0273-2289/00/84–86/0801/$12.00

Inhibition of Microbial Xylitol Production by Acetic Acid and Its Relation with Fermentative Parameters

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Abstract

Precipitated sugarcane bagasse hemicellulosic hydrolysate containing acetic acid was fermented by *Candida guilliermondii* FTI 20037 under different operational conditions (pH 4.0 and 7.0, three aeration rates). At pH 7.0 and k_La of 10 (0.75 vvm) and 22.5/h (3.0 vvm) the acetic acid had not been consumed until the end of the fermentations, whereas at the same pH and k_La of 35/h (4.5 vvm) the acid was rapidly consumed and acetic acid inhibition was not important. On the other hand, fermentations at an initial pH of 4.0 and k_La of 22.5 and 35/h required less time for the acid uptake than fermentations at k_La of 10/h. The acetic acid assimilation by the yeast indicates the ability of this strain to ferment in partially detoxified medium, making possible the utilization of the sugarcane bagasse hydrolysate in this bioprocess. The effects on xylitol yield and production are reported.

Index Entries: Xylitol; hydrolysate; acetic acid; k_La; pH.

Introduction

The aim of biotechnology is to process materials through the action of biological agents and to obtain marketable products by means of technologies that employ, in a practical and economical way, residues from different sources. Lignocellulosics, the most numerous forest and agricultural residues, are of great technological interest, because they are abundant renewable sources of sugar. The relatively low polymerization degree and the open and heterogeneous structure *(1,2)* of hemicellulose (an important lignocellulose component) facilitate the release of fermentable carbohydrates, allowing its utilization in biotechnological processes.

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The degradation of hemicellulose can be performed by physical, chemical, or biological methods *(3)*, such as dilute acid pretreatment, an efficient chemical method that has been largely employed to produce xylose from hemicellulose. The fermentation activity can be inhibited by metals from equipment corrosion, products derived from the hemicellulose hydrolysis, or lignin degradation such as acetic acid, furfural, hydroxymethylfurfural (HMF), phenolic compounds, aromatic acids, and aldehydes *(4)*. The maximum allowable concentration for each inhibitor is dependent on the microorganism utilized and its degree of adaptation, the fermentation process employed, and the simultaneous presence of several other inhibitors *(5)*.

Acetic acid, a compound released from the acetyl groups of lignocellulosics *(4)*, has been considered a strong inhibitor of xylose metabolism by cells *(6,7)*, because besides its interference with the xylose uptake through the cytoplasmatic membrane, it also can inhibit the activity or biosynthesis of xylose reductase *(8)*, which is fundamental to the metabolism of this carbohydrate. The inhibitory effect of acetic acid is a function of its undissociated form (which is therefore pH dependent) *(5)*, aeration rates *(9)*, origin of the culture medium *(10)*, and the presence of other inhibitory compounds *(11)*.

Owing to these compounds, the fermentation media composed of hemicellulosic hydrolysates must be detoxified by the adaptation of the microorganism *(12,13)*, by treatment with molecular sieves, ion-exchange resins or charcoal *(7,14,15)*, by steam stripping and overtitration *(16)*, and by the choice of suitable fermentative parameter values.

One of the fermentative processes that can be affected by the presence of inhibitors *(10,11,17,18)* is xylitol production. Xylitol, a polyol with high added value, has anticariogenic properties *(19)*, high sweetening power *(20)*, and can be utilized as a food ingredient, because it neither undergoes Maillard reaction nor causes obesity *(21)*.

The pH and the aeration rate, which can be expressed as the volumetric oxygen transfer coefficient (k_La), are considered important process parameters for determining the toxic effect of acetic acid on cell metabolism.

This article deals with sugarcane bagasse hemicellulosic hydrolysate fermentation by *Candida guilliermondii* in medium containing acetic acid. The effect of this acid on the microbiological xylitol production rates, under different operational conditions, is also described.

Materials and Methods

Raw Material and Hydrolysate Concentration

Hemicellulosic hydrolysate was obtained through the extraction of sugarcane bagasse (supplied by Usina Santa Bárbara, Santa Bárbara d´Oeste, São Paulo), as described by Rodrigues *(22)*. To triple the xylose content the hydrolysate was concentrated under vacuum at 70°C and stored at 8°C. The hydrolysate was composed of the following: 56.56 g/L of xylose,

4.93 g/L of acetic acid, 0.06 g/L of furfural, 0.17 g/L of HMF, 3.91 g/L of glucose, and 5.40 g/L of arabinose.

