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Evaluation of Inoculum of *Candida guilliermondii* Grown in Presence of Glucose on Xylose Reductase and Xylitol Dehydrogenase Activities and Xylitol Production During Batch Fermentation of Sugarcane Bagasse Hydrolysate

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Abstract

The effect of glucose on xylose-xylitol metabolism in fermentation medium consisting of sugarcane bagasse hydrolysate was evaluated by employing an inoculum of *Candida guilliermondii* grown in synthetic media containing, as carbon sources, glucose (30 g/L), xylose (30 g/L), or a mixture of glucose (2 g/L) and xylose (30 g/L). The inoculum medium containing glucose promoted a 2.5-fold increase in xylose reductase activity (0.582 IU/mg_{prot}) and a 2-fold increase in xylitol dehydrogenase activity (0.203 IU/mg_{prot}) when compared with an inoculum-grown medium containing only xylose. The improvement in enzyme activities resulted in higher values of xylitol yield (0.56 g/g) and productivity (0.46 g/[L·h]) after 48 h of fermentation.

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

Introduction

In xylose-utilizing yeasts, the xylose reductase (XR) (EC 1.1.1.21) and xylitol dehydrogenase (XDH) (EC 1.1.1.9) enzymes play a major role in

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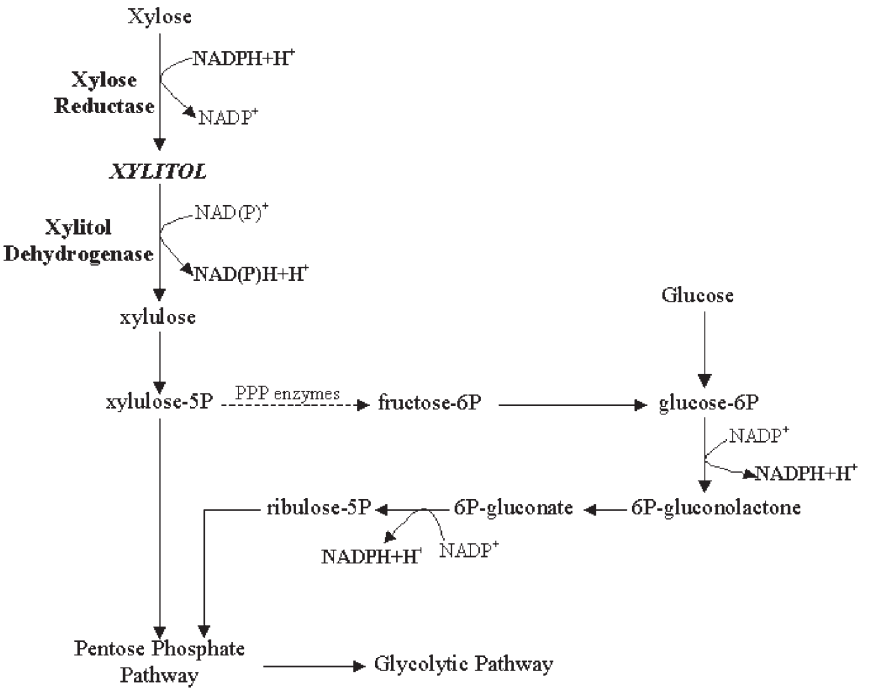

Fig. 1. Scheme of xylose-xylitol metabolism in *C. guilliermondii.*

xylose-to-xylitol bioconversion. XR reduces xylose to xylitol, which is oxidized to xylulose by XDH. Xylulose is then phosphorylated to xylulose-5-phosphate, which can be converted into pyruvate through connection of the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway (Fig. 1).

In *Candida guilliermondii* FTI 20037, XR is exclusively NADPH dependent and XDH has a dual dependence on both NAD and NADP *(1)*. According to Barbosa et al. *(2)*, accumulation and excretion of xylitol are associated with the regeneration of NADPH. In addition, the xylitol flux linearly increases as a function of the dependence of the oxygen limitation and accumulation of NADH *(3)*, because at low O_2 transfer rates, the respiratory chain cannot oxidize the excess NADH, thus preventing the oxidation of xylitol to xylulose and leading to accumulation of xylitol *(4)*. According to Sene et al. *(5)*, XR requires about 10-fold less xylose and cofactor than XDH for the condition in which the reaction rate is half of the V_{max}, and this would explain why xylitol can be produced when the XR/XDH ratio is the lowest.

