Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 23, No. 01, pp. 9 - 21, January - March, 2006

EVALUATION OF THE ACTIVATED CHARCOALS AND ADSORPTION CONDITIONS USED IN THE TREATMENT OF SUGARCANE BAGASSE HYDROLYSATE FOR XYLITOL PRODUCTION

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(Received: May 31, 2004 ; Accepted: August 20, 2005)

Abstract - Xylitol has sweetening, anticariogenic and clinical properties that have attracted the attention of the food and pharmaceutical industries. The conversion of sugars from lignocellulosic biomass into xylitol by D-xylose-fermenting yeast represents an alternative to the chemical process for producing this polyol. A good source of D-xylose is sugarcane bagasse, which can be hydrolyzed with dilute acid. However, acetic acid, which is toxic to the yeast, also appears in the hydrolysate, inhibiting microbe metabolism. Xylitol production depends on the initial D-xylose concentration, which can be increased by concentrating the hydrolysate by vacuum evaporation. However, with this procedure the amount of acetic acid is also increased, aggravating the problem of cell inhibition. Hydrolysate treatment with powdered activated charcoal is used to remove or decrease the concentration of this inhibitor, improving xylitol productivity as a consequence. Our work was an attempt to improve the fermentation of *Candida guilliermondii* yeast in sugarcane bagasse hydrolysate by treating the medium with seven types of commercial powdered activated charcoals (Synth, Carbon Delta A, Carbon Delta G, Carbon 117, Carbon 118L, Carbon 147 and Carvorite), each with its own unique physicochemical properties. Various adsorption conditions were established for the variables temperature, contact time, shaking, pH and charcoal concentration. The experiments were based on multivariate statistical concepts, with the application of fractional factorial design techniques to identify the variables that are important in the process. Subsequently, the levels of these variables were quantified by overlaying the level curves, which permitted the establishment of the best adsorption conditions for attaining high levels of xylitol volumetric productivity and D-xylose-to-xylitol conversion. This procedure consisted in increasing the original pH of the hydrolysate to 7.0 with CaO and reducing it to 5.5 with H_3PO_4. Next, the hydrolysate was treated under adsorption conditions employing CDA powdered activated charcoal (1%) for 30 min at 60ºC, 100 rpm and pH 2.5. The optimized xylitol volumetric productivity (0.50 g/L h) corresponded to a D-xylose-to-xylitol conversion of 0.66 g/g.
Keywords: Xylitol; sugarcane bagasse; Powdered activated charcoal; Hemicellulosic hydrolysate; Factorial design; Overlaying plot.

INTRODUCTION

Xylitol, a alcohol very sweet, is an active anticariogenic agent suitable for diabetics and patients needing parental nutrition (Ylikarhri, 1979). Owing to its physicochemical and technological properties, xylitol can be used as an ingredient in different food products, alone or in combination with other sugars (Hyvönen *et al.*,1982). Its property of prevention of diseases such as otitis and osteoporosis

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is one of the most recent findings concerning the clinical applications of xylitol (Mattila *et al.*, 1998; Uhari *et al.*, 2001). Xylitol can be produced by chemical reduction of D-xylose, but this method involves expensive purification steps that can be avoided by using fermentation (Melaja & Hamalainen, 1997). More profitable processes are based on acid hydrolysis of the hemicellulosic fraction of some lignocellulosic agro-industrial by-products, which are selectively converted into D-xylose solutions for use as culture media in bioconversion (Felipe *et al.*, 1997; Sene *et al.*, 2000; Alves *et al.*, 2002).

One of the most abundant lignocellulosic agro-industrial by-products in Brazil is bagasse, a fibrous residue resulting from the crushing of sugarcane stalks to extract their juice. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues, including sugarcane bagasse, which consists of approximately 50% cellulose, 25% hemicellulose and 25% lignin. The chemical constituents of bagasse are α-cellulose (about 50%), pentosans (30%) and ash (2.4%). Because of its low ash content, bagasse is more suitable for the microbial bioconversion process than such crop residues as rice straw and wheat straw, whose ash contents are 17.5% and 11.0%, respectively (Pandey *et al.*, 2000). Hydrolysis of pentosan, a component of hemicellulose that contains more D-xylose than L-arabinose, provides a good substrate for microbial cultivation.

Hydrolytic reactions in dilute-acid medium are very complex, mainly because the substrate is in a solid phase and the catalyst is in a liquid phase. The mechanism of hydrolytic reaction includes: (a) diffusion of protons through the wet lignocellulosic matrix; (b) protonation of the oxygen of a heterocyclic ether bond between the sugar monomers; (c) rupture of the ether bond; (d) generation of a carbocation as an intermediate; (e) solvation of the carbocation with water; (f) regeneration of the proton with cogeneration of the sugar monomer, oligomer or polymer, depending on the position of the ether bond; (g) diffusion of the reaction products in the liquid phase when form and size permit (some large oligomers cannot cross the matrix); and (h) return to the second step to begin again (Herrera *et al.*, 2002).

