ELSEVIER

Journal of Biotechnology 64 (1998) 211–218

JOURNAL OF Biotechnology

Inhibition of the fermentation of oak hemicellulose acid-hydrolysate by minor sugars

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Received 16 March 1998; received in revised form 2 July 1998; accepted 13 July 1998

Abstract

Synthetic xylose media and detoxified oak hemicellulose acid-hydrolysates with different starting xylose contents were fermented batchwise by two strains of *Pachysolen tannophilus*. Maximum productivities were calculated from the experimental data of ethanol concentration using a graphical procedure. The kinetic parameters calculated for the fermentations of both carbon sources indicate that a competitive inhibition is exerted by the minor sugars (arabinose, rhamnose, and galactose) that are metabolised only slowly or not at all. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Oak; Hemicellulose hydrolysate; Xylose; Alcohol fermentation; *Pachysolen tannophilus*

1. Introduction

Wood is an abundant and cheap (US$ 60/ton) feedstock that can be used, after preliminary hydrolysis, as a substrate for alcohol fermentation. In fact, it has a high sugar content related to the presence of both hemicellulosic (20–25%) and cellulosic (40–50%) fractions (Wyman, 1994). While D-glucose is quickly metabolised by most yeasts and bacteria (Duff and Murray, 1996), D-xylose, the main component of hemicellulose, is a pentose which can be consumed only slowly, mainly in synthetic substrates, by a few microorganisms, among which *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* (Hinman et al., 1989; Wilson et al., 1989; Perego et al., 1990; Hahn-Hägerdal et al., 1993). Although other yeasts are more suited than *Pachysolen tannophilus* to ferment pure xylose to ethanol, without production of xylitol as a by-product (Hallborn et al., 1991), only a few of them effectively utilise complex carbon sources, such as hemicellulose hydrolysates (Perego et al., 1990; Del Borghi et al., 1992; Duff and Murray, 1996).

Oak can be hydrolysed by a two-stage dilute-acid treatment, that sequentially separates hemi-

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PII S0168-1656(98)00109-6

cellulose from cellulose fractions (Duff and Murray, 1996). The first-stage hydrolysate contains most of D-xylose, but its fermentation is affected by several inhibiting compounds generated by the hydrolytic pretreatment, such as acetic acid (produced from the acetyl groups), furfural and hydroxymethylfurfural (HMF) (generated by pentose degradation), some uronic acids, many aromatic lignin-degradation products, and extractives (Tran and Chambers, 1986; Parajó et al., 1995). Acetic acid shows a less inhibition than furfural (Tran and Chambers, 1985, 1986). The latter, which prevents glucose fermentation at concentrations higher than 0.30–0.46 g l^{-1} (Parajó et al., 1995), does not seem to affect xylose fermentation at concentrations below 1.0 g l^{-1} (Roberto et al., 1991).

To minimise inhibition, optimisation studies have been carried out on the hydrolysis (Parajó et al., 1993, 1994), on the hydrolysate pretreatment (Tran and Chambers, 1985, 1986; Parekh et al., 1987; Perego et al., 1990; Roberto et al., 1991; Ferrari et al., 1992), as well as on the conditions of microorganism acclimation (Deverell, 1983; Chen and Gong, 1985; Tran and Chambers, 1985). Del Borghi et al. (1992) and Perego et al. (1994) demonstrated that passing oak hemicellulose hydrolysate, previously detoxified by precipitation with lime, through a porous SiO_2 and Al_2O_3 material can increase the ethanol yield by 15%.

Hinman et al. (1989) and Wilson et al. (1989) were sceptical about the economics of detoxifying operations for industrial wood fermentation. Nevertheless, co-cultures of hexose- and pentose-fermenting yeasts and recombinant bacteria provide the capacity for pentose utilisation. Even though these are the most promising alternatives, they do not appear, at the moment, to be definitive economic solutions for industrial application.

