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INHIBITORY EFFECT OF ACETIC ACID ON BIOCONVERSION OF XYLOSE IN XYLITOL BY *CANDIDA GUILLIERMONDII* IN SUGARCANE BAGASSE HYDROLYSATE

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ABSTRACT

Sugarcane bagasse hydrolysate (initial acetic acid concentration = 3.5g/L), was used as a fermentation medium for conversion of xylose into xylitol by the yeast *Candida guilliermondii* FTI 20037. Acetic acid (2.0g/L) was added to the medium at different times of fermentation, with the aim of evaluating its effects on the bioconversion process. The addition of acetic acid to the medium after 12h of fermentation resulted in the strongest inhibition of the yeast metabolism. In this case, the xylose consumption and cell growth were, respectively, 23.22 and 11.24% lower than when acid was added to the medium at the beginning of fermentation. As a consequence of the inhibitory effect, lower values of the xylitol yield (0.39g/g) and productivity (0.22g/L.h) were observed, corresponding to a reduction of 36 and 48%, respectively, in relation to the values obtained with the addition of acetic acid after other fermentation times. The results obtained allowed to conclude that, under the experimental conditions employed in this work, the inhibitory effect of acetic acid on the xylose-xylitol bioconversion depends on the fermentation time when this acid was added, and not only on its concentration in the medium.

Key words: xylitol, xylose, acetic acid, sugarcane bagasse hydrolysate

INTRODUCTION

Xylitol is an anticariogenic sweetener suitable for diabetic and obese people (10,13) and able to prevent osteoporosis (11) and otitis (19). Nowadays, xylitol is produced by catalytic hydrogenation of pure xylose extracted from wood hydrolysates. However, xylitol can also be obtained from agro-industrial residues by biotechnological process employing yeasts like *Candida guilliermondii,* since they have two key enzymes in the xylose metabolism: NADPH-dependent xylose reductase and NAD^+-dependent xylitol dehydrogenase. The former reduces xylose to xylitol and the latter oxidises xylitol to xylulose and both are induced by xylose (18). The researches on the microbiological production of xylitol have been intensified, not only because of the peculiarities of this sweetener, but mainly due to the possibility of the fermentative process to become an alternative to the chemical synthesis (20) (Fig. 1). However, the production of xylitol through fermentation of hemicelullosic hydrolysates, a substrate rich in xylose, is hampered by the toxic compounds resulting from the hydrolysis of the lignocellulosic materials, such as acetic acid, phenol derivatives, furfural, hydroxymethylfurfural, which are toxic to the yeast (1).

Acetic acid has been pointed out as a strong inhibitor of xylose metabolism of yeast cells (5) but its toxicity to *C. guilliermondii* depends on its concentration (4). According to Maiorella *et al.* (9), acetic acid inhibits the yeast metabolism by chemical interference with the phosphate transport through the cell membrane, which requires expenditure of ATP. Acetic acid interference results in an increase in the ATP required for this maintenance function, as well as interferes with the cell morphology. Lawford and Rousseau (8) reported that acetic acid toxicity is related to the ability of undissociated (protonated)

