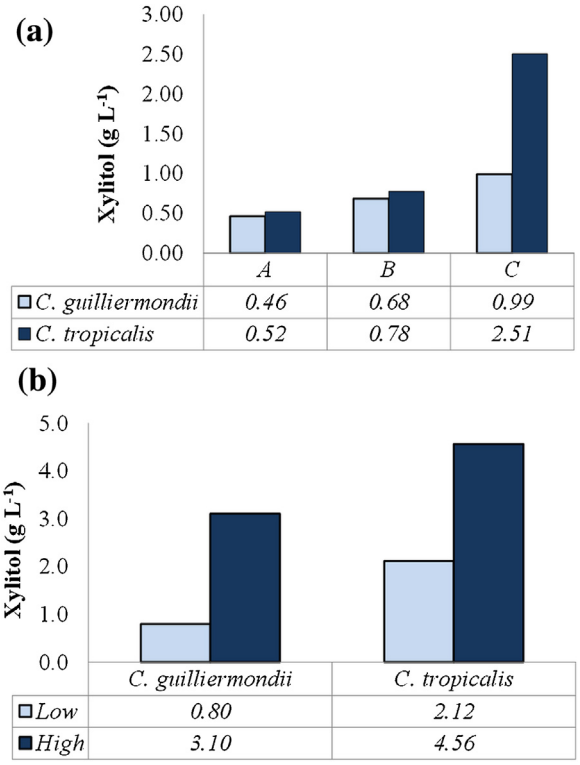

Fig. 3. Xylitol production (a) effect of culture medium and (b) effect of initial cell concentration, *C. guilliermondii:* 0.58 (low) and 3.23 g L^{-1}(high), *C. tropicalis*: 0.23 (low) and 3.89 g L^{-1}(high). Fermentation conditions: initial xylose concentration 30 g L^{-1}, medium C, 120 h.

A detailed scheme of the methodology used to define the best sequence for the conversion of xylans to xylitol (posthydrolysis, detoxification and fermentation) is shown in Fig. 2. Post-hydrolysis treatment (acid hydrolysis) was aimed to convert the *xylo*-oligosaccharides into soluble monomers (xylose, glucose, and arabinose) and dimers (cellobiose) in conditions that maximize xylose concentration by minimizing its degradation to furfural. Based on preliminary post-hydrolysis assays, the maximal xylose concentration in spent liquors (59.3 g L^{-1}) was achieved using 1 wt.% of H_2SO_4 (Table 3). Similar results were obtained by Garrote et al. for *Eucalyptus globulus* using post-hydrolysis with 1 wt.% of H_2SO_4 at 115–125 °C for 1 h (Garrote et al., 2001).

Dilute liquor composition after the sequential stages of concentration and post-hydrolysis is shown in Table 4. The losses of xylose and glucose due to dehydration to furfural and HMF were negligible (<3%).

3.2. Detoxification strategies

The first stage of liquor detoxification was the elimination of sulfuric acid. The use of $Ca(OH)_2$ has proven to decrease toxicity to microorganisms in hemicellulosic hydrolysates for ethanol and xylitol production from different lignocellulosic materials (Martinez et al., 2000; Purwadi et al., 2004). Preliminary overliming treatments with $Ca(OH)_2$ were accomplished to eliminate the H_2SO_4 and partially remove inhibitors. When liquors from Strategy 1 (concentrated and then post-hydrolyzed) were treated, the concentrations of furfural, HMF, and phenolic compounds decreased (35.0%, 18.0% and 18.5%, respectively) when pH increased from 1.3 to 10, without significant changes in xylose concentration (Table 3). These results agree with those of Martinez et al. (2000). However, when treating the spent liquor from Strategy 2 (post-hydrolyzed and then concentrated) with $Ca(OH)_2$, a great amount of gypsum precipitated because of the increase in H_2SO_4 concentration, dragging about 50% of xylose. These results show that the treatment with $Ca(OH)_2$ should be made previous to concentration.