Because it is practically impossible to exactly reproduce the same composition in different detoxified hemicellulose hydrolysates, and because wood composition is variable, only a few attempts have been made to study the kinetics of these fermentations. A further difficulty arises from the presence of several substrates that are simultaneously consumed at different rates.

To shed light on the types of inhibition involved and to estimate the related kinetic parameters, we carried out a set of batch fermentations using the same pretreated batch of hydrolysate for all the runs. Specific productivity can be assessed in such a carbon source only approximately and with difficulty. Nevertheless, under conditions of very slow growth, volumetric productivity can be used in Michaelis–Menten type equation for kinetic purposes. The maximum productivities with synthetic D-xylose media and hemicellulose hydrolysates, at different starting sugar levels, have been calculated by Lineweaver–Burk plots. From a comparison of these kinetic data, we have found that arabinose, rhamnose, and galactose could be responsible for the slower fermentation of detoxified hemicellulose hydrolysate with respect to synthetic medium.

2. Materials and methods

2.1. Preparation of nutrients

Compositions of the oak hemicellulose acid-hydrolysates tested in this work are listed in Table 1. Hydrolysate B consisted of the first stage of dilute acid hydrolysis of oak, while the higher pentose concentration in the hydrolysate A was obtained by washing successive batches of residue through a countercurrent washing scheme (Perego et al.,

Table 1
Average chemical composition of oak hemicellulose acid-hydrolysates

Component	Hydrolysate A[a]	Hydrolysate B[b]
D-Glucose (g l^{-1})	13.2	9.0
D-Xylose (g l^{-1})	106.0	43.5
D-Galactose (g l^{-1})	8.6	3.3
L-Arabinose (g l^{-1})	1.6	2.9
D-Mannose (g l^{-1})	6.8	3.4
L-Rhamnose (g l^{-1})	0.8	1.4
Acetic acid (g l^{-1})	26.6	10.9
Furfural (g l^{-1})	4.2	0.9
HMF[c] (g l^{-1})	0.5	0.3

[a] Hydrolysate submitted to countercurrent operation.
[b] First-stage of dilute acid hydrolysis.
[c] HMF, hydroxymethylfurfural.

1994). Hydrolysates with varying starting xylose concentrations were prepared by diluting hydrolysate A with tap water, up to the selected xylose concentration, and performing the following pretreatment operations (Del Borghi et al., 1992; Perego et al., 1994). The acid hydrolysates were heated at 100°C for 10 min and the consequent volume loss was replaced with distilled water. We overlimed the hydrolysate to pH 10 with $Ca(OH)_2$ and filtered it while still warm. We used concentrated sulphuric acid to adjust the pH to 5.5 and added Na_2SO_3 up to 1.0 g l^{-1}. After nutrient addition up to the following concentrations, 2.0 g l^{-1} urea, 2.0 g l^{-1} yeast extract, and 0.5 g l^{-1} KH_2PO_4, the media were sterilised through 0.45 μm filters.

The synthetic media, used to simulate the composition of hydrolysates lacking in inhibitors, were prepared by adding to 1 l of tap water, 40 g of xylose and the following amounts of mineral salts: 0.4 g $MgSO_4 \cdot 7\ H_2O$, 2.0 g $(NH_4)_2SO_4$, 2.0 g yeast extract, and 5.0 g KH_2PO_4. Once again, sulphuric acid was used to adjust the pH at 5.5 after sterilisation. To prove the competitive inhibition due to minor sugars present in hemicellulose hydrolysate, an additional test was carried out using a synthetic medium containing arabinose, rhamnose, and galactose at the same levels present in hydrolysate A after dilution up to 50 g l^{-1} xylose.

2.2. Microorganisms

The strains of *Pachysolen tannophilus* (NRRL Y-2460 and CBS 4045) used in this study were maintained on agar slants through periodic transfers and subcultures. All slants contained 0.1% yeast extract and 50% of the same carbon source used for batch fermentations. The cells for inocula were grown for 72 h on the same media as used for fermentations.

