

COMPARISON OF BIOETHANOL PRODUCTION FROM ACID
HYDROLYZATES OF WASTE OFFICE PAPER USING *SACCHAROMYCES*
CEREVISIAE AND *SPATHASPORA PASSALIDARUM*

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The aim of this work was to evaluate waste office paper as raw material for bioethanol production using four strains of *Saccharomyces cerevisiae* and *Spathasporapassalidarum* HMD 14.2. Waste paper was hydrolyzed with 1-5% V/V sulfuric acid to 2-10% w/V biomass load for 60-120 min. The most significant variable for the total reduced sugar (TRS) was the biomass load, followed by the acid concentration. The pretreatment time did not exert any significant effect on TRS. The hydrolysate obtained with 5% V/V sulfuric acid, 10% w/V biomass load and 1 hour, containing 8.45 g/L glucose and 9.27 g/L xylose, was chosen for the fermentations. The fermentation with *S. passalidarum* resulted in higher ethanol formation (3.54 g/L) than the fermentation with *S. cerevisiae*, which corresponds to a hypothetical yield of 0.708 g/g glucose. This indicates that *S. passalidarum* produces ethanol not only from glucose, but also from xylose.

Keywords: waste office paper, acid hydrolyzate, ethanol, *Spathasporapassalidarum*, *Saccharomyces cerevisiae*

INTRODUCTION

Compared with traditional biofuels, second generation (2G) biofuels have various benefits, including the use of feedstocks that do not compete with food, the consumption of waste residues, the exploitation of abandoned and/or low productive lands, and the higher reduction of greenhouse gas emission.¹ The production of 2G bioethanol is based on raw materials, such as forest, agricultural and urban residues. Waste paper contained in municipal solid waste is particularly attractive for producing 2G bioethanol, since it is rich in carbohydrates and it is readily available.² Furthermore, the conversion of waste paper into ethanol may offer a useful and valuable alternative route, in addition to/as a complement to recycling and managing that residue.³

The technology for lignocellulosic ethanol production relies mainly on pre-treatment, hydrolysis, fermentation and product separation or

distillation.⁴ However, the cellulose of waste paper is somehow difficult to hydrolyze enzymatically because it is associated with hemicelluloses and lignin, which form barriers that impede enzyme access.² Although dilute-acid hydrolysis is hampered by non-selectivity and by-product formation, it is a fast and easy way to pretreat lignocellulosic materials.⁵

The hydrolysis of cellulose contained in the lignocellulosic materials results in formation of glucose, whereas hemicelluloses yield different sugars, including major amounts of pentoses (xylose and arabinose). *Saccharomyces cerevisiae*, the ethanologenic organism traditionally used in first generation ethanol production,⁶ can ferment glucose, but it cannot ferment pentoses unless it has been genetically modified.⁷

It is a robust microorganism, yielding high ethanol content and productivity, being tolerant to

ethanol⁸ and to the lignocellulose-derived inhibitors.⁹⁻¹⁰

If the fermentation of xylose is not intended or if xylose concentration is low, *S. cerevisiae* is the microorganism of choice for fermenting lignocellulosic hydrolysates. However, according to Galbe & Zacchi,¹¹ the full use of all types of sugars resulting from the hydrolysis of cellulose and hemicelluloses is one of the prerequisites to render lignocellulosic ethanol processes economically competitive. Considering this, recent efforts have been directed towards the development of microorganisms able to ferment pentoses into ethanol.¹²

Recently, an unusual native xylose-fermenting yeast, *Spathasporapassalidarum*, was isolated from the midget of a passalid beetle that preferentially inhabits white-rotted hardwoods.¹³ Further studies with that yeast have revealed that it can simultaneously co-ferment glucose, cellobiose, and xylose with high ethanol yield and productivity.¹⁴ This ability makes *S. passalidarum* an organism of high interest for ethanol production from lignocellulosic materials.