For biotechnological xylitol production, hemicellulosic hydrolysates from lignocellulosic materials have been used as fermentation media. These hydrolysates contain a complex mixture of sugars, which includes pentoses (xylose and arabinose) and hexoses (glucose and mannose).

According to Tavares et al. *(6)*, the general pattern observed for yeast batch cultivation in sugar mixtures is the inhibition or retardation of xylose utilization owing to the presence of glucose in the growth media. However, the various pentose-fermenting yeasts seem to differ in their sensitivity to the regulatory effect of glucose and other hexoses.

Some studies indicate that the presence of glucose in the fermentation medium can improve xylitol production *(6–9)*. The stimulation of xylose metabolism by glucose can be explained by the regeneration of NADPH for the initial steps of xylose metabolism *(10,11)*, because NADPH, the cosubstrate used by XR in *C. guilliermondii* yeast, is generated through the oxidative pentose phosphate pathway. The regulatory effects of D-glucose may be exerted on D-xylose transport or on parts of the D-xylose-inducible pathway *(12)*. Sugai and Delgenes *(13)* related that glucose partially repressed the induction of aldose reductase by D-xylose and the intensity of the catabolite repression correlating with the glucose concentration in the induction system. Additionally, glucose may exert an inducer effect on the induction of aldose reductase activity.

In the present study, the inoculum was pregrown in synthetic media containing glucose, a mixture of glucose and xylose, or only xylose as carbon sources. The effects of this inoculum on XR and XDH activities of *C. guilliermondii* during the fermentation of sugarcane bagasse hydrolysate are described.

Materials and Methods

Microorganism and Preparation of Inoculum

The experiments were conducted with *C. guilliermondii* FTI 20037 maintained at 4°C on malt-extract agar slants. The medium used for the inoculum preparation contained glucose (30.0 g/L) or a mixture of glucose (2.0 g/L) and xylose (30.0 g/L) supplemented with nutrients, namely rice bran extract (20.0 g/L), $(NH_4)_2SO_4$ (2.0 g/L), and $CaCl_2{\cdot}2H_2O$ (0.1 g/L). A control experiment was also performed employing medium containing only xylose (30.0 g/L) and nutrients. Erlenmeyer flasks (125 mL), each containing 50 mL of medium, were incubated on a rotary shaker (200 rpm) at 30°C for 24 h. Afterward, the cells were separated by centrifugation (2000*g*; for 20 min), and rinsed twice with distilled water, and the cell pellet was resuspended in an adequate volume of distilled water. The initial cell concentration for all the experiments was about 1.0 g/L.

Preparation of Sugarcane Bagasse Hydrolysate

Sugarcane bagasse was hydrolyzed in a 250-L reactor at 121°C for 20 min with H_2SO_4 at a 1:10 solid/liquid ratio (100 mg of H_2SO_4/g of dry matter). The hydrolysate was filtered, concentrated at 70°C under vacuum to obtain a threefold increase in the xylose content, and stored

at 5°C. Afterward, it was submitted to a treatment consisting of pH adjustment to 7.0 with CaO (commercial grade) and to 2.5 with H_3PO_4, followed by the addition of 1.0% (w/v) activated charcoal (refined powder) for 30 min under agitation (200 rpm for 60°C). The precipitate formed as a result of this treatment was removed by vacuum filtration and then autoclaved at 111°C, 0.5 atm for 15 min in order to be used as the fermentation medium.

Medium and Fermentation Conditions

Concentrated and treated bagasse hemicellulosic hydrolysate 45 g/L of xylose, 1.75 g/L of glucose, 2.50 g/L of arabinose, 1.99 g/L of acetic acid, 0.011 g/L of furfural, and 0.004 g/L of hydroxymethylfurfural (HMF) was supplemented with nutrients whose contents were 20.0 g/L of rice bran extract, 2.0 g/L of $(NH_4)_2SO_4$, and 0.1 g/L of $CaCl_2{\cdot}2H_2O$. Experiments were performed in a KLF 2000 bench-scale fermentor (Bioengineering Co./Swiss) under the following conditions: working volume of 1.6 L, initial pH adjusted to 5.5 by the addition of an NaOH solution, 500 rpm, and K_La of 17 h^{-1} at 30°C.