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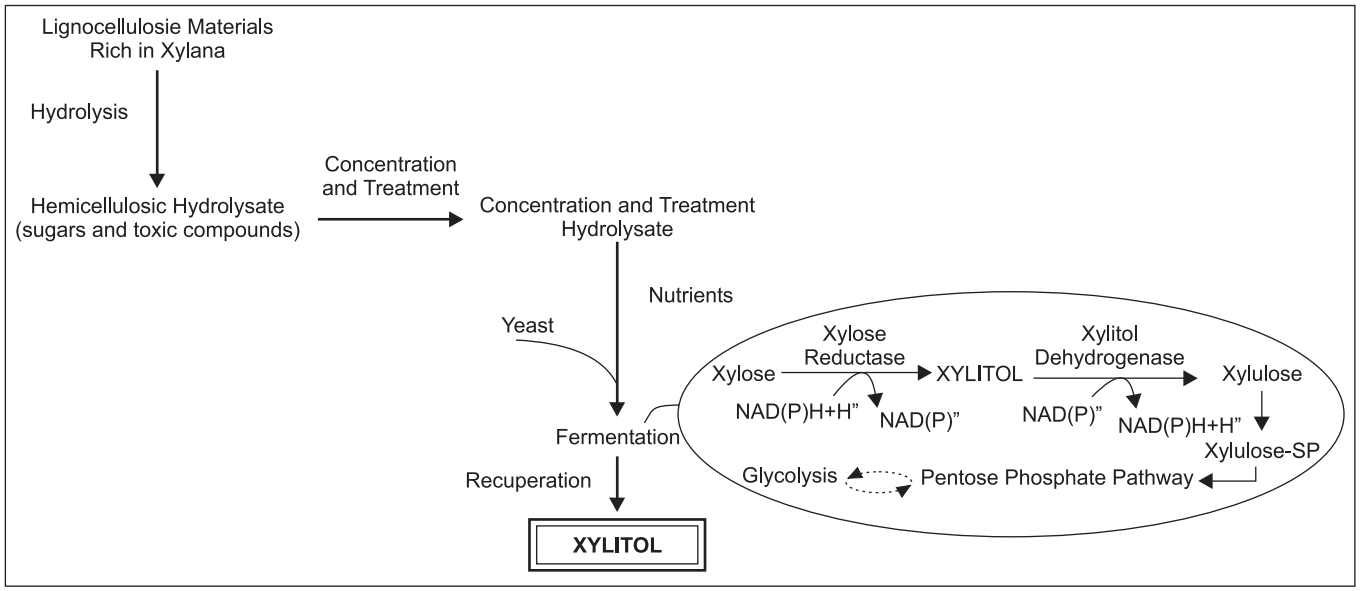

Figure 1. Xylitol production by biotechnological process.

weak acid (pK_a=4.75) to traverse the cell membrane and to act as a membrane protonophore, which causes acidification of the cytoplasm.

A previous study on the bioconversion of xylose to xylitol employing *C. guilliermondii* FTI 20037 cultivated in semi-synthetic medium revealed that the acetic acid concentration determines its degree of toxicity, since a concentration as low as 1.0g/L favoured the bioconversion, while concentrations higher than 3.0g/L inhibited xylose consumption and xylitol formation (4).

In this work, acetic acid was added to fermentation medium based on sugarcane bagasse hydrolysate at different times of fermentation, with the aim of evaluating its effects on the xylose-xylitol conversion by *Candida guilliermondii* FTI 20037 during different phases of the microbial growth.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

The experiments were conducted with *C. guilliermondii* FTI 20037 maintained at 4ºC on malt-extract agar slants. A loop ful of cells, grown on a malt-extract agar slant, was transferred to inoculum preparation medium containing xylose (30.0g/L), rice bran extract (20.0g/L), $(NH_4)_2SO_4$ (2.0g/L) and $CaCl_2.2H_2O$ (0.1g/L). Erlenmeyer flasks (125mL), each containing 50mL medium, were incubated on a rotary shaker (200rpm) at 30ºC for 24h. The cells were separated by centrifugation (2000xg; 20min), rinsed twice with distilled water, and the cell pellet was resuspended in an adequate volume of distilled water. The initial cell concentration for all experiments was around $0.75x10^7$cel/mL.

Preparation of the bagasse hydrolysate

Sugarcane bagasse was hydrolysed in a 250L reactor at 121ºC for 10min with H_2SO_4 (solid:liquid ratio of 1:10). The hydrolysate was first filtered and concentrated at 70ºC under vacuum to obtain a threefold increase in the xylose content. Next, it was submitted to treatment consisting in pH adjustment to 7.0 with CaO (commercial grade) and to 5.5 with H_3PO_4, followed by the addition of 2.4% w/v activated charcoal (refined powder), for 1h, under agitation (200rpm, 30ºC). The precipitate formed as a result of this treatment was removed by vacuum filtration, and then the hydrolysate was autoclaved at 111ºC, 0.5atm and used for medium preparation (14).

Medium and Fermentation Conditions

For fermentation medium preparation, concentrated bagasse hemicellulosic hydrolysate (Table 1) was supplemented with 20.0g/L of rice bran extract, 2.0g/L of $(NH_4)_2SO_4$ and 0.1g/L of $CaCl_2.2H_2O$. To evaluate the inhibitory effect of acetic acid on xylose-to-xylitol bioconversion by *C. guilliermondii,* acetic acid (2.0g/L) was added to the fermentation medium at the beginning of fermentation and also after 12, 24 and 48h. Control experiments employing hydrolysate without the addition of acid were also performed. The media (50mL) were placed in 125-mL Erlenmeyer flasks and fermented at 200rpm for 54h, at 30ºC, with initial pH adjusted to 5.5.