The second stage of detoxification was the removal of calcium ions from the liquor. The use of phosphoric acid acidification resulted in $Ca_3(PO_4)_2$ precipitation, although a fraction of Ca^{++} remained soluble, associated to other acids (phosphoric, acetic, formic, others). The use of H_3PO_4 also involves high consumption of resin in the stage of acetic acid elimination, as it will be seen later. For this reason, Amberlite IRA-120 cationic resin was selected for the removal of calcium ions.

The third stage of detoxification was the treatment with activated charcoal for the removal of inhibiting compounds resulting from biomass treatment. The identification of the variables showing the greatest influence on the adsorption of inhibitors by activated charcoal, obtained by the applied experimental design, is shown in Table 5.

The increase of activated charcoal concentration produced a significant reduction of main inhibitor compounds: HMF, furfural, total phenols, *p*-coumaric acid, ferulic acid, vanillic acid, *p*-OH-benzaldehyde, vanillin, and syringaldehyde (all $p < 0.005$). The concentration of *p*-coumaric acid, vanillic acid, *p*-OH-benzaldehyde, and vanillin increased with the rise of temperature (all $p < 0.05$). The interaction between activated charcoal concentration and temperature affected negatively the concentration of *p*-coumaric acid, vanillic acid, *p*-OH-benzaldehyde, and vanillin (all $p < 0.005$), which means that concentrations of these compounds are significantly affected by temperature at low concentration of activated charcoal, but differences disappeared when treating the liquid with the high concentration of activated charcoal. None of the studied variables affected sugars or organic acids concentration.

After the treatment with 3% activated charcoal at 60 °C for 60 min the total phenol content decreased from 4.90 to 0.13 g eq. vanillin L^{-1} (97.3%). Furfural concentration was reduced from 0.64 to 0.12 g L^{-1} (81%), whereas HMF was not detected (100% removal).

Table 4
Composition of dilute liquor (DL) after sequential stages of concentration, post-hydrolysis, and precipitation ($g\,L^{-1}$).

Compound	Concentration (CF = 3.2)	Post-hydrolysis (1% H_2SO_4)	$Ca(OH)_2$ at pH 10 + H_3PO_4 at pH 5
Cellobiose	**	0.92	1.00
Glucose	0.17	4.79	5.14
Xylose	10.3	57.0	60.0
Arabinose	2.97	3.51	3.67
Formic acid	0.66	0.50	0.50
Acetic acid	2.59	8.02	8.51
Glucans	5.49	und.	und.
Xylan	45.7	und.	und.
Arabinans	0.63	und.	und.
HMF	und.	0.12	0.10
Furfural	und.	1.08	0.64
P-coumaric acid	n.d.	0.03	0.03
Ferulic acid	n.d.	0.07	0.07
Vanillic acid	n.d.	0.02	0.02
p-OH benzaldehyde	n.d.	0.12	0.13
Vanillin	n.d.	0.10	0.10
Syringaldehyde	n.d.	0.03	0.03
Total phenols*	n.d.	5.24	4.91

und.: undetected; n.d.: not determined.
* g equivalents of vanillin L^{-1}.
** Quantified as glucans.

Table 5
Composition of post-hydrolysis liquor treated with activated charcoal.

Coded variable			Fermentable sugars			Inhibitors										
			Monosacharides			Organic acids		Furans		Phenolic compounds						
X_1	X_2	X_3	G*	X*	A*	Fa*	Aa*	H*	F*	PT*	C**	Fl**	V**	p**	Vn**	S**
−1	−1	−1	5.02	59.3	3.68	0.49	8.36	0.08	0.43	1.23	4.20	9.20	8.00	27.7	10.2	2.60
−1	−1	+1	5.12	60.4	3.77	0.52	8.50	0.07	0.39	0.81	2.80	4.90	7.60	21.3	6.80	1.30
+1	−1	−1	5.06	58.5	3.70	0.54	8.13	und.	0.11	0.17	und.	und.	und.	2.00	0.40	und.
+1	−1	+1	5.02	57.7	3.64	0.53	8.03	und.	0.11	0.16	und.	und.	und.	1.30	0.50	und.
−1	+1	−1	5.15	60.0	3.73	0.53	8.45	0.08	0.39	0.93	10.4	11.2	18.2	36.0	13.3	2.30
−1	+1	+1	5.29	61.0	3.84	0.54	8.77	0.09	0.38	0.87	14.5	13.0	19.6	37.3	14.5	2.90
+1	+1	−1	5.21	59.2	3.68	0.54	8.23	und.	0.11	0.14	und.	und.	und.	2.20	0.70	und.
+1	+1	+1	5.23	59.4	3.69	0.56	8.25	und.	0.12	0.13	und.	und.	und.	1.60	0.60	und.