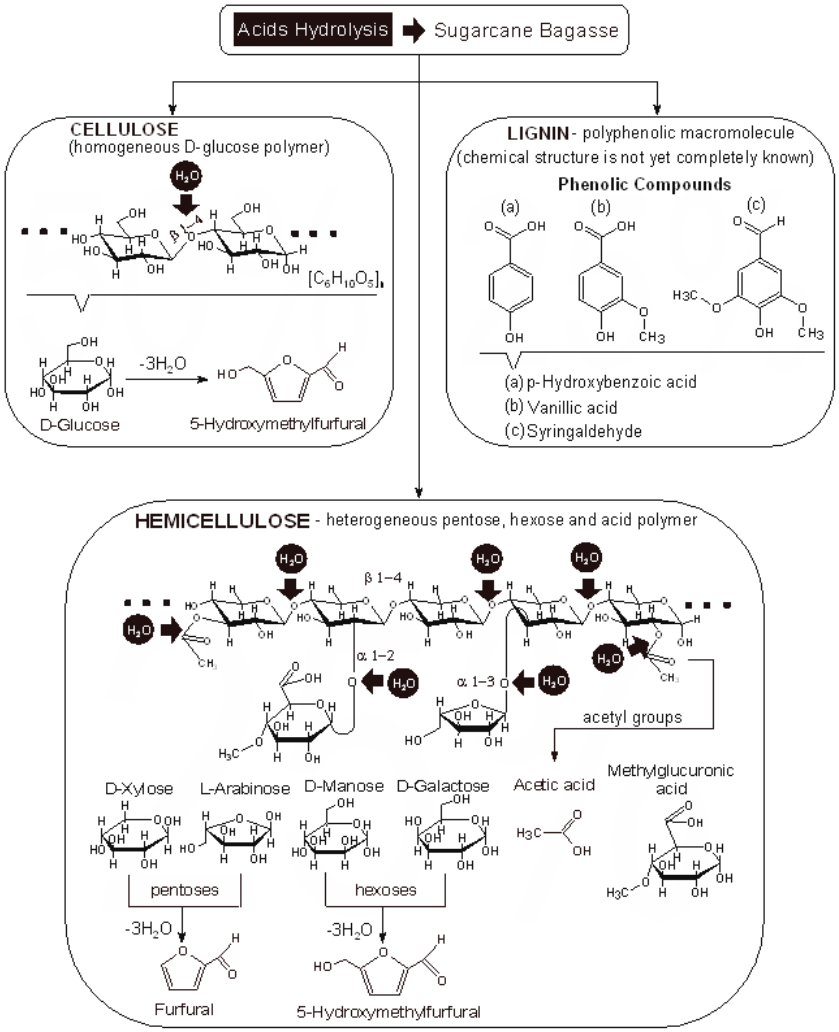

Figure 1: Sketch of sugarcane bagasse degradation by acid hydrolysis

The main problems in the bioprocess utilizing hydrolysates are the individual and interactive toxic effects of some compounds occurring during the hydrolytic process (Figure 1). These compounds are divided into four groups: (1) substances that are released by the hemicellulosic structure, such as acetic acid, which originates in the deacetylation of xylan; (2) phenolic compounds and other aromatic compounds derived from the partial degradation of lignin; (3) the furan derivatives, furfural and 5-hydroxymethylfurfural, resulting from the degradation of pentoses and hexoses, respectively; and (4) metals like chromium, copper, iron and nickel leached from the equipment (Parajó *et al.*, 1998; Palmqvist *et al.*, 2000). All these toxic compounds need to be eliminated or their concentrations reduced so that the hydrolysates can be effectively used in bioconversion processes.

This work focuses on the treatment of sugarcane bagasse hydrolysate by adjustment of pH and adsorption on activated charcoal, employing different charcoals under different adsorption conditions in order to improve the bioconversion of D-xylose into xylitol and to obtain empirical models able to describe the interrelationship between the operational adsorption conditions and the fermentative parameters derived from the experimental data.

MATERIAL AND METHODS

Microorganism and Preparation of Inoculum

The yeast *Candida guilliermondii* FTI20037 was used for bioconversion of D-xylose into xylitol. A stock culture, which was maintained at 4ºC on an agar malt extract slant, was transferred to a 125mL Erlenmeyer flask containing 50mL liquid medium (30.0g/L D-xylose, 2.0g/L $(NH4)_2SO_4$, 0.1g/L $CaCl_2.2H_2O$ and 20.0g/L rice bran extract solution and incubated at a stirring rate of 200rpm at 30ºC for 24h. The cells were then centrifuged at 2000xg for 15 min and washed in sterile distilled water. A suspension was prepared with the cell mass in sterile water and utilized as inoculum. For the experiments, the initial cell concentration was 1.0g/L (about $3x10^7$cells/mL).

Preparation and Treatment of Hemicellulose Hydrolysate

Sugarcane bagasse was introduced into a 350L reactor and mixed with concentrated H_2SO_4 (100mg of acid per gram of dry matter) at a solid:liquid ratio of 1:10 (Pessoa Junior *et al.*, 1997) After hydrolysis (121ºC, 10min), the hydrolysate containing (g/L) D-glucose (0.84), D-xylose (17.85), L-arabinose (1.68), acetic acid (3.15), phenolic compounds (2.20), furfural (0.06) and 5-hydroxymethylfurfural (0.008) was filtered and concentrated under vacuum at 70ºC to quadruple the D-xylose concentration. The hydrolysate thus obtained had the following composition (g/L): D-glucose (3.68), D-xylose (69.23), L-arabinose (7.15), acetic acid (6.02), phenolic compounds (7.88), furfural (0.021) and 5-hydroxymethylfurfural (0.024). The hydrolysate was treated as follows: the initial pH (0.92) was raised to 7.0 with commercial CaO and the hydrolysate was then acidified with concentrated H_3PO_4 to pH 5.5 with the subsequent addition of powdered activated charcoal. The charcoal was mixed with the hydrolysate under different adsorption conditions. In all the treatments the precipitates resulting from adjustment of pH and from addition of activated charcoal were removed by vacuum filtration. For this study seven different types of powdered activated charcoals were used, Synth (Synth, São Paulo, Brazil), CDA and CDG (Brasilac, Paraná, Brazil), C117, C118L and C147 (Carbomafra, Paraná, Brazil) and Carvorite (Carvorite, Paraná, Brazil). These treatments resulted in several hydrolysates that were autoclaved at 111ºC for 15min, before they were used as culture media.