Analytical Methods

The concentrations of sugars and toxic compounds were quantified by chromatography as described by Rodrigues *et al.* (14). Xylose, glucose, arabinose, xylitol and acetic acid were

Table 1. Characteristics of sugarcane bagasse hydrolysate used as the fermentation medium.

Component	Concentration (g/L)
D-Xylose	40.0
D-Glucose	2.20
L-Arabinose	3.70
Acetic acid	3.40
Furfural	0.002
Hydroxymethylfurfural	<0.003
Phenols	0.089

determined by HPLC Shimadzu (Kyoto, Japan) provided with a refractive index (RI) detector and Bio Rad (Hercules, CA) Aminex HPX-87H column (300x7.8mm) at 45ºC, using 0.01N H_2SO_4, a flow rate of 0.6mL.min^{-1}, and a sample injector volume of 20mL. Furfural and hydroxymethylfurfural concentrations were also determined by HPLC Shimadzu provided with a dual l absorbance detector (SPD-10^{A}uv-vis) at 276nm wavelength and a Hewlett-Packard RP 18 (200 mm) column at 25ºC, using a 1:8 acetonitrile:water ratio, 1% acetic acid as the eluent, a flow rate of 0.8mL.min^{-1} and a sample volume of 20mL (17). Total phenols concentration was determined by spectrophotometry (Beckman DU 640B spectrophotometer) as described by KIM and YOO (7), using phenol as the standard compound. Cell number was determined directly by counting in a Neubauer chamber (area=1/400mm^2; height=0.100mm).

Statistical Methods

Statistically significant differences between the conditions evaluated were determined by the t-Student test.

RESULTS AND DISCUSSION

Fig. 2 illustrates the consumption of xylose, arabinose and acetic acid by *Candida guilliermondii* during fermentation of sugarcane bagasse hydrolysate. Acetic acid was added to the fermentation medium at different times of fermentation. Addition of acetic acid to the medium after 12 hours of fermentation resulted in a xylose consumption 23.22% lower than the control. Assimilation of arabinose was slow and similar to xylose consumption. Acid addition after 12h resulted in lower arabinose consumption (4.13%), corresponding to a 84.54% reduction in relation to the control, after 54h of fermentation. Felipe *et al*. (2) also observed inhibition of xylose and arabinose consumption by *C. guilliermondii* when the acetic acid content in sugarcane bagasse hydrolysate was 4.5g/L at the beginning of fermentation. In the present experiment, glucose (2.20g/L) was totally consumed within 12h of fermentation (data not shown), which was also reported by other authors in studies using the same yeast strain and hydrolysate (2,12,16).

The addition of acetic acid in the medium after 12h of fermentation resulted in the consumption of 30.58% of this acid by yeast, which is 40.09% and 27.19% lower than the amounts consumed in the control and in the hydrolysate containing acetic acid added at the beginning of fermentation, respectively. It is already known that the toxicity of the acetic acid is related to its concentration and to the pH of the medium (2,4). However, in this work it is possible to observe that the effect of the acetic acid in the xylose-xylitol bioconversion by *C. guilliermondii* can also be attributed to the fermentation time when this acid was added. This means that depending on the moment that acetic acid was added to the medium (Fig. 2), it interfered with the assimilation of xylose and of the acid itself. Besides, in all conditions evaluated yeast was able to assimilate the acetic acid with eventual increase in the pH of the medium (data not shown), which agrees with results observed by other authors (2,16).

In relation to cell growth, an inhibitory effect of acetic acid on biomass formation occurred in all conditions evaluated, regardless the moment of addition of the acid was to the medium. The lowest biomass formation (6.63x10^{7}cel/mL), observed when the acetic acid was added to the medium after 12h of fermentation, was 26.82% lower than the control (Fig. 3). It was also verified that acetic acid added at the beginning of fermentation (Fig. 3B) resulted in a period of 12h without cell growth, like the control, probably because xylose metabolism depends on induced enzymes.