2.3. Experimental set-up and fermentation conditions

A 1.0-l working volume CSTR, stirred at 300 rpm, was employed for fermentations. The pH of the fermentation media was automatically regulated, to an accuracy of 0.1 pH units. The temperature was kept at 32 ± 0.1°C. A few millilitres of a thick suspension of the selected microorganism was added to the medium up to the selected starting biomass concentration.

2.4. Analytical procedures

Ethanol, furfural, and hydroxymethylfurfural concentrations were determined by gas chromatography. The concentrations of all sugars were measured by HPLC, using a chromatograph (Type Waters ALC 201) with RI detector. A Bio-Rad HPX-42 column was used with 70:30 acetonitrile/water as the mobile phase at a flow rate of 0.8 ml min^{-1}. Cell concentration was determined, by dry weight, using 0.45 μm autoclavable filters. A turbidimetric assay was utilised at 595 nm wavelength to determine the biomass content of a thick cell suspension in water used for the inoculum. Acetic acid concentration was determined at 500 nm by the colorimetric method of Montgomery et al. (1962).

3. Results and discussion

3.1. Preliminary results

The main results of batch fermentations, partially presented in a previous paper (Perego et al., 1987) and summarised in Table 2, are now compared and discussed to highlight the different behaviour of *P. tannophilus* in synthetic medium and hemicellulose hydrolysate. Fermentations were carried out at low starting biomass levels (0.01 $g_{dry}\ l^{-1}$) and using, as carbon sources, either the hemicellulose hydrolysate coming from the first stage of dilute acid oak hydrolysis or a synthetic medium having nearly the same xylose content.

No relevant difference in xylose consumption by both tested strains of *P. tannophilus* was detected (Table 2), so subsequent tables refer only to *P. tannophilus* NRRL Y-2460. These data suggest that glucose contained in hemicellulose hydrolysate was almost completely consumed by *P. tannophilus* before D-xylose could be metabolised.

Table 2
Experimental data of batch fermentations of synthetic xylose medium and oak hemicellulose acid-hydrolysate B by *P. tannophilus*

	t (h)							
	12.0	24.0	36.0	48.0	60.0	72.0	120.0	168.0
Medium: xylose								
Conditions: $S_o = 40$ g l^{-1}								
Inoculum: 0.01 g l^{-1}								
Ethanol (g l^{-1})	1.8	3.0	5.5	11.0	12.0			
Xylose (g l^{-1})	36.3	28.2	21.4	9.2	0.1			
Medium: hemicellulose hydrolysate								
Conditions: $S_o = 43.5$ g_{xylose} l^{-1}, 9.0 g l^{-1} glucose								
Inoculum: 0.01 g l^{-1}								
Ethanol (g l^{-1})			2.7			6.5	10.4	11.2
Glucose (g l^{-1})			0.0			0.0	0.0	0.0
Xylose (g l^{-1})			42.2			27.7	12.8	9.7

This behaviour (Beck and Strickland, 1984) could be the combined result of a natural slowness of pentose fermentation by *P. tannophilus* (Jeffries et al., 1985) and a less efficient transport of xylose with respect to glucose (Batt et al., 1986).

Moreover, D-xylose in the hydrolysate was used at about one-third the rate as that observed in the synthetic solution. Analyses of the other minor sugars listed in Table 1 demonstrated that, while D-mannose was completely consumed within 24 h, no less than 85% L-arabinose and nearly all D-galactose and L-rhamnose were unfermented in the spent medium. These findings are consistent with the results of Neirinck et al. (1982) on the fermentation of spent sulfite liquor. They suggest that inhibition can take place during the fermentation of hemicellulose hydrolysate, in addition to the well-known effects of the above by-products of acid hydrolysis. This inhibition can be ascribed to the presence of recalcitrant (L-arabinose) or unfermentable (D-galactose and L-rhamnose) sugars.

