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Sugar cane bagasse as feedstock for second generation ethanol production. Part II: Hemicellulose hydrolysate fermentability

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Abstract Sugar cane bagasse is produced in Brazil as waste of the sugar and ethanol industries. This lignocellulosic material is a potential source for second-generation ethanol production; however a pretreatment stage is essential, which aims at removing the hemicellulose component by disorganizing the lignocellulosic complex. In this work sugar cane bagasse was pretreated by diluted acid hydrolysis resulting in xylose-rich hydrolysates, which could be fermented to ethanol by a strain of the yeast *Pichia stipitis*. Statistical approach was used to investigate the effects of factors associated with the diluted acid hydrolysis process (acid concentration, solid:liquid ratio and time of exposure) on the fermentability of different hydrolysates. The statistical analysis was useful for determining the effects of the individual factors and their interactions on the response variables. An acid concentration of 1.09% (v/v), a solid:liquid ratio of 1:2.8 (g:ml), and an exposure time of 27 min were established and validated as the optimum pretreatment conditions for ethanol production from hemicellulose hydrolysates of sugar cane bagasse. Under these conditions, a hydrolysate with 50 g/l of xylose, 6.04 g/l of acetic acid, 0.55 g/l of hydroxylmethylfurfural and 0.09 g/l of furfural was obtained and its fermentation yielded roughly 20 g/l of ethanol in 40 hrs.

Keywords: bioethanol, *Pichia stipitis,* xylose fermentation.

INTRODUCTION

The growth of population and the associated demand for fuel and goods coupled with more restrictive environmental regulations have intensified the research and development of renewable energy feedstocks to substitute for and/or to complement fossil fuel sources (Pereira Jr. et al. 2008). Lignocellulosic materials, especially agroindustrial residues, have been the subject of intense research since they are renewable sources of carbon and energy available in large amounts. Sugar cane bagasse is the main Brazilian agroindustrial residue, being produced at approximately

250 kg per ton of sugar cane (Zanin et al. 2000; Wyman et al. 2005). This lignocellulosic material is mainly composed of two polysaccharide fractions (cellulose and hemicellulose) and a polyphenolic macromolecule (lignin). Sugar cane hemicellulose is the second predominant fraction (23-30%), and it is composed of heteroxylans, with a predominance of xylose, which is configured in a chain that can be chemically hydrolyzed (Sun and Cheng, 2002; Ververis et al. 2007). Diluted acid hydrolysis pretreatment of sugar cane bagasse allows the generation of a liquid phase (hemicellulose hydrolysate) rich in xylose, which can be used as substrate (building-block) for biotechnological and chemical processes (Lavarack et al. 2002; Fogel et al. 2005; Gámez et al. 2006).

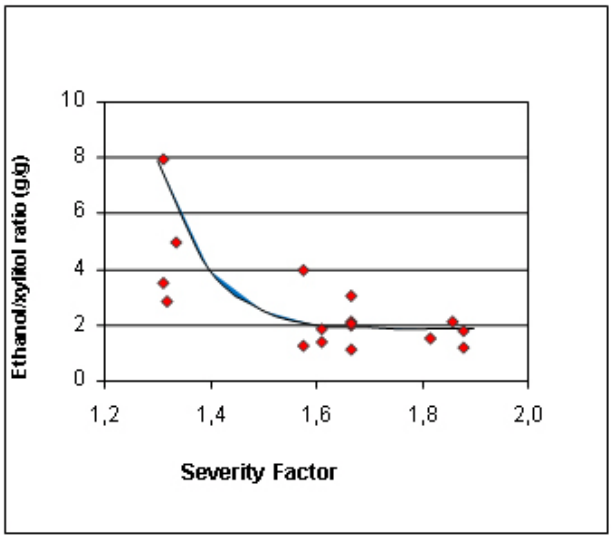

Fig. 1 The influence of the severity factor on ethanol/xylitol ratio.