Enzyme Assays

The cells obtained in the experiments were harvested by centrifuging at 2700*g* for 15 min and resuspended in 0.1 *M* phosphate buffer (pH 7.2). The final suspension (15.0 g/L) was stored in a freezer. The cell suspension was thawed, and the 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 XDH activities. Enzyme activities were determined spectrophotometrically at 340 nm at room temperature *(5)*. One XR or XDH unit (IU) was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADP/min or 1 μmol of NADPH/min, respectively.

Analytical Methods

Xylose, glucose, arabinose, xylitol, glycerol, ethanol, acetic acid, furfural, and HMF concentrations were determined by high-performance liquid chromatography *(14)*. Cell growth was monitored by measuring the absorbance at 600 nm. Cell concentration (g/L) was calculated based on the relation of optical density and cell dry weight through a calibration curve. The volumetric oxygen transfer coefficient (K_La) was determined by the gassing-out methodology *(15)*. Specific activities were obtained by determining the protein content according to the method of Bradford *(16)* using bovine serum albumin as the standard and were expressed as international units per milligram of protein.

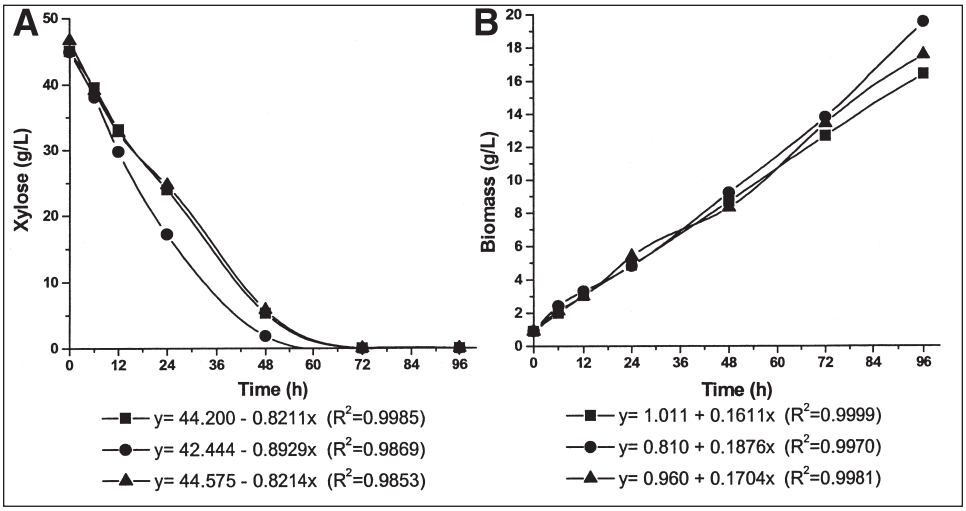

Fig. 2. Effect of inoculum growth medium on **(A)** xylose consumption and **(B)** cell growth during fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii:* (■) medium containing glucose; (●) medium containing mixture of xylose and glucose; and (▲) medium containing xylose, as carbon source.

Results

Xylose Consumption and Cell Growth

To evaluate the effect of glucose on the formation of xylitol in *C. guilliermondii*, fermentations were carried out utilizing inoculum pregrown in synthetic media containing glucose, a mixture of glucose and xylose, or only xylose. Xylose consumption (Fig. 2A) was affected by the carbon source employed in the inoculum growth medium. After 48 h of fermentation, more than 85% of xylose was consumed in all conditions, but when utilizing cells pregrown in a mixture of glucose and xylose, xylose consumption rate was favored. Xylose consumption was similar when cells pregrown in medium containing only glucose or only xylose were employed. These observations are confirmed by the analysis of angular coefficients of the straight lines, using the range of higher linearity [0,48], which correlates to the decrease in xylose levels and fermentation time.