Although office paper has a relatively low content of xylan,³ we were interested in researching the use of *S. passalidarum* as an efficient means of fermenting hydrolysates of that material. The current work aims to compare bioethanol production from dilute acid-pretreated waste office paper using four industrial strains of *S. cerevisiae* and one strain of *S. passalidarum*.

EXPERIMENTAL

Preparation of acid hydrolysates

Waste office paper collected at the Department of Antibiotics from the Federal University of Pernambuco, Brazil, was used. The paper was cut into pieces of approximately 2 cm. The material was mixed with 100 mL sulfuric acid solution in 500-mL Erlenmeyer flasks. The flasks were placed in an autoclave, where hydrolysis was carried out at 121°C under different acid concentrations, reaction times and liquid-to-solid ratios. The hydrolysis conditions were varied in an experiment including a 2³ factorial design and three replicates at the central point (Table 1). The acid hydrolysates were separated by filtration, analyzed for sugar concentration and supplemented with nutrients. The pH was adjusted to 4.5 with NaOH at room temperature. The hydrolysates were enriched with yeast extract (4 g/L), (NH₄)₂SO₄ (2 g/L), KH₂PO₄ (2 g/L) and MgSO₄·7H₂O (0.75 g/L).

Microorganisms

Four industrial strains of *S. cerevisiae* (UFPEDA 1238, UFPEDA 1326, UFPEDA 1337 and UFPEDA

1324), kindly provided from the culture collection of the Department of Antibiotics of the Federal University of Pernambuco, Brazil, were used. The strain of *S. passalidarum* (HMD 14.2) was provided by the Department of Microbiology, Institute of Biological Sciences of the Federal University of Minas Gerais, Brazil. The strains of *S. cerevisiae* were maintained in a solid medium containing (in g/L) glucose (20), yeast extract (4), peptone (3) and agar (15), at pH 7.0, and the *S. passalidarum* was maintained in a solid medium containing (in g/L) glucose (20), yeast extract (10), peptone (20) and agar (15), at pH 7.0.

Fermentation

For preparation of precultures of all the strains, a loopful of cells of each strain was transferred from the agar slants to 500-mL flasks containing 100 mL of culture medium, and incubated at 30°C for 12 h. Cells were harvested by filtration (0.45 µm filter), suspended in sterilized water and used to inoculate the fermentation medium containing: (NH₄)₂SO₄ 2 g/L; KH₂PO₄ 2 g/L; MgSO₄·7H₂O 0.75 g/L; yeast extract 4 g/L. Triplicate fermentations were carried out at 34°C, 80 rpm for 24 h using 0.3 to 0.5 g/L of initial biomass concentration in 250-mL flasks with working volumes of 100 mL. Samples were withdrawn, filtered (0.45 µm filter), and submitted to HPLC analysis.

Analytical methods

Glucose, xylose, carboxylic acids, glycerol, ethanol and furan aldehydes were quantified by HPLC (Agilent HP 1100, Germany) in an Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) column at 60°C, using 5 mM H₂SO₄ at a flow rate of 0.6 mL/min as the mobile phase, and detected using an RI-detector (Agilent).¹⁵ The content of total reducing sugars (TRS) in the acid hydrolysates was measured using the 3,5-dinitrosalicylic acid (DNS) method.¹⁶

Sugar yield and conversion

The sugar yield (Y) from waste office paper and the conversion of cellulose and hemicelluloses in total reducing sugar (C) were calculated using Eqs. 1 and 2, respectively:

$$Y (\%) = \frac{TRS \cdot V}{m} * 100 \quad (1)$$

$$C (\%) = \frac{TRS \cdot f}{C_{paper} * CH} * 100$$

(2)

where: TRS (g/L) – concentration of total reducing sugars; V (L) – volume of reaction mixture; m (g – mass of paper; f – conversion factor (cellulose and hemicelluloses to TRS); C_{paper} (g/L) – concentration of paper; CH (%) – content of cellulose and hemicelluloses in paper.