The yeast *Pichia stipitis* is one of the best xylose-fermenting organisms to produce ethanol. Whereas in most bacteria the xylose metabolism proceeds via direct isomerization to xylulose, in yeasts and filamentous fungi the formation of xylulose occurs via a two-step reaction in which xylose is first reduced to xylitol by NADPH-dependent xylose reductase, followed by oxidation of xylitol to xylulose by NAD^+-dependent xylitol dehydrogenase. It is evident that under anaerobic conditions there will be an overproduction of NADH, resulting in a redox imbalance, which blocks the metabolic activity, since it can not be reoxidized in the absence of oxygen. However, in xylose-fermenting yeasts, such as cells of *Pichia stipitis,* these two first enzymes of xylose catabolism display dual coenzyme specificity. Thus, the reducing equivalents produced in the second reaction can be used for the initial step of xylose metabolism, therefore reducing the overproduction of NADH, and consequently alleviating the cell redox imbalance. Nonetheless, micro aeration conditions will be necessary, since this dual specificity does not occur in the same extent. In the next step, phosphoketolase incorporates inorganic phosphate into xylulose to produce xylulose-5-P, which is converted into glyceraldehyde 3-P and fructose 6-P in the pentose phosphate pathway. Both are converted into pyruvate through the glycolytic pathway, which gives origin to ethanol through two sequential reactions (decarboxylation and

reduction) (Toivari et al. 2001; Jin and Jeffries, 2004; Jeffries, 2006; Pereira Jr. et al. 2008).

Table 1. Levels for each factor in the experimental design.

Factor	Axial point (-α)	Low level (-1)	Central level (0)	High level (+1)	Axial point (+α)
Time of exposure (min)	27	40	60	80	93
Acid concentration (% vv)	0.50	0.75	1.13	1.50	1.75
Solid:liquid ratio (g:ml)	1:3.3	1:3.0	1:2.5	1:2.0	1:1.7

Yeast growth and sugar fermentation can be inhibited by different compounds generated during the acid hydrolysis of hemicellulose. Xylose, the main hemicellulose-derivative pentose, and hexoses may be dehydrated during acid hydrolysis into furfural and 5-hydroxymethyl furfural, respectively, which have been reported as potent inhibitors of yeast enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Modig et al. 2002; Balat et al. 2008). Also, Pampulha and Loureiro-Dias (1990) showed the action of the undissociated form of acetic acid that diffuses into yeast cells, reducing the cytoplasm pH and modify the control of glycolysis involving enolase and phosphorilating enzymes. Phowchinda et al. (1995) showed the inhibitory effect of acetic acid on the growth and fermentation activity of *Saccharomyces cerevisiae* and indicated that it had more impact on biomass synthesis than on ethanol production. Nonetheless, the simultaneous presence of these compounds has a greater inhibitory effect on yeast metabolism than do their individual presence (Palmqvist et al. 1999).

Thus, this work aimed at characterizing the individual effects of sulfuric acid concentration, solid:liquid (S:L) ratio and the time of exposure, as well as their synergic effects on the hydrolysate fermentability and its relation with the concentrations of inhibitors by a strain of the yeast *Pichia stipitis*.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The fermentation assays were carried out with the yeast *Pichia stipitis* 5774 from the Central Bureau voor Schimmelcultures (CBS) - The Netherlands. The strain was stored at 5ºC in agar slant medium containing xylose (5 g/l), yeast extract (2 g/l) and peptone (5 g/l) (Pereira Jr. and Bu'lock, 1994). Cell propagation was performed in two stages using hydrolysate obtained in pretreatment condition of 121ºC during 40 min with a solid:liquid ratio of 1:2 (g of bagasse:mL of 1% sulfuric acid solution). In the first stage, cells were transferred from the agar slant to liquid medium containing 25% hydrolysate, and in the second stage the hydrolysate content was increased to 50%.