Glucose, arabinose, and acetic acid were totally consumed in all fermentations, but it was not possible to establish a correlation between the assimilation of these compounds and the carbon sources employed in the inoculum media (data not shown).

Similar to xylose consumption, cell growth was improved by inoculum grown in medium containing a mixture of glucose and xylose (Fig. 2B). The highest biomass concentration (19.56 g/L) obtained from inoculum grown in the mixture of sugars presented increases of 19 and 11%, in relation to

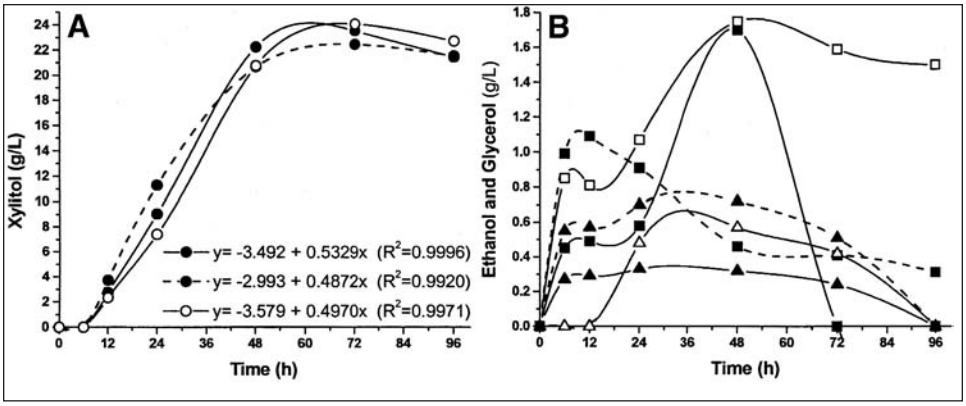

Fig. 3. Effect of inoculum growth medium on **(A)** xylitol formation (●) and **(B)** glycerol (▲) and ethanol (■) concentrations during fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii:* solid symbols, medium containing glucose; dashed line, medium containing mixture of xylose and glucose; and open symbols, medium containing xylose, as carbon source.

the results observed for the inoculum grown in glucose and in the control medium, respectively.

Formation of Xylitol and Byproducts (Glycerol and Ethanol)

Xylitol formation was also improved by inoculum grown in the presence of glucose (Fig. 3A). When employing cells pregrown in a mixture of glucose and xylose, the xylitol concentration (11.29 g/L) was 52.77% higher than that observed in the control medium, after 24 h of fermentation, but this improvement did not continue during the fermentation. After 48 h of fermentation, the highest accumulation of xylitol (22.23 g/L) was observed with cells pregrown in medium containing only glucose. Cells pregrown in glucose also improved the xylitol formation rate, which can be confirmed by the analysis of angular coefficients of the straight lines, using the range of higher linearity [6,48] (Fig. 3A). Figure 3A shows a decrease in xylitol concentration especially after 72 h, when the carbon sources (xylose, glucose, arabinose, and acetic acid) were totally consumed.

The presence of glucose in the inoculum growth medium also favored xylitol yield and productivity (Fig. 4). After 48 h of fermentation, when >85% of xylose was assimilated (Fig. 2A), the highest values of fermentative parameters were observed for all the conditions evaluated. Using only glucose as the carbon source in inolucum growth medium resulted in the highest values of xylitol yield (0.56 g/g), xylitol productivity (0.46 g/L·h]), and conversion efficiency (61.07%) (Fig. 4).

Besides xylitol, glycerol and ethanol, which are byproducts of the yeast metabolism, were produced (Fig. 3B). At the beginning of the fermentations, glycerol and ethanol production rates were higher, independent of the

