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Mixed inhibitions by methanol, furfural and acetic acid on xylitol production by *Candida guilliermondii*

Attilio Converti[1,*], Patrizia Perego[1], Paolo Torre[1] & Silvio Silverio da Silva[2]
[1]*Department of Chemical and Process Engineering 'G.B. Bonino', University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy*
[2]*Department of Biotechnology, Chemical Engineering Faculty of Lorena (FAENQUIL), Rodovia Itajubá-Lorena, km 74.5, 12600-000, Lorena, SP, Brazil*
[*]*Author for correspondence (Fax: +39 0103532586; E-mail: converti@unige.it)*

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Abstract

Xylose production by *Candida guilliermondii* FTI 20037 was carried out in a synthetic medium in the presence of 0–100 g methanol l^{-1}, 0–0.7 g furfural l^{-1} or 0–1.3 g acetic acid l^{-1}. Kinetic results show a mixed inhibition mechanism in all three cases. Maximum specific productivity and saturation constant for product formation were, in the absence of inhibition, 3.6 g_P ${g_X}^{-1}$ h^{-1} and 232 g_S l^{-1}, respectively, while the inhibition constants, K_i and K_i', were 17 and 50 g methanol l^{-1}, 0.62 and 7.0 g furfural l^{-1}, 0.69 and 3.5 g acetic acid l^{-1}, which suggests the following order of inhibition: furfural > acetic acid > methanol.

Introduction

Xylitol, a polyol with sweetness comparable with that of sucrose, has found increasing use in the food and pharmaceutical industries, due to its sweetening and anticariogenic properties. In addition, it is tolerated by diabetics, has been recommended for parenteral nutrition, does not cause acid formation, and has low viscosity and negative heat effect when dissolved in a solution (Parajo *et al.* 1998).

Xylitol is currently manufactured by the catalytic hydrogenation of xylose present in lignocellulosic hydrolysates. However, there are some yeast strains which can produce xylitol directly under milder conditions than those of chemical process (Kosaric *et al.* 1983, Parajo *et al.* 1998), among which *Candida guilliermondii* is one of the most efficient (Silva *et al.* 1997).

The fermentation of hydrolysates is hindered by the formation of inhibitors during chemical hydrolysis, such as furfural and hydroxymethylfurfural (generated by degradation of sugars), acetic acid (liberated from the acetyl groups of the raw materials), lignin degradation products and compounds derived from wood extractives (primarily composed of phenolic compounds and methanol), and inhibitors derived from the metals or minerals in wood, soil or hydrolysis equipment (Converti *et al.* 1999b). In order to minimise their effects, several technologies have been employed, including the adaptation of yeasts (Chen & Gong 1985), addition of reducing substances (Perego *et al.* 1990), neutralisation and overliming (Mayerhoff *et al.* 1997, Converti *et al.* 1999a, b), evaporation and steam stripping (Perego *et al.* 1990), solvent extraction (Parajó *et al.* 1997) and charcoal adsorption (Converti *et al.* 1999b).

The present work deals with a kinetic study of three well-known inhibitors of microbial xylitol production (methanol, furfural and acetic acid), which are usually present in hardwood lignocellulose hydrolysates before detoxification and whose negative effects on productivity, rather than on product yield, were previously evidenced (Silva *et al.* 1997). With the aim at shedding light on the mechanisms of their action, the kinetic parameters were estimated from the experimental data of three series of batch fermentations

by *C. guilliermondii*. Synthetic media containing increasing levels of each inhibitor were used in order to avoid any interference of other inhibitors present in the hydrolysates and/or synergistic effects among the compounds under consideration.

Materials and methods

Microorganism and inoculum cultivation

Candida guilliermondii FTI 20037, isolated in the Biotechnology Department of FAENQUIL, was maintained at 4 °C on agar slants containing 10 g yeast extract l^{-1}, 20 g peptone l^{-1} and 20 g D-glucose l^{-1}. A loopful of cells was transferred to 125-ml Erlenmeyer flasks containing 50 ml of a medium constituted by 30 g xylose l^{-1}, 3 g $(NH_4)_2SO_4$ l^{-1}, 0.1 g $CaCl_2 \cdot 2H_2O$ l^{-1} and 20% (v/v) rice bran extract. The inoculum was cultivated in a rotatory shaker at 200 rpm and 30 °C for 24 h. Afterwards, the cells were collected by centrifugation (2000 *g*, 15 min), washed thoroughly with sterile distilled water, centrifuged and resuspended in sterile distilled water.