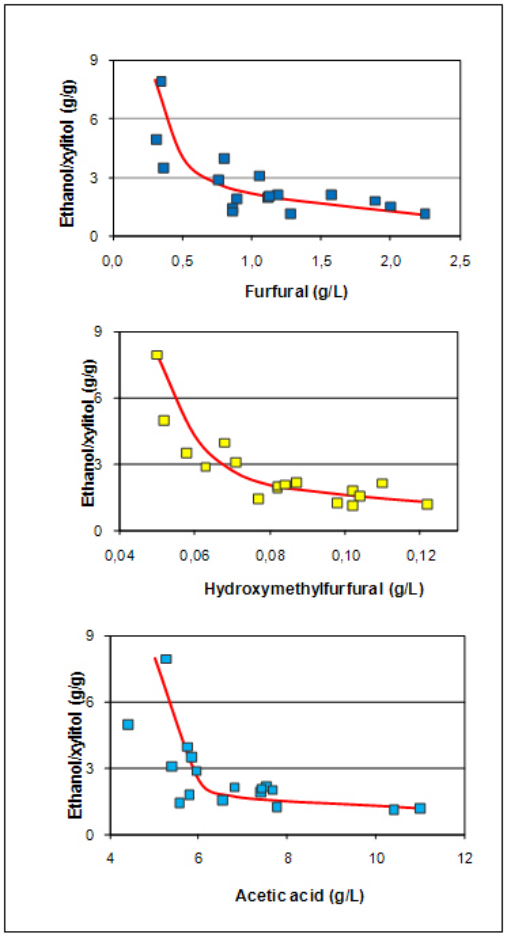

Fig. 2 Effect of inhibitors on the ethanol/xylitol ratio.

Then cells necessary for each fermentation assay was separated from media by centrifugation at 4000 rpm during 4 min. For the propagation stages, the liquid medium was supplemented with urea (2.5 g/l); KH_2PO_4 (2.2 g/l); yeast extract (4 g/l) and 40 ml/l of a solution of salts (Pereira Jr. and Bu'lock, 1994).

Fermentation assays

Hemicellulose hydrolysates were obtained by diluted acid pretreatment using a central composite design with the following factors: time of exposure, concentration of sulfuric acid and solid-liquid ratio for a total of 17 experimental runs (central point performed in triplicate). The temperature was kept constant at 121ºC (Betancur and Pereira Jr., 2010). The levels for each variable are shown in Table 1.

After acid pretreatment, the liquid phase (hemicellulose hydrolysate) was separated from the solid by a pressure filter set within the reaction system (Santa Anna et al. 2007). Then, the pH of the liquid phase was adjusted to 6 by addition of $Ca(OH)_2$, and the generated $CaSO_4$ was separated by filtration. The liquid phase, called hemicellulose hydrolysate, had its sugar and inhibitor contents determined, and was used for fermentation assays.

The fermentations were carried out in conical flasks (500 ml) containing 200 ml of hydrolysate supplemented with the same nutrients as the propagation media. The fermentation media were inoculated with an initial cell concentration of 10 g/l and performed on a rotary shaker (Excella E25-New Brunswick) at 200 rpm for 48 hrs at 30ºC with an initial pH of 6. To validate the fermentability of the hydrolysates in the best pretreatment condition, a fermentation was performed in a 2l-Biostat B fermenter (B. Braun) containing 800 ml of medium with control of agitation (250 rpm), temperature (30ºC) and air supply (0.02 vvm). The pH was maintained at 6 by the addition of 2M NaOH or 2M HCl solutions.

Table 2. Xilose content in the hydrolysate and response variables for the fermentability of hydrolysates by *P. stipitis* after 48 hrs.