Medium and Fermentation Conditions

The hydrolysates obtained from the treatment were supplemented with nutrients the same as those, used in inoculum preparation with the exception of D-xylose. Fermentations were carried out in 125mL Erlenmeyer flasks containing 50mL of culture medium (pH 5.5) on a rotatory shaker at 200rpm and 30ºC for 64h.

Analytical Methods

The concentrations of D-glucose, L-arabionse, D-xylose, xylitol, acetic acid, furfural and 5-

hydroxymethylfurfural were determined by high-performance liquid chromatrography (Rodrigues *et al.*, 2001), and the phenolic concentration by the colorimetric method using $FeCl_3.6H_2O$ and $K_3Fe(CN)_6$ at 700nm (Kim & Yoo, 1996). The cell number was determined directly by counting in a Neubauer chamber (area = 1/400mm^2; height = 0.100mm).

Statistical Analysis

In order to minimize the number of experiments and model the xylitol yield and volumetric productivity, a 2^{5-2} fractionary factorial design (Box *et al.*, 1978) was used. This design also allowed us to evaluate the effects of the independent variables temperature, contact time, shaking, pH and activated charcoal concentration on xylitol yield and volumetric productivity, using the response surface regression procedure. Table 1 shows these variables and their respective maximum (+1) and minimum (-1) coded levels. For statistical calculation, each independent variable was coded according to Eq. (1), where x_i is the coded value, X_i is the real value, X_0 is the real value at the center point and ΔX_i is the step change value. The fermentation parameters of the D-xylose-to-xylitol bioconversion, namely xylitol volumetric productivity (Q_P) and yield, were taken as the dependent variables or responses of the design experiments.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

The experiments were performed in an aleatory order to minimize systematic errors. After statistical analysis, the variables with significant effects were used to compose a 2^3 full factorial design with a centered face and three replicates at the center point. The statistical analyses of the data were performed using the STATGRAPHICS statistical software, version 4.1 and the STATISTICA program, version 5.0. The results were expressed in estimated effects, standard errors, Student's *t* distribution tables and tables of analysis of variance (ANOVA) (degree of freedom, sum of squares, mean squares, *F* - value (Fischer variance ratio) and significance level (*P* - value).

RESULTS AND DISCUSSION

In order to evaluate the efficacy of each powdered activated charcoal employed in the treatment of sugarcane hemicellulosic hydrolysate, a 2^{5-2} fractional factorial design in seven blocks was conducted using xylitol volumetric productivity (Qp) as response. The choice of this fermentation parameter for this analysis was due to the fact that low values have been reported for Qp in recent studies (Rodrigues *et al.*, 2003). In Table 1 the experimental matrix is present as are the values of xylitol volumetric productivity provided by *C. guilliermondii* as a function of different adsorption conditions employed in the treatment of sugarcane bagasse hydrolysate. As the one-way ANOVA (Table 2) showed that there was no significant difference ($P < 0.05$) between the blocks, the choice of type of powdered activated charcoal was based on price. The types with the best prices were CDA and C117, so the two blocks represented by CDA and C117 were again submitted to analysis of estimated effects (Table 3) in order to select the most appropriate for the experiments. According to Table 3, there was no significant difference between the blocks (P=0.5952) represented by CDA and C117, but CDA was chosen because it has a higher iodine number (minimum of 800mg/g), a physical property that measures the adsorption capacity of activated charcoals, than C117 (minimum of 700mg/g). According to Brasilac (*n.d.)* and Carbomafra *(n.d.)*, iodine number is an important physicochemical property that shows by correlation the adsorption surface area of the activated charcoal; it indicates that the larger the iodine number the larger the adsorption area. Danner *et al.* (2003) verified that physicochemical properties of activated charcoal affect the removal of inhibitors such as furan and phenolic compounds from wood hydrolysates for improving the fermentability of this hydrolysate, observing that hydrolysates treated with activated charcoals prepared at a higher temperature (1000ºC) could attain higher fermentability than charcoal prepared at a lower temperature (400ºC).

Table 1: Experimental matrix of a 2^{5-2} fractional factorial design in blocks for xylitol volumetric productivity as a function of operational conditions for adsorption with coded and natural levels of variables.