Statistical analysis

All the experiments were performed in triplicates, and the mean values of the results and the standard deviations were calculated. The Statistica 7.0 software package was used to calculate the effects of three factors on the responses (TRS and Y). The analysis of variance was performed utilizing the Origin 6.0. Statistica 7.0 was used to analyze the experimental results, which were fit to a polynomial linear equation to correlate the process response of the three factors. The general form of the polynomial linear equation is shown in Eq.3:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3$$

where: y —estimated TRS or sugar yields; x_i —factors; b_0 , b_1 , b_2 , b_3 , b_{12} , b_{13} , b_{23} and b_{123} —regression coefficients.

RESULTS AND DISCUSSION

The complete experimental matrix is shown in Table 1, along with the concentrations of total reducing sugars (TRS) and the yield of sugars from initial dry raw material (Y). Depending on the experimental conditions, there was a wide range of sugars released as a result of the dilute acid hydrolysis of the polysaccharides contained in waste paper. The highest concentration of total reduced sugar (TRS) (28.4 g/L) was achieved in the hydrolysis carried out under the harshest conditions and with the highest biomass load (experiment 8), whereas the lowest concentration (2.6 g/L) was detected under the mildest

conditions and with the lowest biomass load (experiment 1). Sugar formation increased as the biomass load increased. In the hydrolyses performed with a 2% biomass load, sugar concentration ranged between 2.6 and 6.6 g/L. In hydrolyses performed with 10 g, concentrations above 24 g/L were achieved; and in those with 6 g, the sugar concentration was around 14.5 g/L.

These results suggest that biomass load and sulfuric acid concentration exerted a more significant effect on the sugar formation than that exerted by the hydrolysis time. This is evident when comparing the pairs of experimental runs performed with different acid concentrations but at the same hydrolysis time and with the same biomass load. A comparison of hydrolyses 5 and 6 shows this clearly. In experiment 6, sugar formation was achieved with an H₂SO₄ concentration of 5%. This was more than three times higher than in experiment 5, which was performed with a 1% (V/V) concentration of H₂SO₄. The statistical processing of the results confirms this, showing that the biomass load (X₃), the sulfuric acid concentration (X₁), and their interaction (X₁X₃) significantly influenced the TRS concentration (Figure 1A). The concentration of solids was the experimental factor exerting the most significant effect.

Table 1
Experimental conditions used in acid hydrolysis, concentration of reducing sugars in the hydrolyzates and achieved sugar yields

Experiment	Experimental conditions			Sugar* concentration (g/L)	Yield (%)
	H ₂ SO ₄ (%)	Time (min)	Biomass load (%)		
1	1	60	2	2.6	13.2
2	5	60	2	5.9	29.6
3	1	120	2	4.2	21.1
4	5	120	2	6.6	33.1
5	1	60	10	6.8	6.7
6	5	60	10	24.5	24.5
7	1	120	10	7.8	7.8
8	5	120	10	28.4	28.4
9**	3	90	6	14.6	24.2
10**	3	90	6	14.6	24.3
11**	3	90	6	14.3	23.9

*Total reducing sugar; **Experiments at the center point (9, 10 and 11) were carried out in replicate to estimate the experimental error

A different pattern was observed for the second response factor, the sugar yield, whose highest values were obtained at low biomass loads. In the hydrolysis performed with the highest sulfuric acid concentration and the longest

reaction time, but at the lowest biomass load (experiment 4), the sugars formed represented 33.1% of the initial dry mass of the raw material (Table 1), which corresponds to the highest yield. The second highest sugar yield (29.6%) was also

obtained at the lowest biomass load (experiment 2). Indeed, the data confirmed that the most significant variable for the sugar yield was the acid concentration, followed by the biomass load (Figure 1B). The pretreatment time did not exert any significant effect on either TRS or yield.

Taking into account that the waste paper used in the experiments contained 56% glucan and 14% xylan, the hydrolytic conversion can be calculated (Equation 2). The highest conversion of the polysaccharides (72.22%) was obtained in the hydrolysis performed with 5% H₂SO₄, 8% w/V biomass load and 120 min (experimental run 8). A relatively high conversion was also obtained in experimental run 6 (62.3%). The calculation of the hydrolytic conversion of each polysaccharide indicates that the hydrolysis of cellulose occurred to a lower extent than that of xylan. For instance in experimental run 8, which produced the highest

sugar concentration, xylan was completely hydrolyzed, whereas only 21.8% of the cellulose was recovered as glucose in the hydrolyzate.