Experiment	Severity Factor	Xylose (g/l)	Ethanol (g/l)	Xylitol (g/l)	$Y_{Ethanol/S}$ (g/g)	$Y_{Xylitol/S}$ (g/g)	Ethanol/ xylitol ratio (g/g)
1	1.31	56.96	17.71	5.04	0.31	0.09	3.51
2	1.61	67.53	22.46	11.91	0.34	0.18	1.89
3	1.31	51.56	18.47	2.33	0.36	0.05	7.94
4	1.61	48.64	14.85	10.47	0.30	0.21	1.42
5	1.58	71.12	19.40	15.49	0.27	0.21	1.25
6	1.88	76.25	13.73	11.72	0.18	0.15	1.17
7	1.58	51.38	18.28	4.60	0.35	0.09	3.97
8	1.88	54.31	17.48	9.71	0.32	0.18	1.80
9	1.34	44.80	18.45	3.70	0.41	0.08	4.98
10	1.81	68.63	20.88	13.64	0.30	0.19	1.53
11	1.67	82.11	16.23	14.39	0.27	0.24	1.13
12	1.67	44.87	18.69	6.09	0.41	0.13	3.07
13	1.32	60.47	20.65	7.23	0.34	0.12	2.86
14	1.86	63.04	22.55	10.61	0.35	0.16	2.13
15	1.67	61.51	18.98	8.87	0.30	0.14	2.14
16	1.67	63.91	20.31	10.16	0.31	0.16	2.00
17	1.67	59.84	20.68	9.99	0.34	0.16	2.07

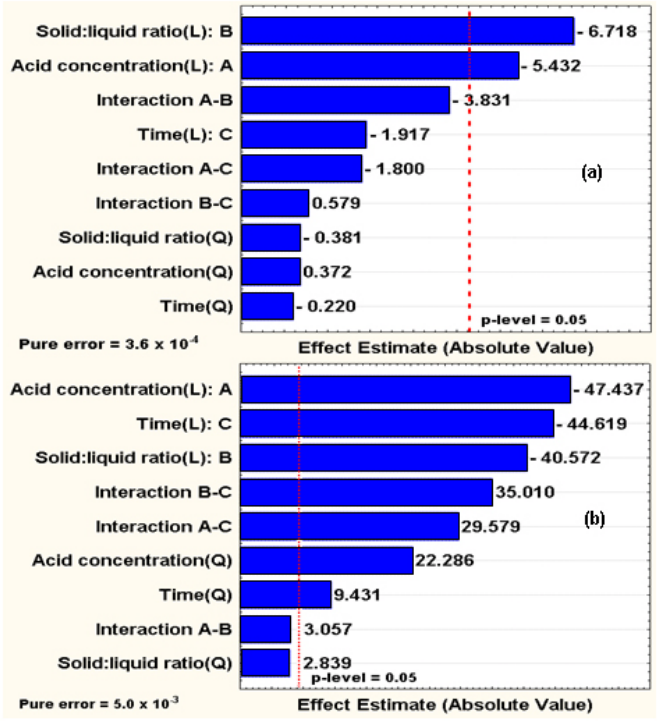

Fig. 3 Pareto charts of standardized effects for (a) ethanol yield and (b) ethanol/xylitol ratio (g/g). (L): Linear effect; (Q): Quadratic effect.

Analytical methods

Xylose, ethanol and xylitol concentrations in the hydrolysates and fermented media were determined by high performance liquid chromatography (HPLC) using an Aminex HPX-87P column (Bio-rad) maintained at 65ºC and with a differential refractive index detector (Waters). Furfural, hydroxymethylfurfural and acetic acid concentration was determined by HPLC using a KC811 column (Shodex) with a UV detector at 230 nm (Waters). The cell concentration was determined by a correlation between cell dry mass and cell suspension absorbance at 570 nm.

The hydrolysis factors were used to determinate the severity factor (Equation 1), as proposed by Schell et al. (2003), and its relation with the response variables was established. Additionally, the effects of each individual factor and its interactions were analyzed using the software STATISTICA 6.0 (StatSoft, 2002).

$$\text{Severity factor} = \text{Log}_{10}\left(\text{time}.\exp\left[\frac{\text{Temperature} - 100}{14.75}\right]\right) - \text{pH}$$

[Equation 1]

RESULTS AND DISCUSSION

The hydrolysates characteristics were discussed previously (Part I of this work). Hydrolysates fermentability was investigated by using a strain of the yeast *P. stipitis* and monitoring the final concentrations of ethanol and xylitol after 48 hrs (Table 2). Total consumption of xylose was achieved for the majority of the experiments, except for those coded as 2, 3, 6, 11 and 15, corresponding to high severity conditions and with residual xylose concentrations below 5 g/l.