The strongest inhibition of xylose assimilation (23.22%) and cell growth (26.82%) occurred when acetic acid was added to the medium after 12h of fermentation. Xylitol formation was 9.47g/L, a reduction of 55.29% when compared to the control, representing a significant difference at a probability level of 95% (Fig. 3). A significant difference (95% probability) in xylitol formation was also observed when acetic acid was added to the medium after 24h of fermentation. However, the inhibitory effect of the acetic acid on xylose consumption (11.23%) and cell growth (12.69%) was lower in comparison with the control (without acid addition).

It is important to emphasise that when the acetic acid inhibits xylitol formation, cell growth also decreases. This probably happens because, at high concentrations, acetic acid either interferes with xylose transport across the plasmatic membrane, or provokes that ATP required for cell growth is used to prevent acidification of the intracellular pH (6,8,9). In our work, the inhibitory effect of the acetic acid during fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* could be due to xylose assimilation by yeast, since independent of the acid addition, cell growth was similar in the first 12h of fermentation, coinciding with glucose consumption.

Values of the fermentation parameters of xylose-xylitol bioconversion in all experiments with *C. guilliermondii*, obtained after addition of acetic acid to the hydrolysate at different fermentation periods, are shown in Table 2.

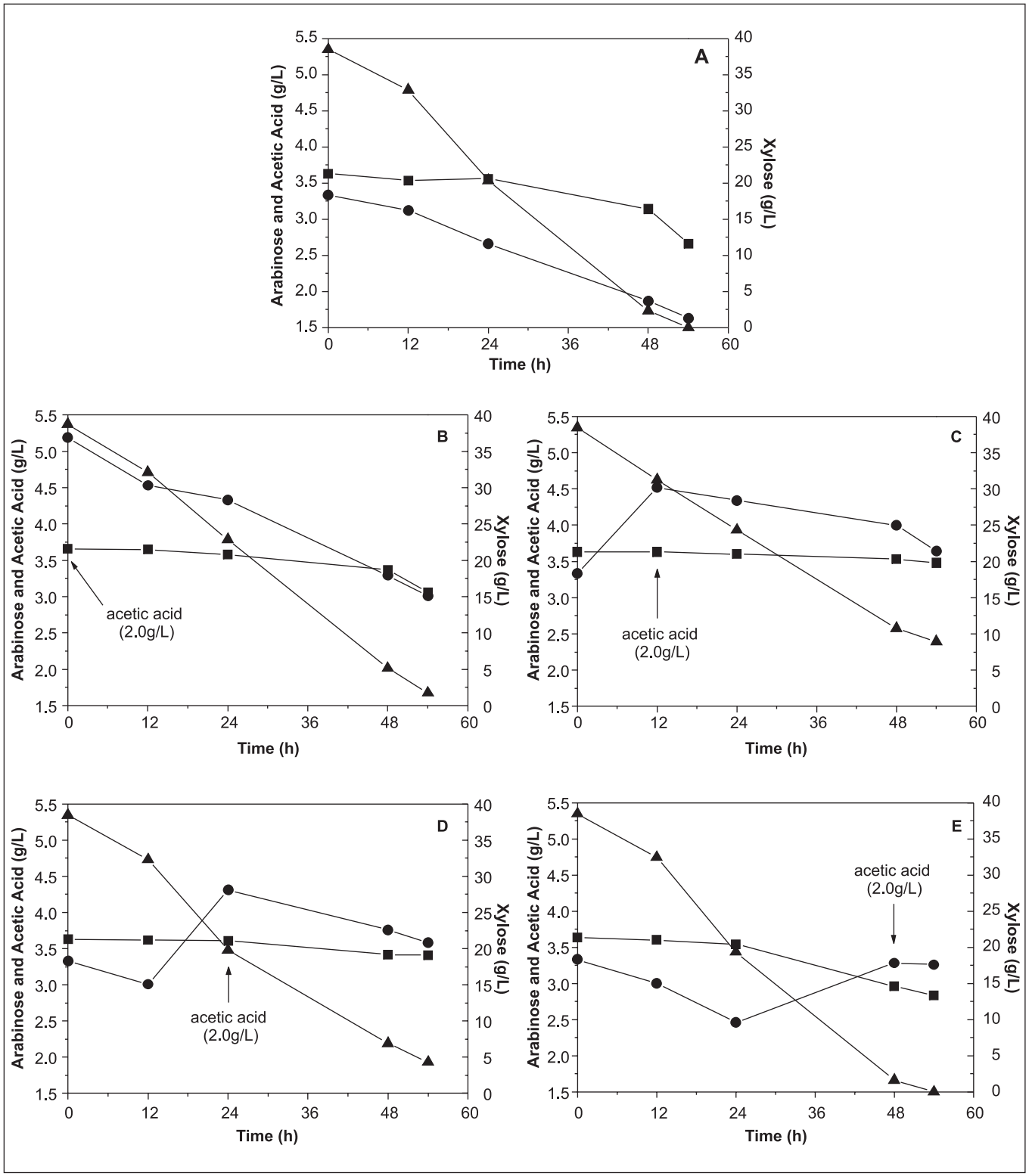

Figure 2. Effect of acetic acid on xylose (▲), arabinose (■) and acetic acid (●) consumption during fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* without (A) and with acetic acid addition (2.0g/L), after 0 (B), 12 (C), 24 (D) and 48 (E) hours of fermentation.