G: glucose, X: xylose, A: arabinose, Fa: formic acid, Aa: acetic acid, H: HMF, F: furfural, PT: phenolic compounds total expressed as g equivalent of vanillin per liter, C: *p*-cumaric acid, Fl: ferulic acid, V: vanillin acid, *p*: *p*-OH benzaldehyde, Vn: Vanillin, S: Siringaldehyde.
und.: undetected.
* $g\,L^{-1}$.
** ppm.

Acetic acid concentration was not affected by the activated charcoal treatment (Table 5), so it had to be removed by another method. The fourth stage of detoxification was then the use of Amberlite IRA-67 resin for acetic acid elimination. Preliminary assays with acetic acid solutions were performed to adjust the treatment conditions, showing that 96% of acetic acid was removed using 10–15 g IRA-67 resin in 100 mL of solution. However, when treating the spent liquor at pH 10, acetic acid was not removed by this treatment, which was attributed to the presence of calcium ions. The liquor was therefore treated with cationic resin IR-120 to remove calcium ions (pH decreased from 10 to 2.8, evidencing that H^+ ions were released as calcium was removed). The subsequent treatment with Amberlite IRA-67 resin allowed the remotion of about 80–85% of acetic acid. Other authors reported analogous results (de Mancilha and Karim, 2003; Nilvebrant et al., 2001), indicating that almost all the aliphatic acids were removed by the anion exchanger.

3.3. Xylitol production strategies

The effect of the composition of fermentation medium on xylitol production is shown in Fig. 3a. Using an initial xylose concentration of 30 $g\,L^{-1}$ and a fermentation time of 96 h, medium C allowed reaching a maximal concentration of xylitol of 0.99 $g\,L^{-1}$ for *C. guilliermondii*, and 2.51 $g\,L^{-1}$ for *C. tropicalis*. This represents an increase of 53% and 31% compared to mediums A and B respectively, when *C. guilliermondii* was used as inoculum, whereas the increase for *C. tropicalis* in both mediums was 79 and 69%, respectively. Based on these results, medium C was selected for fermentation.

From replicas of fermentation of commercial xylose to xylitol at different yeast cell concentration, the average standard deviations were: 0.925 ($g\,L^{-1}$) for yeast cells, 0.783 ($g\,L^{-1}$) for xylose consumption, and 0.663 ($g\,L^{-1}$) for xylitol production. These values were verified by fermentation of a detoxified spent liquor with 52.3 $g\,L^{-1}$ of initial xylose, conducted in duplicate (standard deviations are shown in Fig. 5b).

The effect of inoculum concentration was studied using medium C, an initial xylose concentration of 30 $g\,L^{-1}$, and a fermentation time of 120 h (Fig. 3b). At the highest level of initial cell concentration, xylitol concentrations for *C. guilliermondii* and *C. tropicalis* increased to 3.10 and 4.56 $g\,L^{-1}$ (0.026 and 0.038 $g\,L^{-1}\,h^{-1}$), respectively. All results evidenced that *C. tropicalis* behaved best in all fermentations.

The results of fermentation experiments by *C. tropicalis* of the samples of spent liquor taken at different stages of detoxification (S1–S3 in Fig. 1) and the blank trial using commercial xylose are shown in Fig. 4. Furfural and HMF were not detected in the sterilized samples, presumably removed by steam stripping during the sterilization.