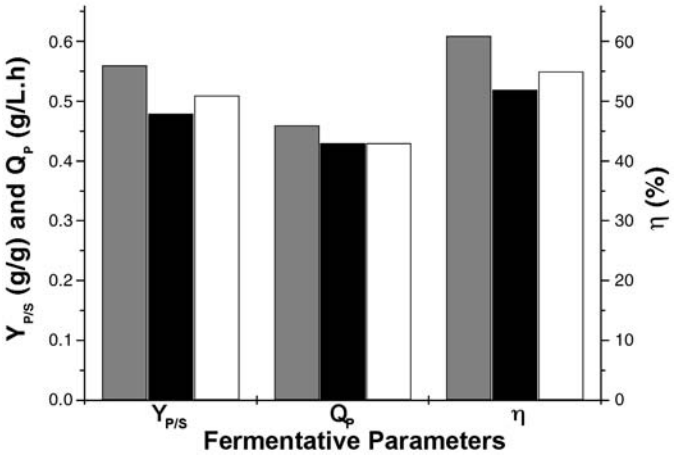

Fig. 4. Effect of inoculum growth medium on xylitol yield ($Y_{P/S}$), productivity (Q_P), and conversion efficiency (η) after 48 h of fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii:* (▪) gray bars, medium containing glucose; (■) black bars, medium containing mixture of xylose and glucose; and (❑) white bars, medium containing xylose, as carbon source.

inoculum growth medium. Like xylitol, these byproducts were assimilated by the yeast, mainly after 72 h, when the carbon sources were totally consumed. However, it was not possible to correlate the carbon source employed in the inoculum growth medium with the formation of these products. The highest ethanol concentration (1.75 g/L) was observed when the inoculum was grown in medium containing only xylose, and the highest glycerol concentration (0.72 g/L) was obtained from inoculum grown in a mixture of xylose and glucose.

Activities of XR and XDH

Table 1 shows the specific activities of XR and XDH during hemicellulosic sugarcane bagasse hydrolysate fermentation by *C. guilliermondii* cells obtained from inoculum pregrown in media containing glucose, a mixture of glucose and xylose, or only xylose as energy and carbon sources.

According to Table 1, the specific activities of XR and XDH were influenced by the carbon source employed in the inoculum growth medium. Maximum specific activity values for XR (0.5817 IU/mg_{prot}) and XDH (0.2029 IU/mg_{prot}) were observed, at 48 h of fermentation, when the inoculum was grown in glucose. These values represent a 2.5- and a 2.0-fold increase, respectively, when compared with those obtained from inoculum medium containing only xylose (control).

Furthermore, after the first 24 h of fermentation the cells pregrown in a mixture of glucose and xylose presented values of XR and XDH activities higher than those observed for cells grown in glucose or xylose, and xylose consumption and xylitol formation were higher.

Table 1
Effect of Inoculum Growth Medium on XR and XDH Specific Activities (IU/mg_{prot}) of *C. guilliermondii* During Fermentation of Sugarcane Bagasse Hydrolysate.

	XR (IU/mg_{prot})			XDH (IU/mg_{prot})		
Time (h)	Glucose	Glucose + xylose	Xylose	Glucose	Glucose + xylose	Xylose
0	0.082	0.190	0.158	0.057	0.333	0.224
6	0.110	0.172	0.137	0.039	0.090	0.120
12	0.244	0.223	0.156	0.092	0.149	0.073
24	0.247	0.379	0.244	0.133	0.199	0.115
48	0.582	0.402	0.232	0.203	0.191	0.098
72	0.408	0.285	0.203	0.111	0.132	0.125
96	0.287	0.203	0.200	0.054	0.039	0.065

Table 2
XR/XDH Ratio During Fermentation of Sugarcane Bagasse Hydrolysate by *C. guilliermondii* Pregrown in Medium Containing Glucose, Xylose and Glucose, or Xylose as Carbon Source

Carbon source for cultivation of inoculum	Time (h)						
	0	6	12	24	48	72	96
Glucose	1.44	2.85	2.65	1.86	2.87	3.70	5.29
Glucose + xylose	0.57	1.92	1.50	1.91	2.10	2.16	5.26
Xylose	0.71	1.14	2.14	2.11	2.36	1.62	3.08

In all the conditions evaluated, enzymatic activities, mainly XR, increased during the first 48 h of fermentation (and decreased afterward), when >85% of xylose was consumed (Fig. 2A).

Independent of the carbon source employed for the inoculum cultivation, there was generally an increase in the XR/XDH ratios during the fermentation (Table 2). After 48 h, the XR activity reached a value about three times higher than the XDH activity value when the inoculum was grown in medium containing glucose. This represented an improvement of 21.6% in the XR/XDH ratio in comparison with the control medium (inoculum growth medium containing only xylose).

