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Xylose Reductase and Xylitol Dehydrogenase Activities of *Candida guilliermondii* as a Function of Different Treatments of Sugarcane Bagasse Hemicellulosic Hydrolysate Employing Experimental Design

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Abstract

The sugarcane bagasse hydrolysate, which is rich in xylose, can be used as culture medium for *Candida guilliermondii* in xylitol production. However, the hydrolysate obtained from bagasse by acid hydrolysis at 120°C for 20 min has by-products (acetic acid and furfural, among others), which are toxic to the yeast over certain concentrations. So, the hydrolysate must be pretreated before using in fermentation. The pretreatment variables considered were: adsorption time (15, 37.5, and 60 min), type of acid used (H2So4 and H3Po4), hydrolysate concentration (original, twofold, and fourfold concentrated), and active charcoal (0.5, 1.75 and 3.0%). The suitability of the pretreatment was followed by measuring the xylose reductase (XR) and xylitol dehydrogenase (XD) activity of yeast grown in each treated hydrolysate. The response surface methodology (2^4 full factorial design with a centered face) indicated that the hydrolysate might be concentrated fourfold and the pH adjusted to 7.0 with CaO, followed by reduction to 5.5 with H_3PO_4. After that it was treated with active charcoal (3.0%) by 60 min. This pretreated hydrolysate attained the high XR/XD ratio of 4.5.

Index Entries: Xylose reductase; xylitol dehydrogenase; sugarcane bagasse hydrolysate; treatment; *Candida guilliermondii*.

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Introduction

The use of sugarcane bagasse, a residue plentiful available in alcohol distilleries, in bioconversions requires a previous acid hydrolysis in order to separate its xylose-rich hemicellulose fraction *(1)*. The hydrolysate can be used as culture medium for growing xylose-metabolizing yeasts in xylitol production *(2,3)*. The fermentative process could become an alternative to the chemical synthesis of this product *(4)*. The xylose–xylitol conversion by xylose-metabolizing yeasts constitutes the first step of their metabolism *(5)*. As xylose induces the synthesis of xylose reductase (EC. 1.1.1.21) and xylitol dehydrogenase (EC. 1.1.1.9) *(6)*, its initial concentration in the medium can affect the overall xylitol yield *(7)*. According to the literature, in synthetic medium as the initial xylose concentration is increased so is the xylitol yield. Probably, it is due to the effect of xylose concentration on the synthesis and activity of xylose reductase (XR) and xylitol dehydrogenase (XD) *(7,8)*. often this result is not observed when sugarcane bagasse hydrolysate is used as culture medium. The reason would be due to the presence of undesirable compounds in the hydrolysate (acetic acid, phenol derivatives, furfural, hydroxymethylfurfural) formed during the hydrolysis procedure. Such compounds could probably affect the yeast metabolism through the inhibition of intracellular enzymes, XR and XD *(9)*. However, the inhibitory effect of those compounds can be reduced by treating the hydrolysate adequately, before its utilization as fermentation medium *(10)*.

It was previously observed that culture media constituted by concentrated hydrolysate submitted to different pretreatments had significant effects on the *C.guilliermondii* fermentative capability as well as on the intracellular XR and XD activities (data not shown). Aiming to attain yeast cells with high XR and low XD activity, several tests were carried out by using pretreated hydrolysates under different conditions as culture media. Moreover, mathematical models derived from the response surface methodology were tried to find some correlation between the enzyme activities and the types of pretreatment under which the sugarcane bagasse hydrolysate was submitted.

Materials and Methods

Microorganism and Inoculum Preparation

The inoculum was prepared with cells of *C. guilliermondii* FTI20037, maintained at 4°C on malt-extract agar. The culture medium containing 30 g/L of xylose was supplemented with the following nutrients: 2.0 g/L of $(NH_4)_2SO_4$, 0.1 g/L of $CaCl_2{\cdot}2H_2O$ and 20.0 g/L of rice bran extract. Fifty milliliters of the medium was placed into 125 mL Erlenmeyer flasks and agitated at 200 rpm, at 30°C for 24 h. For the experiments, the initial cell concentration was 0.5 g/L.