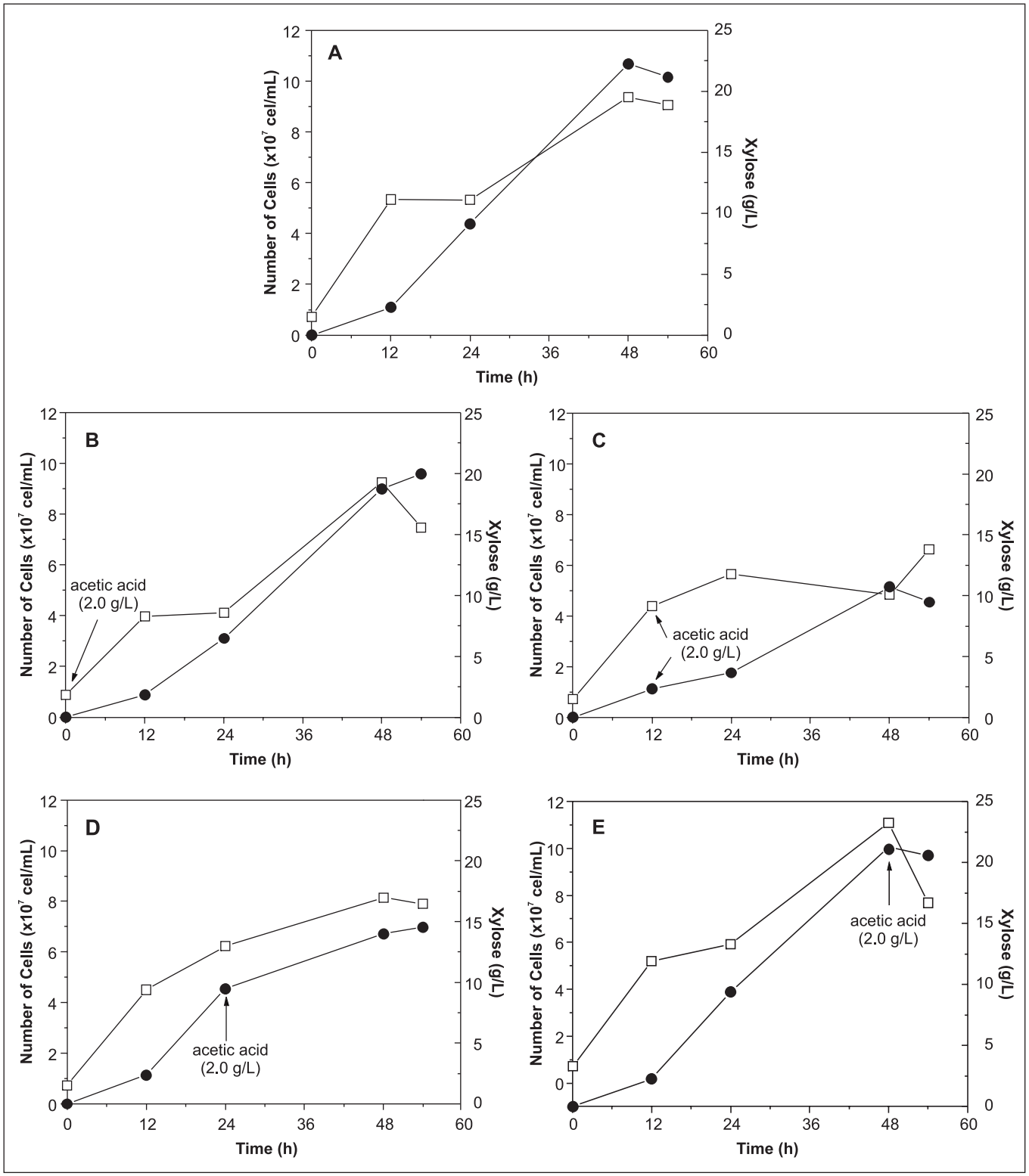

Figure 3. Effect of acetic acid on cell growth (□) and xylitol formation (●) during fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* without (A) and with acetic acid addition (2.0g/L), after 0 (B), 12 (C), 24 (D) and 48 (E) hours of fermentation.

Table 2. Xylitol Yield ($Y_{P/S}$) and Productivity (Q_P) after fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* after addition of acetic acid at different times of fermentation.

Times of acetic acid addition	$Y_{P/S}$ (g/g) Time (h)				Q_P (g/L.h) Time (h)			
	T_{12}	T_{24}	T_{48}	T_{54}	T_{12}	T_{24}	T_{48}	T_{54}
without addition	0.41	0.50	0.62	0.55	0.19	0.38	0.46	0.39
T_0	0.28	0.41	0.56	0.54	0.15	0.27	0.39	0.37
T_{12}	0.32	0.26	0.39	0.32	0.19	0.15	0.22	0.18
T_{24}	0.33	0.23	0.44	0.43	0.20	0.40	0.29	0.27
T_{48}	0.33	0.49	0.58	0.54	0.19	0.39	0.44	0.38

Maximum values of xylitol yield ($Y_{P/S}$=0.62g/g) and productivity (Q_P=0.46g/L.h) were obtained from the control after 48h of fermentation.

Reductions of 37.10% and 52.17% in xylitol yield and productivity respectively occurred when acetic acid was added to the medium after 12h, confirming that the negative effect of the acetic acid is associated with the fermentation time when this acid was added to the medium. These results agree with those previously found for the same yeast strain using synthetic medium (4) or hydrolysates from eucalyptus (3) and sugarcane bagasse (15,17).

Although the addition of acetic acid to the medium at the beginning of fermentation did not cause inhibition of the xylitol production, it is necessary to treat the hydrolysate, due to the presence of other toxic compounds, mainly phenols. These compounds have a synergistic effect which also inhibits the process, even when their concentration in the medium is low.