Discussion

The results presented in this work demonstrate that the presence of glucose in inoculum growth medium improved the xylose-to-xylitol bioconversion. Xylose consumption rate and cell growth were favored

by using cells pregrown in medium containing a mixture of glucose and xylose. However, xylitol formation rate was improved by using cells pregrown in medium containing only glucose.

Kastner et al. *(17)* related a reduction of 5 and 7% in xylose consumption and xylitol formation, respectively, in fermentation of synthetic medium by *Candida tropicalis* pregrown in glucose when compared with cells pregrown in xylose.

A decrease in xylitol concentration mainly after 72 h, when the carbon sources were totally consumed, was also observed, indicating that this polyol was assimilated by the yeast. Its concentration also decreased during the cultivation of *C. guilliermondii* in synthetic medium *(18)* and in sugarcane bagasse hydrolysate *(19,20)*. In addition, experiments conducted in our laboratories revealed that xylitol was utilized as a carbon source by *C. guilliermondii* for cell growth (unpublished results). Xylitol was also used as a carbon source by *Pachysolen tannophilus* cultivated in synthetic medium *(21)*.

It was not possible to establish a correlation between the formation of the subproducts glycerol and ethanol during this metabolism and the presence of glucose in the inoculum growth medium. Glycerol and ethanol can be mainly produced from glucose, but xylose and arabinose metabolism can also contribute to their formation. In addition, both gly cerol and ethanol play an important role in redox regulation, regenerating NAD^+. These byproducts were also assimilated by yeast, and it should be stressed that glycerol and ethanol consumption was accompanied by an increase in cell growth (Fig. 2B). According to Flores et al. *(22)*, glycerol and ethanol can be used as carbon and energy sources by many yeasts.

Some work indicates that xylose induces XR and XDH activities and that glucose can repress this induction *(23,24)*. Thus, utilizing cells pregrown in glucose should result in the lowest values of enzymatic activities, and utilizing cells pre-grown in xylose should give the highest values. However, depending on its concentration, glucose combined with xylose can have an inducing effect on enzyme activity *(9,13)*. Rosa et al. *(9)* observed during the cultivation of *C. guilliermondii* in synthetic medium that XR and XDH activities were sensitive to the glucose content in the medium for a glucose/xylose ratio above 10%.

In the present work, the presence of glucose in the inoculum growth medium improved enzymatic activities. It was observed that after the first 24 h of fermentation, cells pregrown in a mixture of glucose and xylose presented values of XR and XDH activities higher than those observed with cells grown in glucose or xylose, and the xylose consumption and xylitol formation were the highest, coinciding with the lowest XR/XDH ratio. According to Sene et al. *(5)*, XR requires about 10-fold less xylose and cofactor than XDH for the condition in which the reaction rate is half the V_{max}. This would explain why xylitol can be produced when the XR/XDH ratio is the lowest.

It is important to note that sugarcane bagasse hydrolysate is a complex medium containing compounds such as acetic acid and phenols, which are toxic to the yeast. Thus, not only glucose, but the presence of these toxic compounds, can affect enzymatic activities. The influence of toxic compounds was also cited by Sene et al. *(5)*, who affirmed that the best conditions for XR and XDH production did not provide the highest xylitol production, probably because other factors, such as the presence of inhibitors in the hydrolysate, influenced the overall xylose/xylitol conversion.

Conclusion

The use of inoculum of *C. guilliermondii* grown in synthetic medium containing only glucose as a carbon source resulted in the highest XR (0.582 IU/mg_{prot}) and XDH (0.203 IU/mg_{prot}) activities and maximum values of xylitol yield (0.56 g/g) and productivity (0.46 g/[L·h]). However, the use of inoculum grown in medium containing a mixture of glucose and xylose improved xylose consumption rate and cell growth. In the future, we plan to investigate the mechanisms of glucose's influence on xylose-to-xylitol bioconversion, because sugarcane bagasse hydrolysate also contains compounds toxic to the yeast's metabolism.

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