Fermentation conditions

The batch fermentations were carried out in a 2.5-l bench-fermenter containing 1.5 l of the medium at 30 °C, agitation of 300 rpm, aeration of 20 ml min^{-1} and pH 4.0 for 72 h. The tests were performed in a synthetic medium containing the following nutrients: 5.0 g $(NH_4)_2SO_4$ l^{-1}, 1.0 g yeast extract l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.1 g $CaCl_2 \cdot 2H_2O$ l^{-1}, 1.0 g KH_2PO_4 l^{-1}, and about 70 g xylose l^{-1}.

Analytical methods

The xylose and xylitol concentrations were determined by HPLC with a refractive index detector and a sugar-PAK column at 85 °C. Deionized water was used as eluent at a flow rate of 0.5 ml min^{-1}. The cell mass was estimated turbidimetrically at 600 nm and then correlated with the cell dry weight (the washed cells were dried at 90 °C for 24 h).

Results and discussion

Three sets of batch runs were carried out using xylose at 70 g l^{-1}, by progressively increasing the starting concentration of each inhibitor in the medium, in order to investigate their relative inhibition mechanism. In particular, on the basis of the concentrations detected in hemicellulose hydrolysates, the starting levels of these inhibitors were varied from 0 to 100 g methanol l^{-1}, from 0 to 0.7 g furfural l^{-1} and from 0 to 1.3 g acetic acid l^{-1}, respectively.

Table 1. Effect of inhibitors on the apparent maximum specific productivity, ν'_{max}, and saturation constant, K'_S, calculated for xylitol production by *Candida guilliermondii*

I (g_I (l^{-1})	ν'_{max} gP $(g_x\ h^{-1})$	K'_S g_S l^{-1}
0	3.6[a]	232[b]
Methanol		
10	2.2	195
30	1.6	127
60	0.7	114
100	0.5	99
Furfural		
0.1	3.1	203
0.3	2.5	163
0.5	1.9	138
0.7	1.7	120
Acetic acid		
0.2	2.8	190
0.5	2.0	160
1.0	1.6	120
1.3	1.2	111

[a] ν_{max} in the absence of inhibition.
[b] K_S in the absence of inhibition.

The experimental data of product concentration versus time of these batch fermentations allowed the specific productivity, ν, to be calculated as function of the residual substrate concentration, S. Plotting these results according to Lineweaver–Burk, it was possible to estimate the apparent maximum specific productivities, ν'_{max}, and saturation constants for product formation, K'_S, at different concentrations of each inhibitor, I.

The main kinetic results of these tests (Table 1) show, for all three inhibitors, progressive decreases in both apparent ν'_{max} and K'_S with increasing the inhibitor concentration in the medium. Assuming a direct dependence of fermentation kinetics on those of the enzyme controlling the metabolic pathway leading to the product, this kinetic behaviour is consistent, among the various models reported in the literature

for enzyme inhibitions (Roels 1983), with both the uncompetitive and mixed inhibitions, which are based on the hypotheses of stationary state and equilibrium, respectively. This assumption is particularly valid in the case under consideration where only one enzyme (xylose reductase) is involved in product formation.

However, the uncompetitive inhibition mechanism is unlikely in actual systems, implying the same percentage decreases for $\nu_{\max}^{'}$ and $K_S^{'}$, modulated by only one inhibition constant. This does not apply, indeed, to our system, which showed, for all three inhibitors, that $\nu_{max}^{'}$ decreased more quickly than $K_S^{'}$ did. For this reason the kinetic approach of mixed inhibition was used.