			Variables					Coded variables					Qp
	Trial	Run[a]	T[b](ºC)	ct[c] (min)	S[d](rpm)	pH	CC[e] (%)	x_1[f]	x_2[f]	x_3[f]	x_4[f]	x_5[f]	(g/L.h) y[g]
Block 1: **SYNTH**	1	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.37
	2	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.43
	3	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.38
	4	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.42
	5	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.43
	6	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.35
	7	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.47
	8	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.39
Block 2: **CDA**	9	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.50
	10	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.44
	11	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.35
	12	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.40
	13	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.54
	14	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.33
	15	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.43
	16	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.29
Block 3: **CDG**	17	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.37
	18	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.31
	19	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.48
	20	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.43
	21	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.49
	22	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.41
	23	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.45
	24	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.45
Block 4: **C117**	25	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.37
	26	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.43
	27	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.32
	28	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.38
	29	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.42
	30	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.44
	31	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.51
	32	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.48
Block 5: **C118L**	33	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.38
	34	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.37
	35	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.49
	36	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.54
	37	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.52
	38	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.44
	39	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.35
	40	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.39
Block 6: **C147**	41	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.45
	42	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.53
	43	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.44
	44	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.35
	45	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.56
	46	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.41
	47	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.49
	48	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.28
Block 7 :**Carvorite**	49	2	60	30	300	1	10	+1	-1	+1	-1	+1	0.55
	50	4	30	30	100	6	10	-1	-1	-1	+1	+1	0.42
	51	6	60	60	300	6	10	+1	+1	+1	+1	+1	0.46
	52	3	30	60	300	1	1	-1	+1	+1	-1	-1	0.32
	53	7	30	60	100	1	10	-1	+1	-1	-1	+1	0.45
	54	1	60	30	100	1	1	+1	-1	-1	-1	-1	0.50
	55	5	30	30	300	6	1	-1	-1	+1	+1	-1	0.52
	56	8	60	60	100	6	1	+1	+1	-1	+1	-1	0.36

[a]Run: sequence of experiments
[b]T: temperature; [c]tc: contact time ; [d]S: shaking; [e]CC: activated charcoal concentration (w/v)
[f]x_1, x_2, x_3, x_4 and x_5 are dimensionless, normalized and independent variables
[g]y: dependent variable (volumetric productivity)

Table 2: One-way ANOVA for xylitol volumetric productivity obtained with *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate using a 2^{5-2} fractional factorial design in two blocks (CDA and C117).

Source of variation	Sum of squares	Df[a]	Mean squares	*F*-ratio	*P*-value
Between blocks	0.01145	6	0.00191	0.376	0.8905
Within blocks	0.24842	49	0.00507		
Total (corrected)	0.25987	55			

[a]Degrees of freedom

Table 3: Estimates, standard errors and Student's *t* test for xylitol volumetric productivity obtained with *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate using a 2^{5-2} fractional factorial design in two blocks (CDA and C117).

Effects	Estimates	Standard errors	*t*-value
Average	0.6338	±0.0126	-
x_1	0.0500	±0.0252	1.9841[a]
x_2	-0.1875	±0.0252	-7.4405[a]
x_3	0.0525	±0.0252	2.0834[a]
x_4	-0.0650	±0.0252	-2.5794[a]
x_5	-0.0150	±0.0252	-0.5952
$x_2x_3+x_4x_5$	-0.0275	±0.0252	1.0913
$x_2x_5+x_3x_4$	0.0050	±0.0252	0.1984
Between blocks	0.0150	±0.0252	0.5952

[a]confidence level of 90% ($t_{10\%}$=1.753)

Analysis of the estimated effect for xylitol volumetric productivity (Table 3) shows that temperature, contact time, shaking and pH had significant effects ($P < 0.10$). As the effect of charcoal concentration (CC) was not significant ($P > 0.10$), this variable was eliminated and other experiments were conducted with a view to attaining a 2^4 full factorial design using the responses xylitol volumetric productivity (Qp) and yield ($Y_{P/S}$) (Table 4).

Table 4 contains the experimental matrix as well as the results achieved for the fermentation parameters of the D-xylose-to-xylitol bioconversion by *C. guilliermondii* as a function of different adsorption conditions employed in the treatment of sugarcane bagasse hemicellulosic hydrolysate. After the 2^4 full factorial design, the fermentation parameters were analyzed by response surface methodology. Analysis of the estimated effects (Table 5) shows that temperature, contact time and interaction between temperature and pH had significant effects ($P < 0.05$) on xylitol volumetric productivity (Qp). With respect to yield, temperature, contact time pH, interaction between temperature and contact time and interaction between temperature and pH also had significant effects ($P < 0.05$). Neither the shaking parameter nor its interactions with other variables had significant effects on yield and xylitol volumetric productivity; thus treatment at the lower level (100 rpm) was preferred, because it consumes less energy.

A 2^3 full factorial design with three replicates at center point was used to obtain the mathematical model representing this fermentation process by the response surface methodology. The response values used in runs 1 to 8 were the average of the runs at the same level for the parameters temperature, contact time and pH of the 2^4 full factorial design. The matrix for this design and for the fermentation parameters (Qp and $Y_{P/S}$) is shown in Table 6. Firstly an ANOVA of the experimental data was conducted to estimate the quadratic model. This analysis showed that for xylitol volumetric productivity (Qp), the curvature was significant at the 10% probability level ($P < 0.10$).

However, this same analysis for D-xylose-to-xylitol yield did not have a significant effect at the 10% probability level ($P > 0.10$) (data not shown). For these reasons, the xylitol volumetric productivity experiments were combined (Table 7), obtaining a 2^3 full factorial design with a centered face and three replicates at the center point.

As is evident in Table 7, run 2 provided the highest xylitol volumetric productivity (0.52 g/L.h), whereas run 3 provided the lowest productivity (0.31 g/L.h). This difference is equivalent to 40%. As for D-xylose-to-xylitol yield Table 6 shows that the variation between maximum and minimum values (0.77 g/g for run 2 and 0.51 g/g for run 8) corresponded to a decrease of 34%, which confirms that the different adsorption conditions employed in the treatment of sugarcane bagasse hydrolysate all had an effect on the D-xylose-to-xylitol bioconversion.