Hydrolysate Treatment

The hydrolysate pH was adjusted to 7.0 by the addition of commercial CaO. The resulting precipitate was removed and the supernatant pH was reduced to 5.5 with H_3PO_4, originating a new precipitate, which was also removed. Activated charcoal was then added to the supernatant and the hydrolysate was submitted to agitation at 30°C at 200 rpm (rotatory agitator; New Brunswick Scientific, Edison, NJ) for 1 h. The activated charcoal was removed by vacuum filtration and the clarified hydrolysate was autoclaved at 111°C for 15 min.

Microorganism and Growth Conditions

Cultures of the yeast *C. guilliermondii* FTI 20037, supplied by FAENQUIL, Biotechnology Department, Lorena, São Paulo, Brazil, were maintained in tubes containing malt-extract agar at 4°C. The cell inoculum was prepared from a suspension of yeasts grown in 125-mL Erlenmeyer flasks containing 50 mL of the following: 30 g/L of xylose, 5 g/L of $(NH_4)_2SO_4$, 0.1 g/L of $CaCl_2$, and 10 g/L of rice bran extract at 30°C at 200 rpm for 24 h.

Medium and Cultivation Conditions

Bioconversion experiments were performed in a BIOFLO III bench-scale fermentor (New Brunswick Scientific, Edison, NJ) with 1.0 L of medium composed of treated sugarcane bagasse hemicellulosic hydrolysate (the same nutrient used to prepare the inoculum). The same nutrients were utilized to prepare the inoculum, except for xylose, at 30°C and 300 rpm, initial pH 4.0 and 7.0, and k_La of 10, 22.5, and 35/h. Initial cell concentration in the fermentation medium was fixed at 0.2 g/L.

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 RP 18 column at 25°C with acetonitrile:water (1:8) and 1% acetic acid as the eluent, and a 0.8 mL/min flow rate was employed for determination of furfural and HMF in a visible ultraviolet-light detector (SPD-10A UV-VIS).

Cell growth was monitored by measuring absorbance at 600 nm (Beckman-DU 640B spectrophotometer). Cell concentration was calculated based on the relation of optical density and cell dry weight through a calibration curve. The k_La was determined by the gassing-out methodology *(23)*.

Results and Discussion

Acetic acid has been described as a potent inhibitor of the xylose metabolism *(7,9,10)*. It has been postulated that, in yeasts such as *Saccharomyces cerevisiae (24)* and *C. guilliermondii (25)*, cell growth and xylose metabolism inhibition by acetic acid is caused by its penetration into cells, resulting in intracellular acidification *(24)*. This leads to a decoupling of energy production and of the transport of various nutrients *(26)*. In agreement with our results, at pH 4.0 and k_La of 10/h (Fig. 1A) the acetic acid was completely consumed in about 100 h, and cells reached a final concentration of 4.3 g/L. A reduction in time and an increase in cell production coincided with an increased aeration rate of k_La = 22.5 and 35/h (Fig. 1B,C). The long time observed for acetic acid uptake at the lowest oxygen transfer rate (k_La = 10/h) was owing to the very low cell volumetric productivity attained (Q_x = 0.035 g/[g·h]). A lag phase of about 24 occurred in all the fermentations conducted at pH 4.0 (Fig. 1A–C). Roberto et al. *(27)* and Mohandas et al. *(28)* also observed a lag phase of *C. guilliermondii* and *Pichia stipitis* cultivated in hemicellulosic hydrolysates at pH 4.5 and 5.0, respectively. Figure 2C shows that at pH 7.0, and with a greater oxygen supply (k_La = 35/h), the time required for the acetic acid to be totally consumed was shorter than in other aeration conditions (Fig. 2A,B). Final biomass concentration at pH 7.0 was also higher at k_La of 35/h. Meinander et al. *(29)* reported that under aerobic conditions cells were capable of assimilating acetic acid and of producing cell mass. Pessoa et al. *(30)* also reported a faster uptake of acetic acid at higher aeration rates and initial pH 6.0 in hemicellulosic hydrolysate fermentation by *C. tropicalis*.

Although acetic acid had been consumed more rapidly in experiments with a high oxygen supply, greater xylitol yield factors were obtained for both pH values when the low aeration rate was utilized (*see* Table 1). Table 1 also shows that xylitol volumetric productivities were low at initial pH 4.0 in all aeration rates and that its values increased with an increase in pH.