Table 1
Partial Composition of the Sugarcane Bagasse Hemicellulosic Hydrolysate Obtained by Acid Hydrolysis

	Hydrolysate		
Composition (g/L)	Original	Concentrated twofold	Concentrated fourfold
Xylose	16.40	30.70	63.30
Glucose	1.28	2.03	4.28
Arabinose	1.94	2.36	5.43
Acetic acid	4.11	5.42	6.21
Furfural	0.24	0.16	0.13
Hydroxyimethylfurfural	0.30	0.54	0.70
Phenol	1.06	2.10	2.72

Hydrolysate Preparation

Sugarcane bagasse was submitted to acid hydrolysis, at 1:10 solid/liquid ratio (100 mg of H_2SO_4 per gram of dry matter), at 121°C for 20 min. After hydrolysis, the hydrolysate was filtered and part of the volume obtained was concentrated under vaccuum at 70°C to increase the xylose concentration twofold and fourfold. The composition of the hydrolysates thus obtained is presented in Table 1. Both the original and concentrated hydrolysates were treated as follows: the initial pH (1.18, 0.85, and 0.51) was raised to 7.0 with CaO (commercial powder) and reduced to 5.5 with concentrated H_2SO_4 or H_3PO_4, with the subsequent addition of 0.5, 1.75, or 3.0% (w/v) activated charcoal (refined powder), under agitation (200 rpm) at 30°C, for 15 min, 37.5 min or 60 min, following experimental design. In all the treatments, the precipitate resulting from pH adjustment and from addition of activated charcoal was removed by vacuum filtration. The hydrolysates obtained as a function of the different treatments were all autoclaved at 111°C for 15 min, to be used as culture media.

Medium and Fermentation Conditions

The fermentation media were prepared from the original and concentrated hydrolysates, treated and supplemented with the same nutrients described in the inoculum preparation. The original and the twofold and fourfold concentrated hydrolysates were fermented for 20 h, 34 h and 48 h, respectively, in the 125 mL Erlenmeyer flasks each containing 50 mL of medium (pH 5.5), on a rotary shaker at 200 rpm at 30°C.

Enzyme Assay

The cells obtained in the experiments were harvested by centrifugation at 2700*g* for 15 min, and resuspended in 0.1 *M* phosphate buffer (pH 7.2). Cells were disrupted by sonication in 1 s pulses for a period of

35 min using a disrupter (VC-100; Sonics & Materials, Newton, CT). The samples were centrifuged at 6700*g* (MR 1812; Jouan, Winchester, VA) at 4°C for 10 min and the supernatant was utilized for determination of the XR and XD activities. Enzyme activities were determined spectrophotometrically at 340 nm at room temperature *(12)*. Specific activities were obtained by determining the protein content according to the method of Bradford *(13)*, using bovine serum albumin as the standard. The enzymatic activity was defined as the amount of enzyme necessary to catalyze the oxidation/reduction of 1 nmol of NAD(P)H or NAD^+ per min.

Analytical Methods

Xylose, xylitol, acetic acid, glucose, and arabinose were quantified by high-performance liquid chromatography (Waters, Milford, MA) with a refraction index detector on a Bio-Rad Aminex HPX-87H at 45°C, with 0.01 *N* H_2SO_4 as the eluent at a 0.6 mL/min flow rate.

A Hewlett-Packard RP18 column at 25°C with acetonitrile:water (1:8) and 1% acetic acid as the eluent, and 0.8 mL/min flow rate was employed for determination of furfural and hydroxymethylfurfural in a visible ultraviolet-light detector (SPD-10A UV-VIS). The concentrations of phenols were determined by spectrophotometry, as described by Kim and Yoo *(11)*. Cell concentration was determined by optical density at 600 nm (Beckman-DU 640B spectrophotometer).

Experimental Design and Statistical Analysis

The application of a 2^4 full factorial design with a centered face and six replicates at the center point *(14,15)* was used to obtain the mathematical models that represent the variations observed in the activities of XR and XD enzymes (Table 2). The variables codified as low level (–1), central level (0) and high level (+1) were [HC] hydrolysate concentration (original, twofold and fourfold concentrated), [A] acid utilized in treatment (H_2SO_4 and H_3PO_4), [AC] activated charcoal (0.5, 1.75 and 3.0%), and [AT] adsorption time (15, 37.5 and 60 min).

The statistical analysis was performed using the STATGRAPHICS 6.0, STATISTICA 5.0 and DESIGN-EXPERT 5.0 programs.