Table 4: Experimental matrix for xylitol volumetric productivity (Q_P) and yield ($Y_{P/S}$) obtained with *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate treated with CDA activated charcoal at natural and coded levels, using a 2^4 full factorial design with three replicates at the center point.

				Variables		Coded variables				Responses	
		T(°C)[c]	tc(min)[d]	S(rpm)[e]	pH	x_1[f]	x_2[f]	x_3[f]	x_4[f]	Qp	$Y_{P/S}$
Trial[a]	Run[b]	x_1	x_2	x_3	x_4					(g/L.h)	(g/g)
	1	60	30	300	6.0	+1	-1	+1	+1	0.39	0.59
10		30	30	100	6.0	-1	-1	-1	+1	0.44	0.62
	2	30	30	100	1.0	-1	-1	-1	-1	0.37	0.56
15		30	30	300	6.0	-1	-1	+1	+1	0.43	0.66
11		60	60	100	6.0	+1	+1	-1	+1	0.35	0.52
	3	60	60	300	1.0	+1	+1	+1	-1	0.43	0.59
16		30	60	300	1.0	-1	+1	+1	-1	0.29	0.58
	4	30	30	300	1.0	-1	-1	+1	-1	0.38	0.58
	5	30	60	300	6.0	-1	+1	+1	+1	0.43	0.61
12		60	60	300	6.0	+1	+1	+1	+1	0.40	0.50
	6	60	60	100	1.0	+1	+1	-1	-1	0.39	0.58
09		60	30	300	1.0	+1	-1	+1	-1	0.50	0.78
13		60	30	100	1.0	+1	-1	-1	-1	0.54	0.77
	7	60	30	100	6.0	+1	-1	-1	+1	0.44	0.58
14		30	60	100	1.0	-1	+1	-1	-1	0.33	0.58
	8	30	60	100	6.0	-1	+1	-1	+1	0.39	0.57
	9	45	45	200	3.5	0	0	0	0	0.43	0.59
	10	45	45	200	3.5	0	0	0	0	0.42	0.61
	11	45	45	200	3.5	0	0	0	0	0.44	0.59

[a]Experiments from 2^{5-2} fractional factorial design
[b]Run: sequence of experiments
[c]T: temperature, [d]tc: contact time and [e]S: shaking
[f]x_1, x_2, x_3, x_4 and x_5 are dimensionless, normalized and independent variables
[g]y_1 and y_2: dependent variables (xylitol volumetric productivity and yield, respectively)

Table 5: Effect estimates, standard errors and *t*-test for xylitol productivity and yield in the D-xylose-to-xylitol bioconversion obtained with *C. guilliermondii*, using a 2^4 full factorial design with three replicates at the center point.

		Productivity (Qp)			Yield ($Y_{P/S}$)	
Effects	Estimates	Standard errors	*t*-values	Estimates	Standard errors	*t*-values
Average	0.410	±0.006	-	0.603	±0.007	-
x_1	0.047	±0.013	3.615[a]	0.187	±0.017	11.000[a]
x_2	-0.060	±0.013	-4.615[a]	-0.076	±0.017	-4.471[a]
x_3	0.000	±0.013	0.000	0.014	±0.017	0.823
x_4	0.005	±0.013	0.385	-0.046	±0.017	-2.706[a]
x_1x_2	-0.015	±0.013	1.154	-0.056	±0.017	-3.294[a]
x_1x_3	0.000	±0.013	0.000	-0.011	±0.017	0.647
x_1x_4	-0.075	±0.013	5.769[a]	-0.086	±0.017	-5.059[a]
x_2x_3	0.023	±0.013	1.769	-0.006	±0.017	-0.353
x_2x_4	0.027	±0.013	2.077	0.014	±0.017	0.823
x_3x_4	0.007	±0.013	0.538	0.004	±0.017	0.235

[a]Significant at a probability level of 5% ($t_{5\%}$=2.131)

Table 6: Experimental matrix for xylitol volumetric productivity (Q_P) and yield ($Y_{P/S}$) obtained with *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate treated with CDA activated charcoal at natural and coded levels, using a 2^3 full factorial design with three replicates at the center point.

	Variables			Coded variables			Responses	
	Temperature (ºC)	Contact time (min)	pH	x_1	x_2	x_4	Q_P	$Y_{P/S}$
Run	x_1	x_2	x_4				y_1[a]	y_2[a]
1	30	30	1.0	-1	-1	-1	0.37	0.57
2	60	30	1.0	+1	-1	-1	0.52	0.77
3	30	60	1.0	-1	+1	-1	0.31	0.58
4	60	60	1.0	+1	+1	-1	0.41	0.59
5	30	30	6.0	-1	-1	+1	0.43	0.64
6	60	30	6.0	+1	-1	+1	0.41	0.59
7	30	60	6.0	-1	+1	+1	0.41	0.59
8	60	60	6.0	+1	+1	+1	0.37	0.51
9	45	45	3.5	0	0	0	0.43	0.59
10	45	45	3.5	0	0	0	0.42	0.61
11	45	45	3.5	0	0	0	0.44	0.59

[a]y_1 and y_2: dependent variables (xylitol volumetric productivity and yield, respectively)

Table 7: Experimental matrix for xylitol volumetric productivity (Q_P) obtained with *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate treated with CDA activated charcoal at natural and coded levels, using a 2^3 full factorial design with centered face and three replicates at the center point.