The final ethanol concentration varied between 13.73 g/l and 22.55 g/l with xylitol production as an intermediate metabolite in concentrations ranging from 2.33 g/l to 15.49 g/l. The highest ethanol concentrations (experiments 2, 10, 13, 14, 16 and 17) were not coincident with the highest xylose content in the hydrolysates (experiments 5, 6 and 11). Xylitol synthesis increased as xylose content increased in the hydrolysate, especially in experiments 5 and 11. This result confirms that hydrolysis under severe conditions (1.7), as used in experiment 11, leads to high xylose content in the hydrolysate, yet no efficient ethanol production is achieved during the fermentation. Additionally, the two best ethanol/xylitol ratios (7.94 g/g and 4.98 g/g) were obtained from pretreatments carried out under moderate severity conditions (1.31 and 1.34 for experiments 3 and 9, respectively).

Table 3. Predicted process conditions, using empirical models, for process optimization and their experimental validation.

Pretreatment conditions	**Predicted conditions**	
Acid concentration (% vv)	1.09	
S:L ratio (g:ml)	1:2.8	
Time of exposition (min)	27	
Response variables for the fermentation process	**Predicted values**	**Experimental values**
Ethanol concentration (g/l)	20.58 ± 0.80	19.05
Xylitol concentration (g/l)	2.77 ± 0.49	2.86

No correlation was found between the severity factor and ethanol concentration or yield. In contrast, the ethanol/xylitol ratio decreased dramatically when the severity factor increased (Figure 1). It is well known that xylitol is a fungal-compatible solute (Davis et al. 2000), and its accumulation during fermentation is considered to be a cellular response to the medium's adversities, be it by the redox imbalance that can be caused under oxygen restriction, high substrate concentration or the presence of inhibitors that may be generated during the pretreatment stage, particularly if high severity conditions are imposed.

Figure 2 shows the effect of inhibitors on the yeast metabolic product distribution during fermentation. The ethanol/xylitol ratio decreases when the concentration of inhibitors (furfural, hydroxymethylfurfural and acetic acid) increases. Initially, the ethanol/xylitol ratio decreases sharply when the concentration of furfural increases from 0.4 g/l to 0.8 g/l, and then it continues to diminish, but more slowly. The same performance was observed for hydroxymethylfurfural and acetic acid, with sharp declines for concentrations up to 0.07 g/l and 6 g/l, respectively. Both furans and acetic acid are reported to be inhibitors of *Pichia stipitis* yeast (Modig et al. 2002; Balat et al. 2008). The increase in xylitol yield as the severity factor rises can be

ascribed to the increasing concentration of inhibitors during acid pretreatment that are generated in harsher conditions. It seems that xylitol, apart from being a compatible solute, has other protective functions for the cell.

The linear effect of the S:L ratio and the acid concentration were the most important factors determining ethanol yield (Figure 3a) and the only ones with statistical significance ($p < 0.05$). All factors during the acid pretreatment showed statistically significant effects on the ethanol/xylitol ratio, except for the quadratic effect of the S:L ratio and its interaction with the acid concentration (Figure 3b). The negative values of the effects indicate that high ethanol/xylitol ratios, ideal for the ethanol fermentation of hemicellulose hydrolysates, require short exposure times and low acid concentrations and S:L ratios, within the range evaluated. However, these conditions lead to the generation of hydrolysates with low xylose content and consequently low ethanol concentration in the fermentation process, which is not technologically sound. Figure 3b also shows that the linear effect of time of exposure is negative, which means that as exposure time gets shorter the ethanol/xylitol ratio gets higher, within the range evaluated.