Fitted linear models (Equation 3) were obtained for both of the responses (Table 2). The values in bold relate to the significant effects. The R² values for the two models are 0.9590 and 0.9619, indicating that the three factors accounted for over 95% of the variation in the extent of TRS or sugar yield. This indicates that the fitted model is adequate. The use of graphical representations of the empirical models to facilitate interpretation of factor effects on TRS and sugar yield is discussed below. The three dimensional response surface plots are obtained by plotting the response (TRS or sugar yield) on the Z-axis against any two factors, while keeping the other two factors at their '0' coded levels (Figures 2A and B).

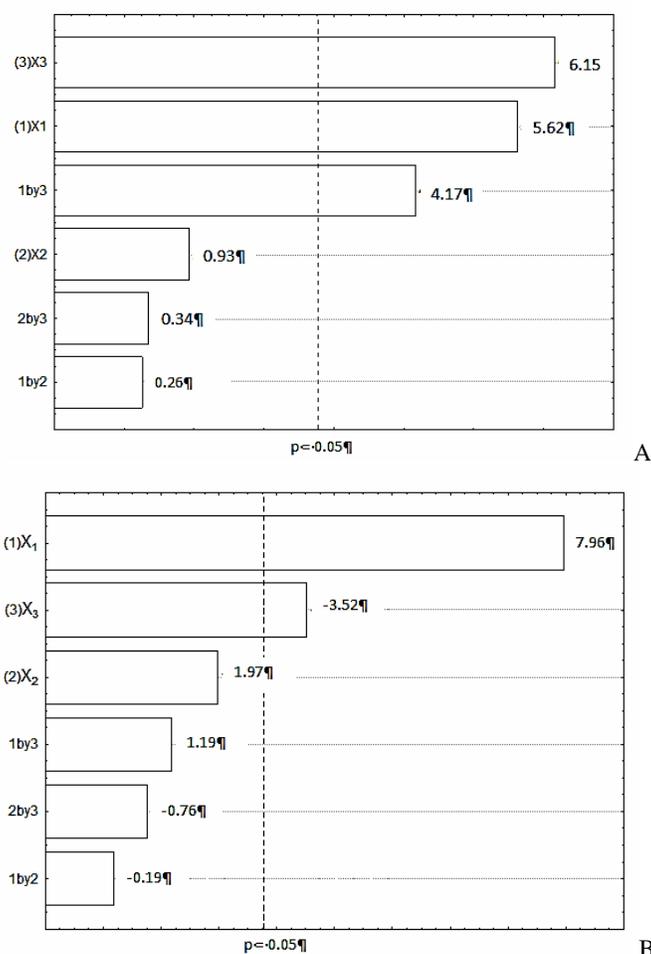


Figure 1: Analysis of significance of independent factors presented as standardized Pareto charts for concentration of total reducing sugars (A) and sugar yield (B) in the acid hydrolysis of waste office paper

Table 2
Fitted values of model coefficients

Coefficient	TRS	Sugar yield
b_0	11.85	10.77
b_1	5.49	8.34
b_2	0.90	2.06
b_3	6.02	-3.69
b_{12}	0.25	-0.20
b_{13}	4.07	1.24
b_{23}	0.33	-0.80
b_{123}	0.47	0.89