It is clear that the toxic effect of acetic acid on the xylose metabolism depends on the fermentation conditions and on the yeast strain employed *(10)*. High yields of xylitol occurred without acetic acid with a xylose utilization rate of 0.74 g/(L·h) *(31)*, and with 1 g/L of the same acid *(32)* in fermentations performed in a semisynthetic medium at pH 2.5 and oxygen-limited conditions with *C. guilliermondii*. When sugarcane bagasse hydrolysate was used for cultivation of this yeast at pH values lower than 4.5 and with about 4.5 g/L of acetic acid, the uptake of xylose and other sugars and the production of xylitol were strongly inhibited *(10)*. The pattern of the fermentation conducted by *C. parapsilosis* in aspenwood hydrolysate was not disturbed at pH 4.75 in the presence of 3 g/L of acetate *(11)*. Almeida e Silva et al. *(33)* observed xylitol formation assistance at low concentrations of acetic acid in experiments conducted with *Paecilomyces variotii* in eucalyptus hydrolysate. A possible reason that xylose fermentation is stimulated by acetic acid at low concentrations is that part of the acetic acid

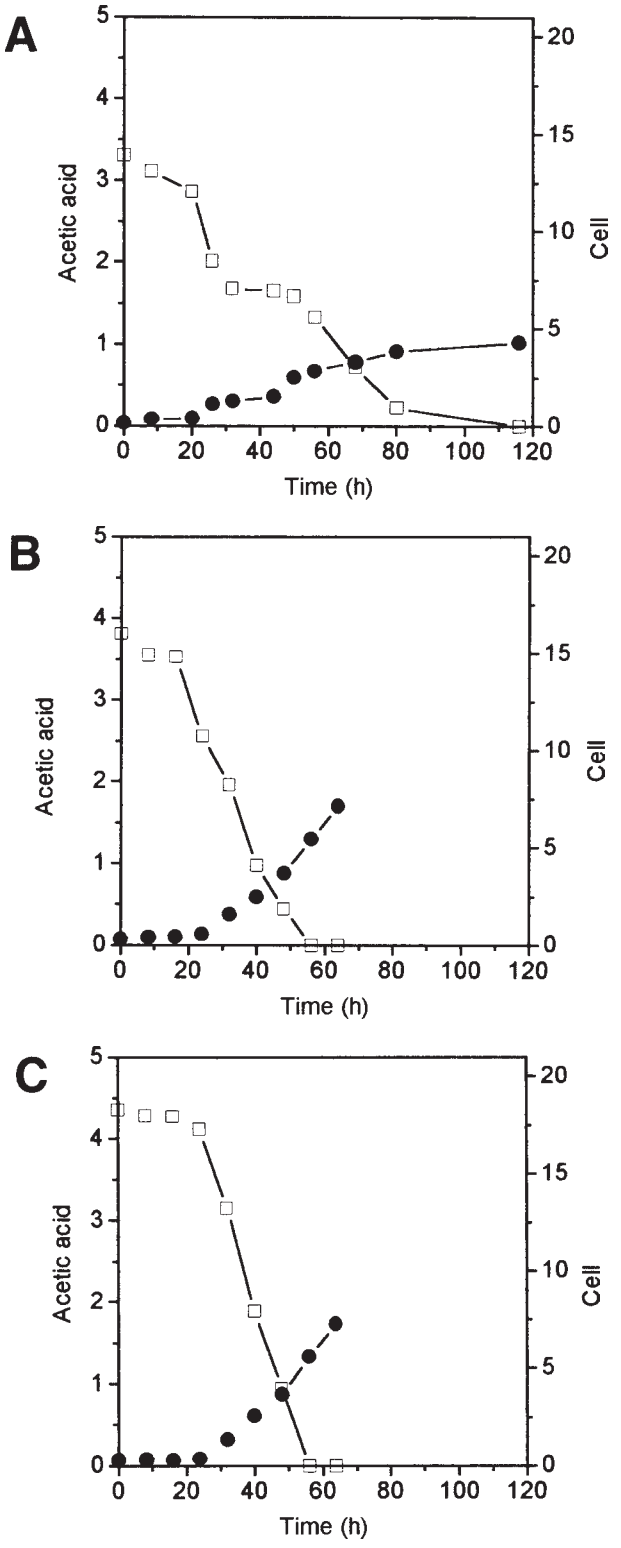

Fig. 1. Acetic acid (□) and cell (●) concentrations (g/L) in fermentations conducted at pH 4.0 and $k_L a$ of 10/h **(A)**, 22.5/h **(B)**, and 35/h **(C)**.