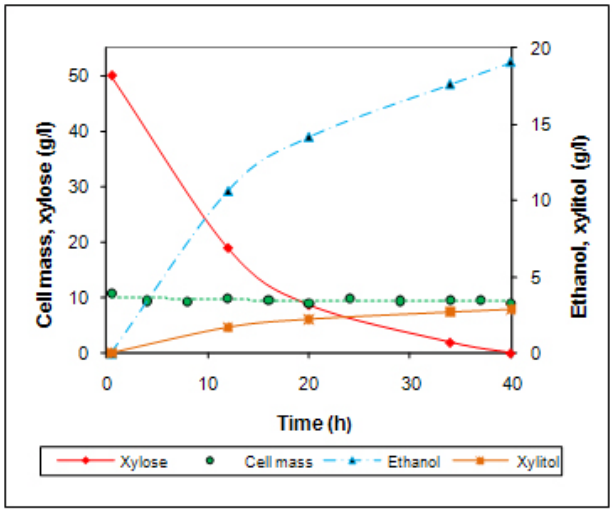

Fig. 4 Fermentation performance of hemicellulosic hydrolysate obtained from optimized conditions.

Empirical models for each response variable (Equation 2) were obtained and used to determine the conditions for achieving the best performance of the pretreatment hydrolysis and the fermentation process. The final ethanol concentration model was not statistically significance, which resulted in a low coefficient of determination (R^2 = 0.47). On the other hand, the empirical model for xylitol concentration had a very good coefficient of determination (R^2 = 0.92). In the case of the ethanol and xylitol yields during the fermentation process, the empirical models had coefficients of determination of 0.73 and 0.88, respectively, corroborating the greater mathematical model fit for xylitol than for ethanol yield. For the ethanol/xylitol ratio, the empirical model showed statistical significance and a good coefficient of determination (R^2 = 0.88).

Response variable = $\beta_o + \beta_1$ (Acid concentration) + β_{11} (Acid concentration)^2
+ β_2 (Solid:liquid ratio) + β_{22} (Solid:liquid ratio)^2
+ β_3 (*time*) + β_{33} (*time*)^2
+ β_{12} (Acid concentration)(Solid:liquid ratio)
+ β_{13} (Acid concentration)(time)
+ β_{23} (Solid:liquid ratio)(time)

[Equation 2]

β: Empirical Coefficient

The pretreatment conditions yielding the best process performance were determined using the desirability function (Equation 3) (StatSoft, 2002) under optimization criteria to maximize the xylose concentration, ethanol concentration and ethanol/xylitol ratio and to minimize the xylitol concentration and yield. The experimental results of the optimized conditions (Table 3) were within the predicted confidence intervals for the xylitol and ethanolconcentrations, even without statistical significance for the ethanol concentration model.

$D = (d_1(Y_1) \times d_2(Y_2) \times ... \times d_k(Y_k))^{1/K}$ **[Equation 3]**

D: overall desirability
Y_i: response variable
$d_i(Yi) = 0$ for undesirable value and increase to$d_i(Yi) = 1$ for desirable value
k: number of response variables

Figure 4 shows the hydrolysate fermentation carried out under the pretreatment predicted conditions. The process began with fast xylose uptake and ethanol production. Both rates diminished after 15 hrs and total xylose consumption was achieved at the end of 40 hrs cell growth was not observed, which may be due to the hydrolysate composition and fermentation conditions, especially the oxygen supply, which was low enough (Q_V=0.02 vvm) to assure ethanol fermentation in detriment to cell growth (Jeffries et al. 2007).

CONCLUDING REMARKS

The severity factor showed inversely proportional effect on ethanol/xylitol ratio, whilst no tendency was observed with the ethanol concentration. Furfural, hydroxymethylfurfural, and acetic acid concentrations have strong influence on the metabolic distribution products during the hydrolysate fermentation. The use of statistical analysis allowed the individual effect of every acid pretreatment factor on hydrolysates fermentability to be determined. Also, the conditions dictated by the statistical analysis were validated experimentally with good agreement with the predictions. In the experimental validation a hydrolysate with 50 g/l of xylose, 6.04 g/l of acetic acid, 0.55 g/l of hydroxylmethylfurfural and 0.09 g/l of furfural was obtained and final ethanol concentration of 19.1 g/l was achieved during fermentation.

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