Results and Discussion

Table 2 presents the design matrix and the experimental responses related to the activity of the enzymes XR and XD from *C. guilliermondii* as a function of the concentration and the different combinations of treatment of the sugarcane bagasse hydrolysate. The results show the highest variation between maximum and minimum values obtained, which indicates the influence of the studied factors in the enzymatic activity.

The analysis of the estimated effect (Table 3) indicates that all the factors studied showed significant effect, principal or the interaction, on the activity of the enzymes. For xylose reductase activity, at 5% probability

Table 2
Design Matrix and Attained Responses for the Activity of the Enzymes XR and XD in the Bioconversion of Xylose into Xylitol by *C. Guilliermondii* as a Function of Different Treatments of Bagasse Hydrolysate According to a 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

Treatment	[HC]	[A]	[AC]	[AT]	Spec. activ. XR ($nmol/mg_{prot} \cdot min$)	Spec. activ. XD ($nmol/mg_{prot} \cdot min$)
1	− 1	− 1	− 1	+ 1	594.47	372.23
2	+ 1	− 1	− 1	− 1	690.51	394.63
3	− 1	+ 1	− 1	− 1	572.07	203.39
4	+ 1	+ 1	− 1	+ 1	1103.79	289.00
5	− 1	− 1	− 1	− 1	650.41	255.67
6	+ 1	− 1	− 1	+ 1	776.39	504.52
7	− 1	+ 1	− 1	+ 1	638.26	265.45
8	+ 1	+ 1	− 1	− 1	797.49	431.57
9	− 1	− 1	+ 1	− 1	780.35	278.25
10	+ 1	− 1	+ 1	+ 1	782.61	312.09
11	− 1	+ 1	+ 1	+ 1	645.62	305.83
12	+ 1	+ 1	+ 1	− 1	794.45	218.56
13	− 1	− 1	+ 1	+ 1	768.56	415.98
14	+ 1	− 1	+ 1	− 1	845.35	291.19
15	− 1	+ 1	+ 1	− 1	720.87	396.68
16	+ 1	+ 1	+ 1	+ 1	1075.93	325.31
17	0	− 1	0	0	514.65	365.30
18	0	− 1	0	0	397.53	—
19	0	− 1	0	0	491.53	396.72
20	0	+ 1	0	0	459.27	353.24
21	0	+ 1	0	0	495.64	234.18
22	0	+ 1	0	0	440.18	—
23	− 1	− 1	0	0	570.16	392.51
24	+ 1	− 1	0	0	695.74	400.51
25	0	− 1	0	0	617.17	385.93
26	0	− 1	0	0	601.57	350.76
27	0	− 1	− 1	0	727.64	425.01
28	0	− 1	+ 1	0	626.65	376.96
29	0	− 1	0	− 1	625.84	369.72
30	0	− 1	0	+ 1	647.70	371.24
31	− 1	+ 1	0	0	658.38	410.33
32	+ 1	+ 1	0	0	587.40	228.38
33	0	+ 1	0	0	571.67	273.29
34	0	+ 1	0	0	464.69	258.36
35	0	+ 1	_ 1	0	656.74	215.98
36	0	+ 1	+ 1	0	631.46	232.04
37	0	+ 1	0	− 1	613.54	200.74
38	0	+ 1	0	+ 1	542.00	178.34

According to a 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

Table 3
Estimates, Standard Errors and Significance Levels for the Activity of the Enzymes XR and XD in the Bioconversion of Xylose into Xylitol by *C. guilliermondii* as a Function of Different Treatments of Bagasse Hydrolysate, According to a 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

	XR			XD		
Factors and Interactions	Estimate	Standard errors	*p*	Estimate	Standard errors	*p*
Average	525.219	± 23.248		320.979	± 16.608	
[HC]	155.051	± 40.163	0.0007[a]	9.944	± 26.701	0.7171
[A]	3.401	± 29.137	0.9093	−91.031	± 19.901	0.0001[a]
[AC]	46.408	± 40.163	0.2593	−20.456	± 26.701	0.4598
[AT]	48.445	± 40.163	0.2395	29.959	± 26.701	0.2739
[HC] × [A]	69.721	± 40.163	0.0954[b]	−27.716	± 26.701	0.3105
[HC] × [AC]	−41.254	± 44.903	0.3771	−96.571	± 29.852	0.0038[a]
[HC] × [AT]	85.964	± 44.903	0.0676[b]	−16.316	± 29.852	0.5960
[A] × [AC]	−26.412	± 40.163	0.5240	35.062	± 26.701	0.2027
[A] × [AT]	52.991	± 40.163	0.1995	−47.361	± 26.701	0.0899[b]
[AC] × [AT]	−33.841	± 44.903	0.4664	3.574	± 29.852	0.9071
$[HC]^2$	155.831	± 76.833	0.0538[a]	86.393	± 51.293	0.1063[b]
$[AC]^2$	221.236	± 76.833	0.0082[a]	−4.477	± 51.293	0.9322
$[AT]^2$	114.531	± 76.833	0.1491	−69.452	± 51.293	0.1895