	Variables			Coded variables			Response
Run	Temperature (ºC) x_1	Contact time (min) x_2	pH x_4	x_1	x_2	x_4	Q_P (g/L.h) y_1
1	30	30	1.0	-1	-1	-1	0.37
2	60	30	1.0	+1	-1	-1	0.52
3	30	60	1.0	-1	+1	-1	0.31
4	60	60	1.0	+1	+1	-1	0.41
5	30	30	6.0	-1	-1	+1	0.43
6	60	30	6.0	+1	-1	+1	0.41
7	30	60	6.0	-1	+1	+1	0.41
8	60	60	6.0	+1	+1	+1	0.37
9[a]	30	45	3.5	-1	0	0	0.42
10[a]	60	45	3.5	+1	0	0	0.49
11[a]	45	30	3.5	0	-1	0	0.46
12[a]	45	60	3.5	0	+1	0	0.48
13[a]	45	45	1.0	0	0	-1	0.38
14[a]	45	45	6.0	0	0	+1	0.36
15	45	45	3.5	0	0	0	0.43
16	45	45	3.5	0	0	0	0.42
17	45	45	3.5	0	0	0	0.44

[a]runs in the centered face experiments

The residual analysis obtained by plotting the residuals versus the values estimated by the proposed model showed that the residuals varied randomly, which means they did not have unexplained systematic tendencies, so the curve represented the points satisfactorily (data not shown). Application of the response surface methodology, on the basis of the coefficient estimate, provides an empirical relationship between the values of xylitol volumetric productivity (y_1) and the coded variables tested (x_i) by means of regression equation 2 by replacing the qualitative variable x_2 by its lowest coded level (-1):

$$\hat{y}_1 = 0.477 + 0.026x_1 - 0.0218x_4 - \\ -0.0361x_1x_4 - 0.0482x_4^2 \quad (2)$$

where y_1 represents the xylitol volumetric productivity (Qp), x_1 the initial coded temperature and x_4 the coded pH. Solving this mathematical model by means of the coded levels for the temperature 60ºC (+1) and the pH 2.5 (-0.4) makes it possible to predict 0.518 g/L h of xylitol volumetric productivity. The model's optimal region can be observed in Figure 2, which depicts the response surface and contour lines described by the y_1 model. When temperature level +1 (60ºC) decreases to -1 (30ºC) and the pH level -1 (1.0) increases to +1 (6.0), xylitol volumetric productivity decreases by 25 and 18%, respectively (Figure 2).

In Table 9 the regression coefficients, standard errors, *t*-values and significance level for the model representing the D-xylose-to-xylitol yield at a significance level of 5% are presented. In this case, contact time (x_2), interaction between temperature and contact time and interaction between temperature and pH had significant effects.

Table 8: Regression coefficients, standard errors, *t*-test and significance levels for the model representing the xylitol volumetric productivity obtained with *C. guilliermondii*, using a 2^3 factorial design with centered face and three replicates at the center point.

Variables	Coefficients	Standard errors	*t*-values	*P*-values
Constant	0.4477	±0.0125	-	-
x_1	0.0260	±0.0105	2.9213[a]	0.0130[a]
x_4	-0.0218	±0.0117	-2.4494[a]	0.0310[a]
x_1x_4	-0.0361	±0.0099	-3.6331[a]	0.0035[a]
x_4x_4	-0.0482	±0.0164	-3.4676[a]	0.0047[a]

[a]a confidence level of the 95% ($t_{5\%}$=2.201)
$R^2_{adjusted}$= 0.69

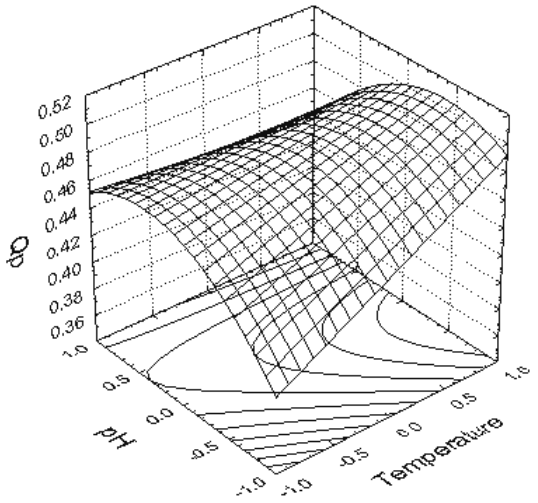

Figure 2: Response surface and contour lines described by the y_1 model representing the xylitol volumetric productivity obtained with *C. guilliermondii* in sugarcane bagasse hemicellulosic hydrolysate.

Table 9: Regression coefficients, standard errors, *t*-test and significance levels for the model representing the D-xylose-to-xylitol yield obtained with *C. guilliermondii* using a 2^3 factorial design with centered face and three replicates at the center point.