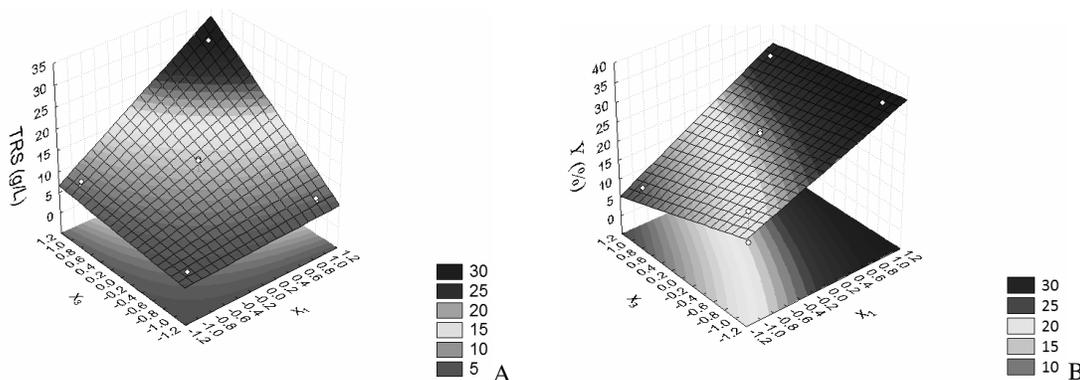


Figure 2: Response surface for the dependence of TRS concentration (A) and sugar yield (B) on biomass load and H_2SO_4 concentration during acid hydrolysis of waste office paper. The hydrolysis time was kept constant at the central point

As shown in the response surface diagram, the increase of the acid concentration (X_1) and of the biomass load (X_3) led to an increase of the concentration of total reducing sugars (Figure 2A). On the other hand, with the increase of the acid concentration, the yield also increased independently of the biomass load (Figure 2B).

These findings are in close agreement with the results reported by Dubey *et al.*,⁴ who found a maximum recovery of reducing sugars from waste paper using 0.5 N H_2SO_4 at 120°C for 2 h reaction time at a biomass:acid ratio of 1:10 (w/v). However, their highest sugar concentration (12.4 g/L) is lower than our values.

Since the hydrolyzate obtained by hydrolysis with 5% V/V sulfuric acid, at 10% w/V biomass load and for 1 hour contained higher sugar concentration, this was chosen for the fermentation experiments with the five yeast strains. That hydrolyzate contained 8.45 g/L glucose, 9.27 g/L xylose and 0.26 g/L furfural, whereas organic acids and hydroxymethylfurfural were not detected.

The initial concentration of total reducing sugars was about 5 g/L glucose and 8 g/L xylose in all fermentations, due to dilution of the

hydrolyzate while adjusting the pH with NaOH 5 M and to addition to the inoculum. The sugar consumption profile during fermentation revealed major differences between *S. cerevisiae*, which consumed only glucose and *S. passalidarum* HMD 14.2, which depleted both glucose and xylose. This confirms previous information about sugar utilization by these two yeasts. It is well known that baker's yeast does not consume xylose⁷ and that *S. Passalidarum* can simultaneously coferment glucose, cellobiose, and xylose with high ethanol yield and productivity.¹⁴ Xylose consumption by *S. passalidarum* was relatively low at the beginning of fermentation, and it remained around 43% of the initial content during the first 12 h, but when the glucose was depleted, xylose utilization accelerated and was completely consumed after 24 h.

The ethanol concentrations achieved in the fermentation with the five yeasts are shown in Figure 3. The fermentation with *S. passalidarum* resulted in higher ethanol formation (3.54 g/L ethanol) than the fermentation with *S. cerevisiae*. In general, these results are higher (345 to 796%) than those achieved by Dawson & Boopathy¹⁷ in fermentation with *S. cerevisiae* of acid

hydrolyzates of sugarcane bagasse (0.395 g/L) using hydrolysis processes relatively close to

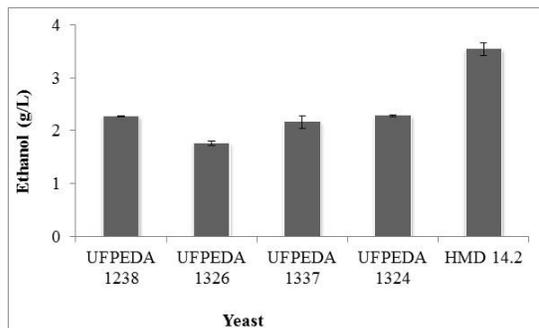


Figure 3: Ethanol concentration in the fermentations of waste office paper with the five yeasts