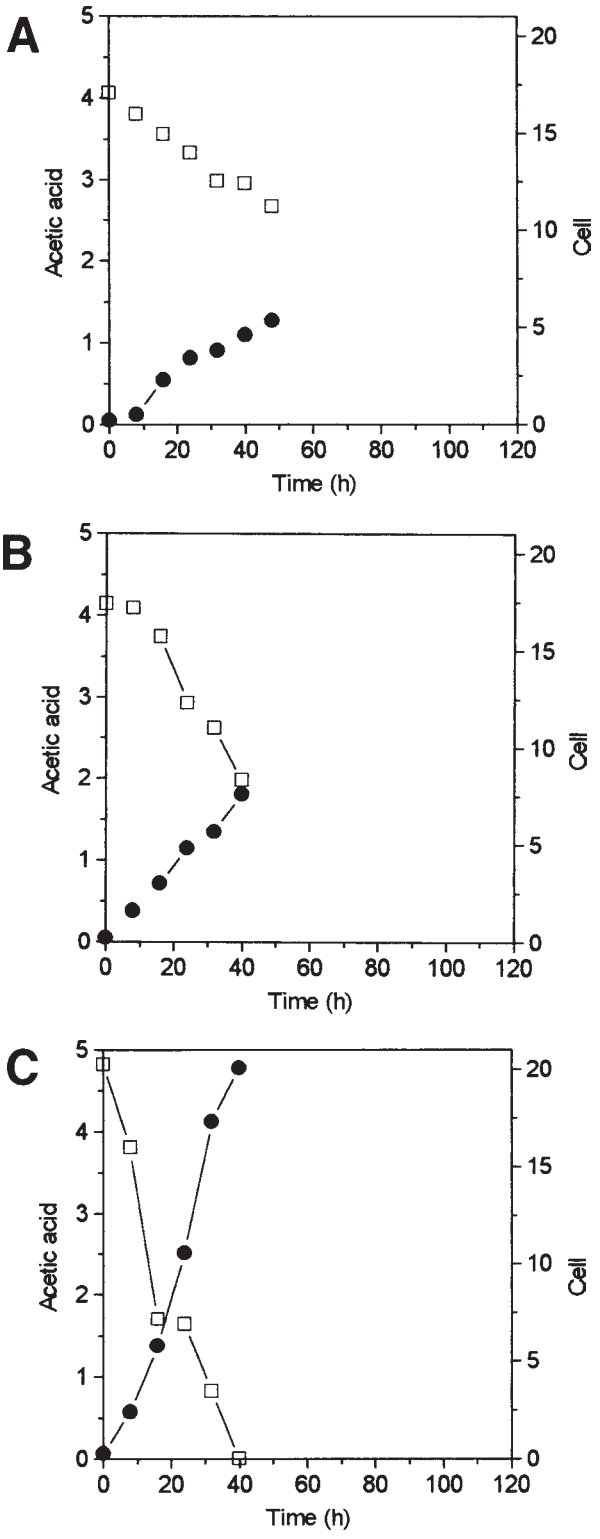

Fig. 2. Acetic acid (□) and cell (●) concentrations (g/L) in fermentations conducted at pH 7.0 and $k_L a$ of 10/h **(A)**, 22.5/h **(B)**, and 35/h **(C)**.

Table 1
Xylitol Volumetric Productivities, Xylitol Yield Factors, Xylose Uptake Rate, and Final Xylitol Concentration Obtained in Sugarcane Bagasse Hydrolysate Fermentations

pH	K_La	Q_p [a]	$Y_{P/S}$	ΔS	P
4.0	10	0.26	0.69	99	30.21
7.0		0.56	0.79	87	26.64
4.0	22.5	0.38	0.61	93	24.53
7.0		0.72	0.71	95	28.64
4.0	35	0.45	0.64	87	28.60
7.0		0.56	0.35	98	22.35

[a]Q_p, xylitol volumetric productivity (g/[L·h]); $Y_{P/S}$, xylitol yield factor (g/g); ΔS, xylose consumption (%); and P, final xylitol concentration (g/L).

directly enters the Krebs cycle via acetyl-CoA, whereas at acid concentrations of >1.0 g/L, part of the acid continues to be directed toward the Krebs cycle and the remainder may be utilized by another energy-consuming metabolic pathway. This may result in the lack of energy for the maintenance of the overall metabolism of the yeast, leading, e.g., to reduced cell growth *(25)*.

The inhibitory effect of the acetic acid depends not only on the fermentation conditions and yeast strain, but also on the presence of other compounds in the culture medium such as furfural, HMF, and phenolic compounds. Preziosi-Belloy et al. *(11)* verified no growth in a semidefined medium containing low concentrations of furfural, HMF, vanillin, syringaldehyde, and acetic acid. They hypothesized that these compounds act cumulatively (synergistic effect) to inhibit the fermentative activity by interfering with sugar uptake or other essential elements. The synergistic effect of inhibitors was also reported by Tran and Chambers *(20)* in red oak hydrolysates and by Lohmeier-Vogel et al. *(34)* in a semisynthetic medium.

According to our results, the inhibitory effect of the acetic acid can be overcome by a simple and economical sugarcane bagasse hydrolysate treatment and by the use of suitable pH and aeration values, making possible the utilization of sugarcane bagasse in a large-scale microbial xylitol production. We also have shown a relation among acetic acid and xylose uptake rates and xylitol formation.

Acknowledgments

We thank Maria Eunice M. Coelho for revising the manuscript and acknowledge financial assistance from FAPESP.

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