The results of this study showed that the inhibitory effect of acetic acid on xylose-to-xylitol bioconversion was dependent on the time of exposion of the cells to the acid. Stronger inhibition was observed when acid was added after 12h, when the yeast metabolic activity was maximum.

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RESUMO

Efeito inibitório do ácido acético na bioconversão de xilose em xilitol por *Candida guilliermondii* em hidrolisado de bagaço de cana

Hidrolisado de bagaço de cana-de-açúcar contendo uma concentração inicial de ácido acético de 3,5g/L foi utilizado como meio de fermentação para a bioconversão de xilose em xilitol pela levedura *Candida guilliermondii* FTI 20037. Ácido acético (2,0g/L) foi adicionado ao meio em diferentes tempos de fermentação, com o objetivo de avaliar o efeito deste ácido neste bioprocesso. O maior efeito inibitório deste ácido na bioconversão de xilose em xilitol pela levedura ocorreu quando este foi adicionado ao meio após 12h de fermentação. Nesta condição observou-se uma redução de 23,22% e 11,24%, respectivamente, no consumo de xilose e no crescimento celular em relação à fermentação em que a adição deste ácido ocorreu no tempo inicial de incubação. Como conseqüência do efeito inibitório, observou-se os menores valores de rendimento (0,39g/g) e produtividade (0,22g/L.h) de xilitol, correspondendo a uma redução de 36 e 48%, respectivamente, em relação aos valores obtidos com a adição de ácido acético nos outros tempos de fermentação. Os resultados obtidos permitem concluir que, nas condições experimentais empregadas neste trabalho, o efeito inibitório do ácido acético sobre a bioconversão de xilose em xilitol é dependente do tempo de fermentação em que a adição do ácido foi feita e não apenas de sua concentração no meio.

Palavras-chave: xilitol, xilose, ácido acético, hidrolisado de bagaço de cana

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