The fermentation with *S. passalidarum* resulted in higher ethanol formation than the fermentation with *S. cerevisiae*. That value corresponds to a hypothetical yield of 0.708 g/g glucose (Figure 5), which is higher than the maximal theoretical yield (0.511 g/g) expected from glucose. This indicates that *S. passalidarum* produces ethanol not only from glucose, but also from xylose, whereas *S. cerevisiae* produces it only from glucose, which is a consequence of the above-discussed sugar consumption pattern. This confirms previous reports on the ethanol-producing ability of *S. passalidarum*.¹⁴ However, the yields of ethanol from glucose for the five strains of *S. cerevisiae* varied from 0.31 to 0.39 g/g (Figure 4). It has previously been shown that *S. cerevisiae* UFPEDA 1337 and UFPEDA 1238 are rather resistant to contamination and stresses found in the industry.¹⁸⁻¹⁹ In fermentations of enzymatic hydrolysate from sugar cane bagasse with *S. cerevisiae* UFPEDA 1238, a 0.39 g/g ethanol yield from glucose was obtained, when the sugar cane bagasse was delignified.¹⁹ The ethanol yields from TRS for all yeasts varied between 0.24 and 0.31 g/g. The lower yield in ethanol from TRS for *S. passalidarum* (0.31 g/g) in relation to the ethanol yield from glucose indicates that although *S. passalidarum* produced ethanol from glucose and xylose, the values achieved are far from the optimal. This could be a consequence of the partial diversion of xylose towards the formation of other products, such as xylitol.²⁰ Xylitol is an intermediary product of xylose metabolism, and it is known that for some xylose-utilising yeasts, as for instance *Pichia stipitis*, xylitol can be accumulated depending on

those employed in the present work.

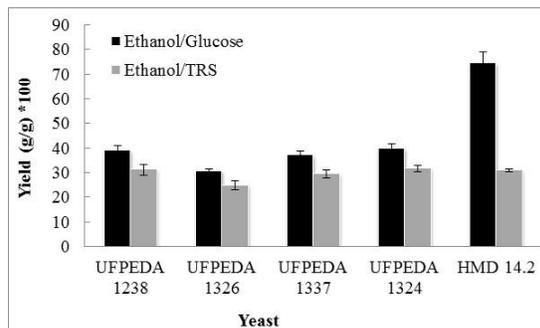


Figure 4: Ethanol yields from glucose and TRS in the fermentations of waste office paper with the five yeasts

the aeration conditions.²¹ Anyway, it should be noted that these results are comparable to those obtained in the fermentation of an acid hydrolysate of waste paper with another xylose-utilizing yeast, *Pichia stipitis*.⁴

The analysis of variance showed that the ethanol yield from glucose for all yeasts is statistically different at 95% confidence level ($F = 93.22$). Likewise, ethanol yield from glucose among the strains of *S. cerevisiae* is statistically different ($F = 13.76$; $\alpha = 0.05$). On the other hand, the ethanol yield from glucose for UFPEDA 1238, UFPEDA 1337 and 1324 is not significantly different at the same confidence level ($F = 1.22$). In relation to the ethanol yield from TRS, the values were significantly different for all the strains ($F = 6.17$). However, the ethanol yields from TRS for three strains of *S. cerevisiae* (UFPEDA 1238, UFPEDA 1337, UFPEDA 1324) and *S. passalidarum* HMD 14.2 were not significantly different ($F = 0.77$).

CONCLUSION

This study showed that relatively high concentrations of sugars can be obtained by the hydrolysis of 10 g of waste office paper using 5% V/v sulfuric acid during 60-120 minutes. The statistical analysis of the results revealed that the reaction time did not exert any significant effect for either sugar concentration or sugar yield. The fermentation of the hydrolysates with different yeast strains confirmed that *S. passalidarum* is able to produce ethanol from xylose. However, the relatively low ethanol yield from total sugars indicated that a part of xylose is diverted towards the formation of other products.

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