3.2. Kinetic study

Additional batch runs were carried out at higher starting concentration (0.20 g_{dry} l^{-1}) of *P. tannophilus* cells, which had been previously submitted to four successive adaptations. Under these conditions the natural lag phase was nearly suppressed and all the batch runs achieved their maximum volumetric productivities nearly at the start of fermentations. We calculated ethanol productivities from the starting tracts of the curves shown in Figs. 1 and 2. These graphs describe ethanol formation, at increasing starting xylose levels, from synthetic xylose media and hemicellulose hydrolysates, respectively. Maximum productivities and saturation constants have been estimated from their respective Lineweaver–Burk

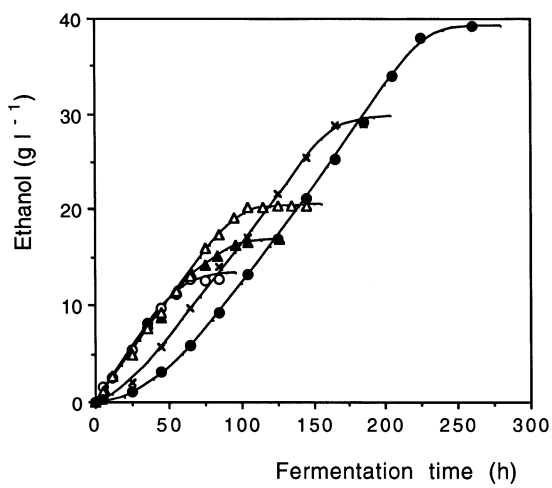

Fig. 1. Alcohol production during batch fermentations of synthetic xylose solutions by *P. tannophilus*. Starting xylose concentration (g l^{-1}): (○) 50; (▲) 75; (△) 100; (×) 150; (●) 200.

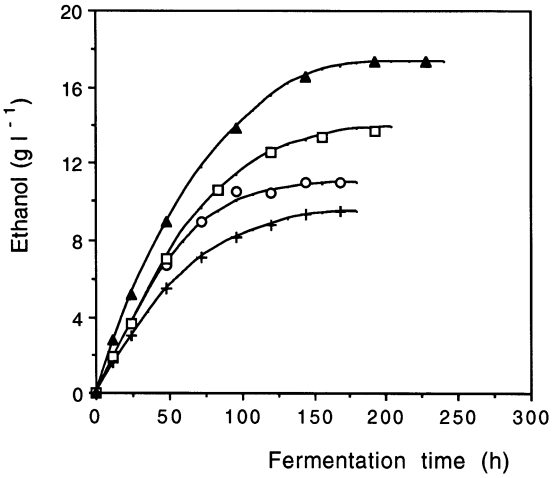

Fig. 2. Alcohol production during batch fermentations of oak hemicellulose acid-hydrolysates by *P. tannophilus*. Starting xylose concentration (g l^{-1}): (+) 40; (○) 50; (□) 65; (▲) 75.

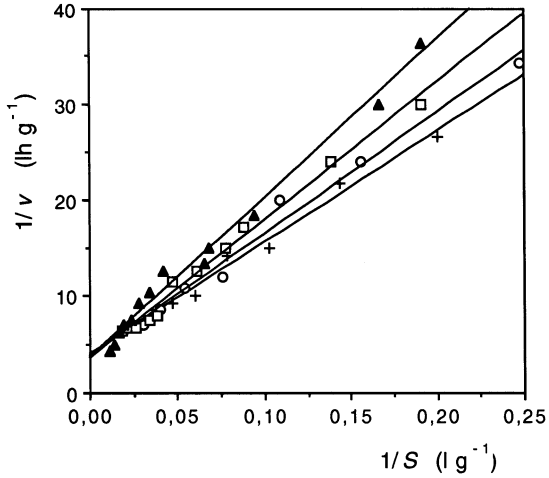

Fig. 4. Lineweaver–Burk plots of batch fermentations of oak hemicellulose acid-hydrolysates by *P. tannophilus*. Starting xylose concentration (g l^{-1}): (+) 40; (○) 50; (□) 65; (▲) 75.

plots in Figs. 3 and 4. Differences between the values of either of these two kinetic parameters have been ascribed to the presence of inhibition factors in the hemicellulose hydrolysate.