[a]Significant at 5% probability level.
[b]Significant at 10% probability level.

level, the hydrolysate concentration and the quadratic terms for the hydrolysate concentration and activated charcoal concentration, presented significant effects. The interactions hydrolysate concentration/acid and hydrolysate concentration/adsorption time presented significant effects, at 10% probability level. The results presented show that hydrolysate concentration has a positive main effect as well as the effects of the interaction between the hydrolysate concentration and the variables type of acid and adsorption time. Therefore, the type of acid (H_3PO_4 in this case) and adsorption time of 60 min caused an increasing of the XR specific activity. According to Alves et al. *(10)*, the treatment of sugarcane bagasse hydrolysate with H_3PO_4 increased the xylitol yield produced by *C. guilliermondii*.

Table 4 presents the regression coefficients, standard errors, *t* values, and significance levels for the model representing XR activity in function of the hydrolysate and activated charcoal concentration. Meanwhile, Table 5 show the results of the quadratic response-surface model fitting, in the form of analysis of variance, denoting that the mathematical model is significant ($p < 0.05$). This is confirmed by the determination coefficient ($R^2 = 0.69$), which indicates that the selected model is suitable for the process and allows an estimation of 69% variance in function of the hydrolysate concentration and activated charcoal concentration.

Table 4
Regression Coefficients, Standard Errors, *t*-Test and Significance Level for the Model Representing the Xylose Reductase Activity of the *C. guilliermondii* in the 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

Factors	Model term	Coefficients	Standard errors	*t*	*p*[a]
Constant		533.28	22.82	23.37	0.0000
[HC]	X_1	155.37	20.27	3.82	0.0006
$[A]^2$	X_1^2	98.89	36.09	2.74	0.0100
$[AC]^2$	X_2^2	131.59	36.09	3.65	0.0009

[a]Significant at 5% probability level.

Table 5
Analysis of Variance for the Model Representing the Xylose Reductase Activity of the *C. guilliermondii* in the 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point[a]

Source of variations	Sum of squares	Degrees of freedom	Mean square	F	Probability *p* (>F)
Model	573196	5	114639.00	13.9477	0.0000
Error	263014	32	8219.19		
Corrected total	836210	37			

[a]$R^2 = 0.69$

The mathematical model for the xylose reductase activity is represented by the equation (1):

$$Y_1 = 533.28 + 155.37\ X_1 + 98.89\ X_1^2 + 131.59\ X_2^2 \quad (1)$$

where Y_1 represents the xylose reductase activity, X_1 the hydrolysate concentration, and X_2 the activated charcoal concentration.

The mathematical model determines, in the study interval, the minimum region for the xylose reductase activity, about 472.27 nmol/mg_{prot}·min, using original hydrolysate treated with 1.75% activated charcoal. However, the response surface and contour lines denote the increase of the enzymatic activity with increase of the concentration of hydrolysate and activated charcoal concentration utilized in the treatment (Fig. 1). Therefore, as can be seen in Fig. 1, it was attained the maximum activity of the xylose reductase (919.13 nmol/mgprotmin) using fourfold concentrated hydrolysate, treated with H_3PO_4 and 3% activated charcoal for 60 min, in the interval considered.