Effects	Estimates	Standard errors	*t*-values
Average	0.6036	±0.0096	-
x_1	0.0225	±0.0226	0.9955
x_2	-0.0775	±0.0226	3.4292[a]
x_4	-0.0475	±0.0226	2.1018
x_1x_2	-0.0575	±0.0226	2.5442[a]
x_1x_4	-0.0875	±0.0226	3.8717[a]
x_2x_4	0.0125	±0.0226	0.5531

[a]confidence level of 95% ($t_{5\%}$=2.228)

In this case, the residual analysis of the proposed model showed that the curve also represented the points satisfactorily (data not shown). Table 10 shows the ANOVA regression and the model's correlation coefficient (R^2 = 0.62) reveals that the mathematical model is significant and can be expressed by equation (3):

$$\hat{y}_2 = 0.60364 - 0.03875x_2 - \\ -0.0575x_1x_2 - 0.04375x_1x_4 \quad (3)$$

where y_2 represents the D-xylose-to-xylitol yield ($Y_{P/S}$), x_2 the initial coded contact time and x_1x_4 the coded interaction between temperature and pH. Solving this mathematical model by means of the coded levels for contact time 30min (+1), temperature 60ºC (+1) and pH 1.0 (-1) makes it possible to predict a xylitol yield of 0.69 g/g. The model's optimal region can be observed in Figure 3, which depicts the response surface and contour lines described by the y_2 of the model. According to Figure 3, when the contact time level +1 (60min) decreases to –1 (30min) and the pH level +1 (6.0) decreases to –1 (1.0), the D-xylose-to-xylitol yield increases by 19 and 33%, respectively.

Table 10: Regression coefficients, standard errors, *t*-test and significance levels for the model representing the xylitol volumetric productivity obtained with *C. guilliermondii*, using a 2^3 factorial design with three replicates at the center point.

Variables	Coefficients	Standard errors	*t*-values	*P*-values
Constant	0.60364	±0.013705	44.0440	0.0000
x_2	-0.03875	±0.016071	-2.4112	0.0424[a]
x_1x_4	-0.04375	±0.016071	-2.7223	0.0262[a]

[a]confidence level of 95% ($t_{5\%}$=2.228)
R^2= 0.62

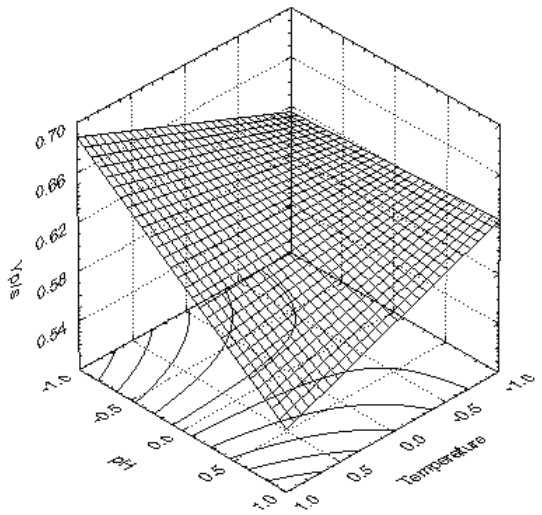

Figure 3: Response surface and contour lines described by the y_2 model representing the yield of D-xylose-to-xylitol bioconversion obtained with *C. guilliermondii* in sugarcane bagasse hemicellulosic hydrolysate.

That both xylitol volumetric productivity (Table 8) and D-xylose-to-xylitol yield obtained (Table 10) shows that the variables temperature and pH were significant at a confidence level of 95%. These two variables were used to determine the optimization condition, which resulted in the following models:

$$Qp: \hat{y}_1 = 0.477 + 0.026x_1 - 0.0218x_4 - \\ -0.0361x_1x_4 - 0.0482x_4^2 \quad (4)$$

$$Y_{P/S}: \hat{y}_2 = 0.64239 - 0.04375x_1x_4 \quad (5)$$

The xylitol volumetric productivity (Qp) is the same as that utilized to plot Figure 2, but in the xylitol yield model the variable x_2 (contact time) was substituted for -1, since this variable has a negative effect at a confidence level of 95% (Table 9), and the value was added to the constant of the model. Based on the two models (Equations 4 and 5), a graphic optimization was conducted using the Design-Expert program. This method consists in overlaying the curves of all the modes according to the criteria adopted. The optimal working conditions to attain high levels of xylitol volumetric productivity and D-xylose-to-xylitol yield were defined using the following criteria: (1) xylitol volumetric productivity higher than 0.45 g/L h and (2) D-xylose-to-xylitol yield higher than 0.60 g/g. The overlaying plot in Figure 4 shows a shaded area where the requirements were satisfied. Therefore, a point corresponding to temperature level +1 (60ºC) and pH level -0.4 (2.5) was assigned as an optimum point. Under these conditions, the model predicted a xylitol volumetric productivity of 0.51 g/L h (with variation from 0.49 to 0.53 g/L h possible) and a D-xylose-to-xylitol yield of 0.66 g/g (with variation from 0.65 to 0.67 g/g possible) in the confidence interval of 95%.

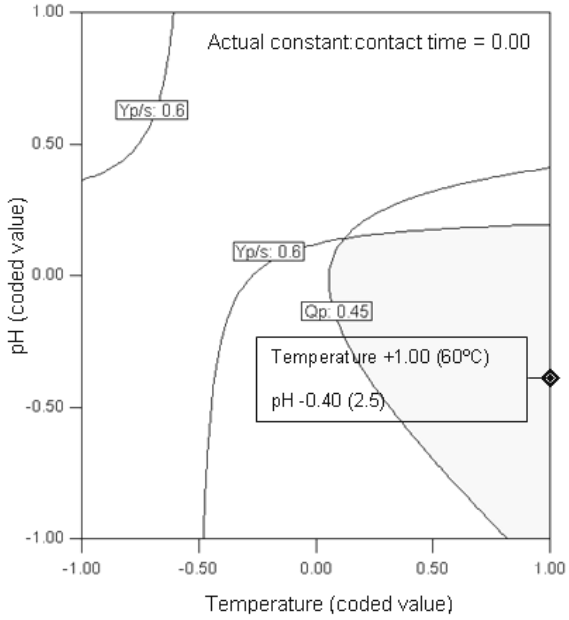

Figure 4: Optimum region obtained by overlaying plots of the two responses ($Y_{P/S}$ and Qp) as a function of temperature and pH.