Estimation of maximum productivity and of saturation constant has been possible for the synthetic xylose medium (Fig. 3) only from the runs carried out at relatively low starting xylose concentrations, namely 50, 75, and 100 g l^{-1}. We found a relatively constant V_{max} (around 0.24 g l^{-1} h^{-1}), whereas the saturation constant linearly increased from 2.61 to 5.60 g l^{-1}, thus showing a nearly constant ratio $K_s/S_o \cong 0.054$ (Table 3). At higher starting sugar contents ($S_o > 100$ g l^{-1}) $1/V$ did not follow a linear behaviour for values of $1/S$ approaching 0, because of the lag phase appearance. Nevertheless, the nearly linear increase in K_s has been ascribed to a simple substrate saturation phenomenon.

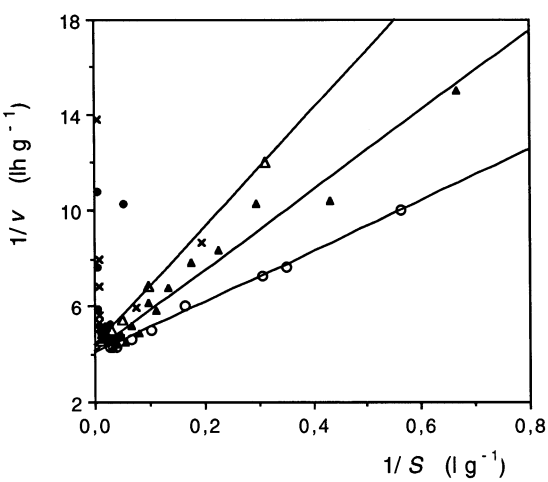

Fig. 3. Lineweaver–Burk plots of batch fermentations of synthetic xylose solutions by *P. tannophilus*. Starting xylose concentration (g l^{-1}): (○) 50; (▲) 75; (△) 100; (×) 150; (●) 200.

These observations on the whole suggest the existence of a gradual substrate saturation up to $S_o \cong 100$ g l^{-1}, which is similar to a competitive-type inhibition. Over this threshold a further inhibition takes place, which is probably related to

Table 3
Kinetic parameters of batch fermentations of synthetic xylose solutions by *P. tannophilus*

	S_o (g l^{-1})		
	50	75	100
K_s (g l^{-1})	2.62	4.06	5.60
V_{max} (g l^{-1} h^{-1})	0.247	0.243	0.228
r^2	0.993	0.966	0.998
K_s/S_o (−)	0.0525	0.0541	0.0560

S_o, starting xylose concentration; r^2, determination coefficient.

Table 4
Kinetic parameters of batch fermentations of oak hemicellulose acid-hydrolysate by *P. tannophilus*

	S_o (g l^{-1})			
	40	50	65	75
K'_s (g l^{-1})	29.37	33.56	40.60	46.56
V_{max} (g l^{-1} h^{-1})	0.253	0.265	0.282	0.275
r^2	0.986	0.988	0.991	0.988
K'_s/S_o (−)	0.734	0.671	0.624	0.621
I (g l^{-1})	4.15	5.19	6.74	7.78

S_o, starting xylose concentration; r^2, determination coefficient.

increases in both osmotic pressure and viscosity. This behaviour is consistent with the observed exponential decrease of specific productivity with S_o (Slininger et al., 1982), which was 'generically' attributed to high xylose levels.

Fig. 4 shows, for hemicellulose hydrolysate, a linear dependence of $1/V$ on $1/S$ at the start of each fermentation within the whole tested range of starting xylose concentration ($40 < S_o < 75$ g l^{-1}). Behaving similarly to the synthetic solution, the straight lines seem to tend to the same point on the ordinate axis, which corresponds to a maximum ethanol productivity only a little higher (0.27 g l^{-1} h^{-1}) than that calculated for the synthetic medium. This variation in V_{max} is too small to be of any significance and to be justified somehow. The linear increase of the saturation constant with S_o, whose values are listed in Table 4, suggests that a similar substrate saturation phenomenon takes place also with this carbon source, within the tested range of S_o. This result too is in good agreement with the observations of Slininger et al. (1982).