In case mixed inhibition takes place, two different sites for the substrate and the inhibitor are present in the controlling enzyme. Contrary to uncompetitive inhibition, however, the inhibitor has affinity not only for the enzyme-substrate (ES) complex but also for the free enzyme (E). In addition, the substrate can react not only with the free enzyme, but also with the enzyme-inhibitor (EI) complex. If the reversible complex-forming reactions are sufficiently quick, one can assume a thermodynamic equilibrium for the mixture of E, S, ES, EI and ESI (Roels 1983).

Combining the expressions of the equilibrium constants for every complexes with the material balance for the enzyme:

$$E_0 = E + EI + ES + ESI \tag{1}$$

and assuming that the rate-controlling step of product formation is the breaking of the enzyme-substrate complex, the rate of substrate consumption is

$$r_S = \frac{r_{S,\max} S}{K_1\left(1 + \frac{I}{K_3}\right) + S\left(1 + \frac{I}{K_4}\right)}, \tag{2}$$

where K_3 and K_4 are the equilibrium constants of the reactions which lead to the formation of E and ES from the EI and ESI complexes, respectively.

This equation can directly be exploited in fermentation systems supposing a direct link between the specific productivity, ν, and the rate of the reaction catalysed by the enzyme controlling the metabolic pathway which leads to product formation. Therefore, we can write:

$$\nu = \frac{\nu'_{\max} S}{K'_S + S}, \tag{3}$$

where

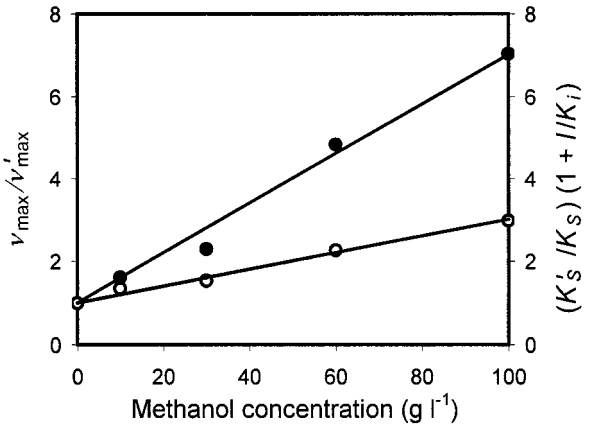

Fig. 1. Graphical estimation of the constants K_i and $K_i^{'}$ for the inhibition of methanol on xylitol production by *C. guilliermondii*. (●) $\nu_{\max}/\nu_{\max}^{'}$; (○) $(K'_S/K_S)\,(1 + I/K_i)$. I = methanol concentration (g l^{-1}).

$$\nu'_{\max} = \frac{\nu_{\max}}{\left(1 + \frac{I}{K_i}\right)} \tag{4}$$

and

$$K'_S = \frac{K_S\left(1 + \frac{I}{K'_i}\right)}{\left(1 + \frac{I}{K_i}\right)} \tag{5}$$

are the apparent maximum specific productivity and saturation constant of product formation in the presence of inhibitor, $\nu_{\max}$ and K_S the same parameters referred to the not inhibited reaction, K_i and $K_i^{'}$ two fermentation inhibition constants related to the equilibrium constants K_4 and K_3, respectively.

According to Equation (4), the values of K_i were estimated for methanol (Figure 1), furfural (Figure 2) and acetic acid (Figure 3) from the slopes of the straight lines obtained plotting the experimental values of $\nu_{\max}/\nu_{\max}^{'}$ listed in Table 1 versus I. Analogously, the corresponding values of $K_i^{'}$ were estimated by the slopes of the straight lines obtained plotting $(K'_S/K_S)\,(1 + I/K_i)$.

From the values of K_i and $K_i^{'}$, listed in Table 2 for each inhibitor, and those of $\nu_{\max}^{'}$ of Table 1, a comparative analysis of their respective inhibiting power can be made. To this purpose, it should be stressed that K_i and $K_i^{'}$ are related to the equilibrium constants (K_4 and K_3) of the ESI and EI complexes-breaking reactions leading to ES and E, respectively. This means that lower values of these constants are indicative of