With respect to xylitol dehydrogenase activity, at 5% probability level, the acid and the interaction between hydrolysate concentration and

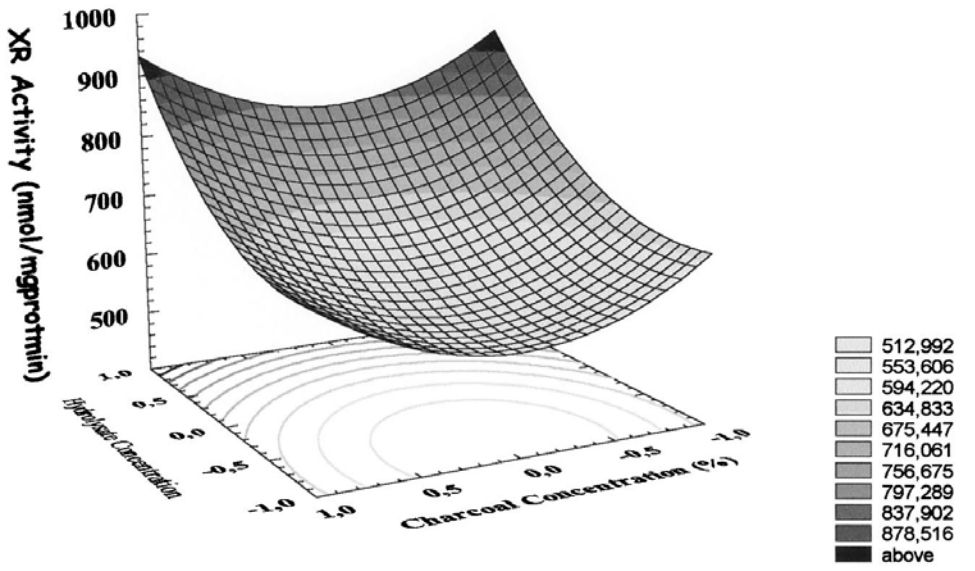

Fig. 1. Response surface and contour lines described by the Y_1 model representing the XR activitity of *C. guilliermondii*.

activated charcoal concentration, presented significant effects (Table 3). Besides, taking into account the quadratic terms related to the hydrolysate concentration it becomes clear that the interaction between type of acid and adsorption time presented significant effects at 10% probability level. The results presented show that acid has a negative main effect and the effects of the interaction between that factor and adsorption time are also negative. Therefore, when treatment of the hydrolysate is carried out with H_2SO_4 and adsorption time of 15 min the XD activity increases, whereas using H_3PO_4 and adsorption time of 60 min such activity diminishes. This result allows to determine the condition in which the yeast has simultaneously high XR activity and low XD activity.

Table 6 presents the regression coefficient, standard errors, *t* values, and significance level for the model representing the xylitol dehydrogenase activity in function of the hydrolysate and activated charcoal concentration. Table 7 shows the results of the quadratic response-surface model fitting, in the form of analysis of variance, denoting that the mathematical model is significant ($p<0.05$). The determination coefficient ($R^2 = 0.58$) indicates that the selected model is suitable for the process and allows an estimation of 58% variance in function of the hydrolysate concentration and activated charcoal concentration.

The mathematical model for the xylitol dehydrogenase activity is represented by Eq. (2):

$$Y_2 = 215.82 - 48.29\ X_1X_2 + 42.4\ X_1^2 \quad (2)$$

where Y_2 represents the xylytol dehydrogenase activity, X_1 the hydrolysate concentration and X_2 the activated charcoal concentration.

Table 6
Regression Coefficients, Standard Errors, *t*-Test and Significance Level for the Model Representing the Xylitol Dehydrogenase Activity of the *C. guilliermondii* in the 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

Factors	Model term	Coefficients	Standard	*t*	*p*
Constant		215.82	15.29	20.97	0.0000[a]
[HC][AC]	X_1X_2	– 48.26	14.19	3.40	0.0019[a]
$[A]_2$	X_1^2	42.40	22.79	1.86	0.0727[b]

[a]Significant at 5% probability level.

[b]Significant at 10% probability level.

Table 7
Analysis of Variance for the Model Representing the Xylitol Dehydrogenase Activity of the *C. guilliermondii* in the 2^4 Factorial Design with a Centered Face and Six Replicates at the Center Point

Source of variations	Sum of squares	Degrees of freedom	Mean square	F	Probability *p* (>*F*)
Model	135568	5	27113.70	8.41810	0.0000
Error	96626.5	30	3220.88		
Corrected total	232195	35			

[a]R^2= 0.58

Figure 2 shows the response surface and the contour lines described by the X_2 model. As can be seen, low values for xylitol dehydrogenase activity was obtained using original hydrolysate treated with 0.5% activated charcoal or by using concentrated hydrolysate treated with 3.0% activated charcoal. For these conditions, the mathematical model makes it possible to obtain the minimum values for these variables, in the study interval at least, resulting in a xylitol dehydrogenase activity of 209.93 $nmol/mg_{prot}{\cdot}min$.