The values of the determination coefficient calculated from the straight lines of Figs. 3 and 4 (see r^2 data in Tables 3 and 4) are always very close to 1.000, which proves the validity of the Lineweaver–Burk approach for the kinetic description of the fermentation system under consideration.

A comparison among the values listed in Tables 3 and 4, which refer to the same starting xylose concentration ($S_o = 50$ g l^{-1}), clearly shows for hemicellulose hydrolysate approximately the same maximum productivity as the synthetic solution but a value of the saturation constant a round dozen times higher, which clearly indicates that an additional competitive inhibition occurs. Because furfural and HMF concentrations in the hydrolysate were reduced by the detoxifying pretreatment below the threshold considered inhibitory for microbial metabolism (< 1 g l^{-1}), we thought that recalcitrant sugars present at low concentrations in the hemicellulose hydrolysate could be responsible for this effect. Support to this hypothesis came from a proper additional test carried out using a synthetic xylose medium also containing arabinose, rhamnose, and galactose at the same starting levels as those present in the 50 g l^{-1} xylose hydrolysate. In fact, it showed values of the saturation constant (29.82 g l^{-1}) and the maximum productivity (0.254 g l^{-1} h^{-1}) both in reasonable agreement with those estimated for the hydrolysate (Table 4).

The inhibition constant has been calculated from the Lineweaver–Burk-type plot, as follows. Supposing the existence of a competitive-type inhibition mechanism, the reciprocal of productivity can be described by the equation:

$$\frac{1}{V} = \frac{K_s[1 + (I/K_i)]}{V_{max}S} + \frac{1}{V_{max}} \quad (1)$$

where I is the overall concentration of the remaining inhibitors taken as a whole and K_i the related overall inhibition constant.

A comparison of this equation with that holding in the absence of inhibitors allows to relate the apparent saturation constant for the hemicellulose hydrolysate (K'_s) to the one for the synthetic xylose solution (K_s):

$$K'_s = K_s(1 + I/K_i) \quad (2)$$

where the presence of the inhibition term $(1 + I/K_i)$ is responsible for the higher value of K'_s if compared with K_s.

The overall inhibition exerted by the recalcitrant sugars (rhamnose, galactose, and arabinose) can then be considered as a whole assuming the sum of their concentrations as total inhibitor concentration, I. From the composition of the raw hydrolysate A in Table 1, the values of I listed in the last line of Table 4 have been obtained. Com-

paring only the fermentations of media with the same starting xylose levels, overall inhibitor concentrations of 5.19 and 7.78 g l^{-1} and overall inhibition constants of 0.439 and 0.743 g l^{-1} have been estimated at $S_o = 50$ and 75 g l^{-1}, respectively. As expected by the model of competitive inhibition, not too different values of K_i/S_o have been calculated from these values ($8.8 \cdot 10^{-3}$ and $9.9 \cdot 10^{-3}$, respectively).

4. Conclusions

The present kinetic study confirms that the simultaneous presence of glucose and xylose in hemicellulose hydrolysate is responsible for a sequential mechanism of sugar utilisation, by which glucose is consumed nearly completely before D-xylose could be metabolised at valuable rate.

A graphical method has shown, for the fermentation of oak hemicellulose acid-hydrolysate by *P. tannophilus*, a substrate saturation up to $S_o \cong 100$ g l^{-1} and an additional inhibition over this threshold probably related to increases in both osmotic pressure and viscosity. In addition, a comparison between the kinetic parameters calculated for hemicellulose hydrolysate and synthetic xylose medium with similar starting xylose concentration suggests the presence in the former solution of additional competitive-type inhibitors. Indications come from the present study that these inhibitors may coincide with the fraction of minor sugars contained in wood hemicellulose (L-rhamnose, D-galactose, and L-arabinose) that are metabolised only slowly or not at all.

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