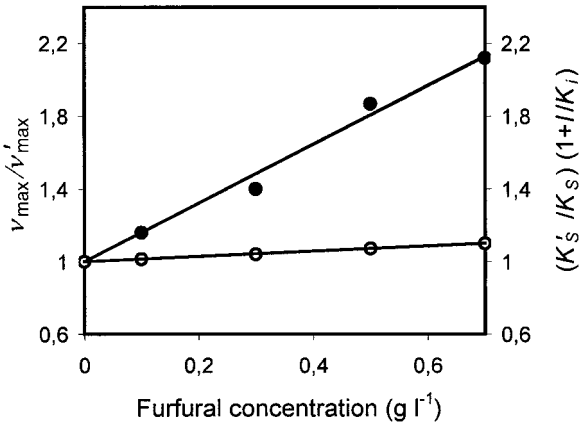

Fig. 2. Graphical estimation of the constants K_i and $K_i^{'}$ for the inhibition of furfural on xylitol production by *C. guilliermondii*. (●) $\nu_{max}/\nu_{max}^{'}$; (○) $(K_S'/K_S)\,(1 + I/K_i)$. I = furfural concentration (g l^{-1}).

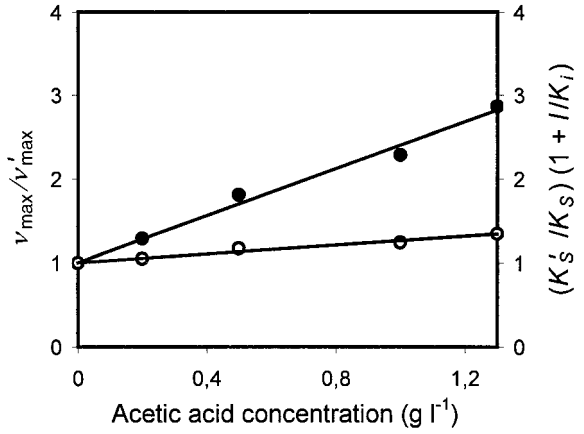

Fig. 3. Graphical estimation of the constants K_i and $K_i^{'}$ for the inhibition of acetic acid on xylitol production by *C. guilliermondii*. (●) $\nu_{max}/\nu_{max}^{'}$; (○) $(K_S'/K_S)\,(1 + I/K_i)$. I = acetic acid concentration (g l^{-1}).

a stronger inhibition effect. In particular, while K_i is the only inhibition constant influencing the apparent maximum specific productivity, the *I*-dependent ratio $(1+I/K_i)/(1+I/K_i)$ should be used to investigate the effect of mixed inhibition on the apparent saturation constant.

Comparing the values of K_i listed in Table 2, it is evident that methanol, which is characterised by the highest value of this constant, was the less powerful inhibitor, being necessary a concentration of 17 g methanol l^{-1} to achieve a specific productivity which was a half of the maximum value in the absence of inhibition. On the other hand, furfural and acetic acid

Table 2. Values of the constants K_i and $K_i^{'}$ estimated for methanol, furfural and acetic acid mixed inhibition of xylitol production by *C. guilliermondii*.

Inhibitor	Methanol	Furfural	Acetic acid
K_i g l^{-1}	17	0.62	0.71
r^2	0.988	0.988	0.988
$K_i^{'}$ (g l^{-1})	50	7.0	3.8
r^2	0.988	0.999	0.973

were very strong inhibitors, being able to halve the specific productivity at concentration only of 0.62 g furfural l^{-1} and 0.71 g acetic acid l^{-1}. As far as the effects of these inhibitors on the apparent saturation constant are concerned, a reduction to a half of the K_S value obtained in the absence of any inhibition was observed at 50 g methanol l^{-1}, 0.75 g furfural l^{-1} and 1.1 g acetic acid l^{-1}.

These results on the whole suggest that, among the inhibitors present in acid hemicellulose hydrolysates tested in this study, furfural was the strongest inhibitor of xylose fermentation by *C. guilliermondii*, followed by acetic acid and, in some extent, by methanol. In addition, all these compounds exerted an inhibition effect which can be described by mixed inhibition mechanism. Particularly in the case of furfural and acetic acid, it seems to be widely justified the great effort made by the scientific community to minimise their formation during the acid hydrolysis of lignocellulose materials or to remove them from the fermentation media.

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