As the hydrolysate concentration had a significant effect on the activity of both XR and XD enzymes, it is justifiable to choose it as the main factor available in this study, because the xylose concentration in the medium is the limiting factor for the induction, synthesis and activity of both enzymes as well as for xylitol production *(7,8)*. According to Nolleau et al. *(7)*, the XR activity of *C. guilliermondii* grown in synthetic medium under microaerobic conditions increased with the increase in initial xylose concentration up to 150 g/L, dropping when the xylose concentration got higher. The XR and XD activities are also influenced by sugars like glucose and arabinose present in the hydrolysate. Kern et al. *(16)* observed that XR and XD enzymes of *C. tenuis* are induced by d-xylose and l-arabinose. According to

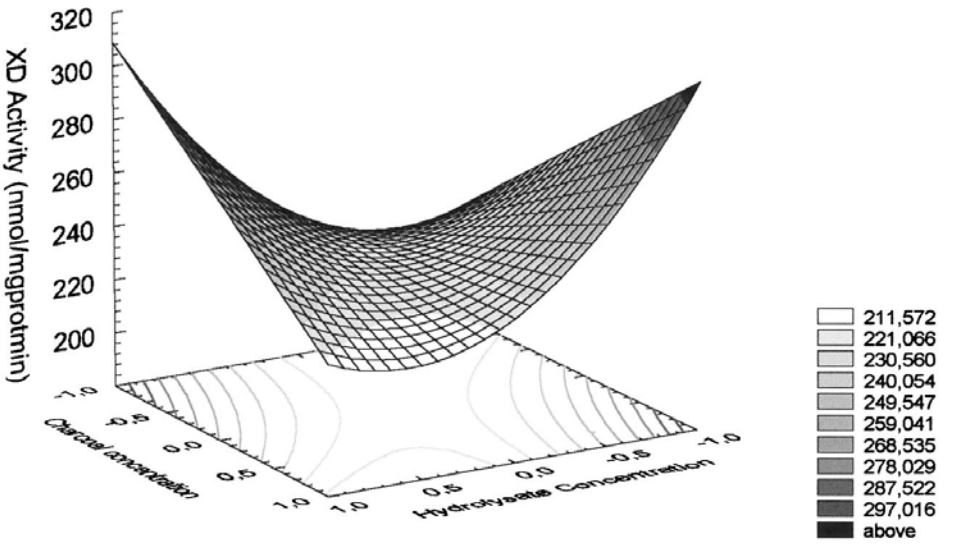

Fig. 2. Response surface and contour lines described by the Y_2 model representing the XD activitity of C. guilliermondii.

these authors, the xylose utilization by this yeast is regulated by induction and catabolic repression of XR and XD. If the medium contains a mixture of sugars (glucose, xylose, arabinose), glucose is metabolized first, leading consequently to the decreasing of XR and XD activities.

According to Parajó et al. *(17,18)*, the activated charcoal treatment of the hydrolysate allows the removal of acetic acid and phenol derivatives, which are inhibitors in several bioconversion processes *(9, 17–19)*. Alves et al. *(10)* reported to have found the maximum xylitol production by treating bagasse hydrolysate concentrated threefold with activated charcoal (2.4%), probably due to the action of this substance in the removal of the inhibitors present in the hydrolysate. According to Lohmeier-Voguel et al. *(20)*, the synergistic effect among these compounds is responsible for this inhibition, being active even when they are present in the medium in low concentration.

Conclusion

From the results obtained it can be concluded that the hydrolysate concentration and the type of treatment influenced the activities of xylose reductase and xylitol dehydrogenase, as shown by the mathematical equations obtained. Thus, *C. guilliermondii* cells with high xylose reductase activity and low xylitol dehydrogenase activity were obtained using fourfold concentrated hydrolysate treated with H_3PO_4 and 3% activated charcoal for 60 min, at least in the interval considered.

Therefore, a study of the influence of treatment of the hydrolysate on the activity of the enzymes acting on the bioconversion of xylose into

xylitol by *C. guilliermondii* is important to make the utilization of sugarcane bagasse hemicellulosic hydrolysate in biotechnological processes viable.

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