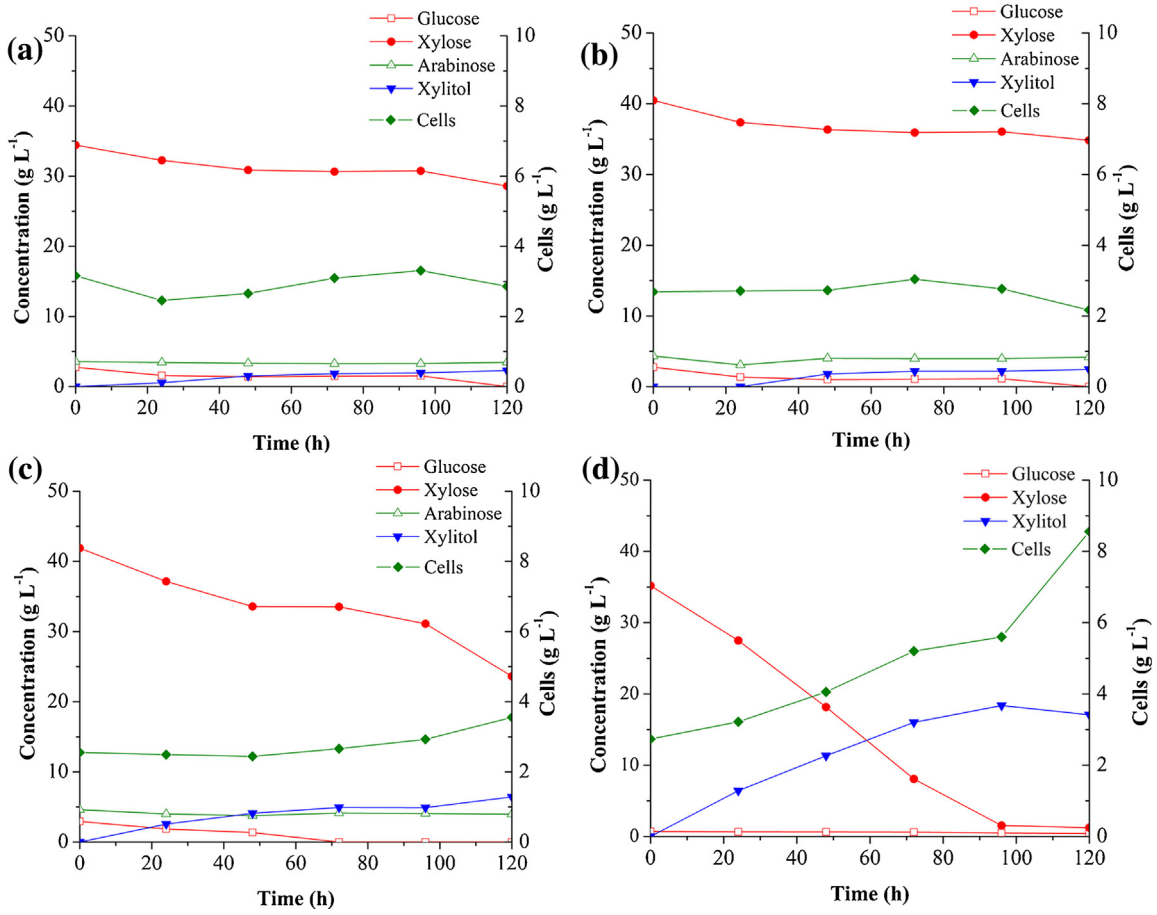

Fig. 4. Fermentation of spent liquor by *C. tropicalis* (a) undetoxified (S1, inhibitors: 6.65 g L^{-1} of acetic acid and 6.90 g L^{-1} of PT), (b) after treatment with $Ca(OH)_2$ at pH 10 (S2, inhibitors: 8.15 g L^{-1} of acetic acid and 3.08 g L^{-1} of PT) (c) after treatment with 3% activated charcoal, 120 rpm, 60 min, at 60 °C (S3, inhibitors: 7.92 g L^{-1} of acetic acid and 0.47 g L^{-1} of PT), and (d) blank trial using commercial xylose.

During fermentation of commercial xylose, 96% was consumed, of which 55% was used to produce xylitol (productivity of 0.14 g L^{-1} h^{-1}, Fig. 4d). However, a small amount of xylose was consumed during fermentation of samples S1 and S2 (17 and 14%, respectively), but only 42 and 47% of consumed xylose was respectively converted to xylitol (productivity of 0.02 g L^{-1} h^{-1}, Fig. 4a and b). In the case of sample S3 more xylose was consumed (44%) and 38% of consumed xylose was converted to xylitol (productivity of 0.05 g L^{-1} h^{-1}, Fig. 4c).