To verify these optimized conditions the variables temperature, shaking, agitation, pH and charcoal concentration were fixed at levels +1.00 (60ºC), -1.00 (30 min), -1.00 (100 rpm), -0.40 (2.5) and -1.00 (1%), respectively, and treatments employing these adsorption conditions were conducted in triplicate. The average values of xylitol volumetric productivity and D-xylose-to-xylitol yield were 0.50 g/L h and 0.66 g/g, respectively. These results showed that the model the experimental data well and thus described well the region under study. Under optimized conditions an average of 76% of phenolic compounds, 44% acetic acid, 60% 5-hydroxymethylfurfural and 58% furfural were removed, and after 64h of fermentation 94% D-xylose, 65% acetic acid and 55% L-arabinose had been consumed. D-xylose consumption (94%) was similar, as also reported by Dominguez et al. (1996) (95%), who cultivated a mutant of *Candida sp* 11-2 in sugarcane bagasse hydrolysate treated with activated charcoal to form xylitol. The partial consumption of L-arabinose (7.15 g/L) and total consumption of D-glucose, in the hydrolysate at low concentrations (3.68 g/L), were detected in all

experiments (data not shown), as also reported by Alves et al. (1998) and Rodrigues et al., (2003), who cultivated the same strain in sugarcane bagasse hydrolysate.

In relation to adsorption conditions, the highest temperature (60ºC) employed favored the improvement of fermentation parameters. However, in the study of adsorption operational conditions for improvement of wood hydrolysate fermentability Parajó et al. (1996b) verified that the effect of activated charcoal concentration is about three times higher than that of temperature on D-xylose-to-xylitol conversion ($Y_{P/S}$). According to these authors, the media treated with an intermediate (205 g/g) or low (400 g/g) hydrolysate:charcoal ratio at 24ºC were sufficient for 87-88% D-xylose consumption in 36h, with volumetric consumption rates in the range of 0.93 to 0.98g/L h and obtained the maximum xylitol yield (0.6 g/g). In this work, the results permitted verification that increasing charcoal concentration, and consequently improving the adsorption surface, favors the desirable removal of all toxic compounds studied, but the highest toxic compound removal was not a proportional to improvement of the fermentation parameters. The highest values for the toxic compound removal, 99% phenolics (trial 33), 58% acetic acid (trial 01), 100% furfural (trial 14) and 100% 5-hydroxymetylfurfural (trial 01) (data not shown) were obtained with a 10% concentration of activated charcoal, while xylitol volumetric productivity for these trials was 0.38, 0.37, 0.33 and 0.37g/L h, respectively (Table 1).

With regard to contact time, the fixed value of 30 min was the same as that in a study on wood hydrolysate treatment with activated charcoal; Parajó *et al.* (1996b) reported that irrespective of the hydrolysate:charcoal ratio, 400g/g or 20g/g, the maximum phenol adsorption on activated charcoal occurred in about 30min of contact time. The same authors observed that, after a fast initial period of up to about 30min and a slow stage, the surface area of the activated charcoal was progressively blocked during the process.

Table 6 shows that for the lowest pH improved the fermentation parameters (trial 02) related to the reduction of the concentrations of acetic acid and phenols under this condition. According to Rodrigues et al. (2001), the removal of acetic acid (pK_a=4.76) by adsorption of activated charcoal is related to the balance between its dissociated and undissociated forms regulated by equilibrium, which is affected by pH. These authors achieved maximum values for removal of acetic acid (62%) and phenols (98%) when activated charcoal (2.4% w/v) was added to a fourfold concentration of sugarcane bagasse hydrolysate at pH 0.92. A possible explanation for the highest values for removal of the toxic acids such as acetic acid and phenolics can be due to their molecular form at a low pH, favoring the London forces (adsorption forces).

CONCLUSIONS

This study shows the application of statistical theories to fermentation processes and proves that treatment of sugarcane bagasse hydrolysate affects the formation of xylitol. The different types of powdered activated charcoals employed did not have a significant effect on the xylitol volumetric productivity obtained with *Candida guilliermondii*. The fermentation parameters of the bioconversion of D-xyloste to xylitol were affect by the adsorption conditions employed during treatment of the sugarcane bagasse hydrolysate. The variables temperature, contact time and pH had significant effects on the parameters xylitol volumetric productivity and xylitol yield. Defining an optimum point made it possible to achieve a xylitol volumetric productivity of 0.50 g/L h and a xylitol yield of 0.66 g/g. Although the xylitol volumetric productivity improved, a much better result could have been achieved if a more effective method of treatment had been employed. Further work will thus be carried out to evaluate the treatment of sugarcane bagasse hydrolysate in a continuous system employing ion-exchange resins and activated charcoal in columns to be used as media in fermentation with adapted cells.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of FAPESP and CNPq. They are also grateful to Maria Eunice M. Coelho for revising this paper.

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