After 120 h of fermentation, cells concentration in samples S1 and S2 decreased 10 and 19% respectively, whilst it showed an increase of 39 and 213% respectively, in S3 and blank samples. These results show the strong inhibitory influence of acetic acid and phenolic compounds.

A sample of detoxified liquor rich in xylose (52.3 g L^{-1}) was used as culture medium for xylitol production using *C. tropicalis* and results were compared with the fermentation of commercial xylose as control (Fig. 5a and 5.b). In this case, both samples showed similar results: about 97.0% of consumed xylose, 35% conversion to xylitol of the consumed xylose, productivity of 0.13 g L^{-1} h^{-1}, and cell growth near 280%.

The increase of initial xylose concentration (104.1 g L^{-1}) enhanced the efficiency of fermentation (Fig. 5c). When initial xylose concentration in detoxified spent liquor increased from 52.3 to 104.1 g L^{-1}, xylose consumption decreased and conversion of xylose to xylitol increased from 34.7 to 45.8% of consumed xylose. These results are consequence of the increase in productivity (0.13 and 0.27 g L^{-1} h^{-1}, respectively).

Maximal xylitol concentration of 32.0 g L^{-1} was achieved (fermentation efficiency of 46%, productivity 0.27 g L^{-1} h^{-1}) using the following conditions: 104.1 g L^{-1} of initial xylose, 2.4 g L^{-1} of yeast cells, 30 °C, and 120 rpm.

The efficiency and productivity of fermentation of detoxified and undetoxified spent liquors from sugarcane bagasse autohydrolysis by *C. tropicalis* were lower than those reported by others authors (Li et al., 2012; Ping et al., 2013; Rao et al., 2006). Considering that detoxified spent liquor showed similar fermentation parameters than the blank trial using commercial xylose, it is possible that the problem was *C. tropicalis* yeast. As a consequence, the fermentative capacity of *C. tropicalis* cells should be optimized by adaptation and screening.

Comparison with results of xylitol production obtained by other authors is difficult since liquors may have different amounts and type of inhibitors, and microorganisms have different tolerances to them. De Albuquerque et al., 2014; reviewed diverse values for xylitol production by *C. tropicalis* yeast from industrial residues as raw material. Results have proven to be very discrepant and were related to microbial tolerances and growth conditions. The ranges of results reported for xylitol production by *C. tropicalis* were 0.49–0.83 g g^{-1} of yield and 0.43–1.01 g L^{-1} h^{-1} of productivity (De Albuquerque et al., 2014) while in our work, only a yield of 0.46 g g^{-1}, and productivity of 0.27 g L^{-1} h^{-1} were achieved. These low results can be attributed mainly to genetic characteristics of *C. tropicalis* considering that detoxified spent liquor showed similar fermentation parameters than the blank trial using commercial xylose. In future work, *C. tropicalis* cells for long-term cell recy-

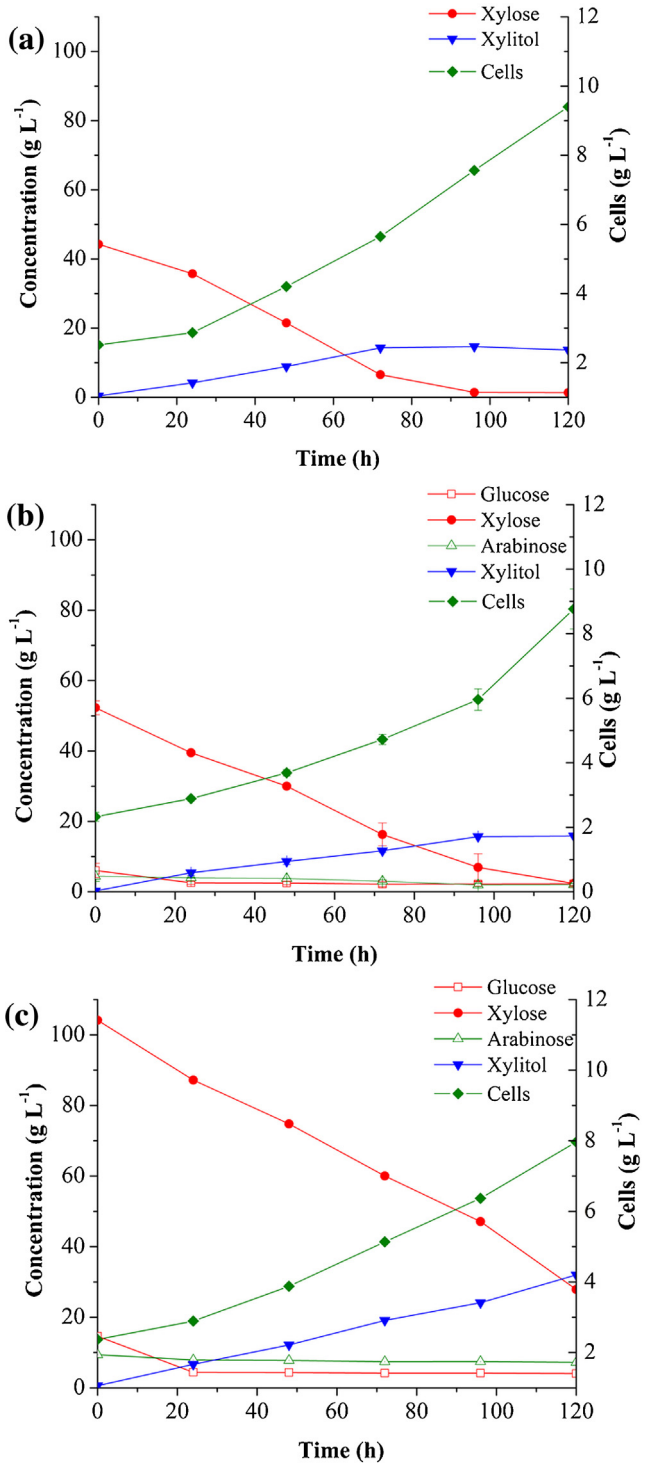

Fig. 5. Fermentation by *C. tropicalis* (a) blank trial using commercial xylose (44.2 g L^{-1} of xylose), (b) detoxified spent liquor 52.3 g L^{-1} of initial xylose (inhibitors: 0.17 g L^{-1} of PT, fermentations were conducted in duplicate), (c) detoxified spent liquor 104.1 g L^{-1} of initial xylose (inhibitors: 0.28 g L^{-1} of PT).

cle fermentations will be used as a method to improve yield and volumetric productivity.

Xylitol production from xylans of sugarcane bagasse in biorefineries is a viable strategy which can combine the production of low value commodities at large-scale (e.g. cellulosic bioethanol with a price up to USD 1.0 per liter) with small-scale production of high value products. The most typical biorefinery scenario is based on the extraction of sugars from biomass to produce cellulosic ethanol, steam and electricity. This classic scheme should be improved to achieve a more efficient use of both, lignocellulosic feedstocks and energy (Clauser et al., 2015).

4. Conclusions

An integral study of the extraction of xylose from sugarcane bagasse, its purification and its biotechnological conversion to xylitol was performed. The presented results determined the technological parameters to obtain a detoxified spent liquor rich in xylose and its bioconversion to xylitol. The detoxification through a sequence of treatments with $Ca(OH)_2$, IR-120 resin, activated charcoal and IRA-67 resin practically removed all inhibitors from the hemicellulosic spent liquors from bagasse autohydrolysis. The maximal concentration of xylitol obtained was 32.0 g L^{-1} by fermentation with *C. tropicalis* (xylose: 104.1 g L^{-1}, yield: 0.46 g g^{-1}, productivity: 0.27 g L^{-1} h^{-1}). The genetic characteristics of *C. tropicalis* should be developed to improve yield and volumetric productivity. Xylitol production from xylans obtained from sugarcane bagasse in biorefineries is a viable strategy to produce low value commodities at large-scale, as bioethanol from cellulose, together with the production of high value products, such as xylitol.

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