Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase

Tobias MODIG, Gunnar LIDÉN¹ and Mohammad J. TAHERZADEH

Department of Chemical Engineering II, Lund University, P.O. Box 124, SE-221 00, Lund, Sweden

The kinetics of furfural inhibition of the enzymes alcohol dehydrogenase (ADH; EC 1.1.1.1), aldehyde dehydrogenase (AIDH; EC 1.2.1.5) and the pyruvate dehydrogenase (PDH) complex were studied *in vitro*. At a concentration of less than 2 mM furfural was found to decrease the activity of both PDH and AIDH by more than 90 %, whereas the ADH activity decreased by less than 20 % at the same concentration. Furfural inhibition of ADH and AIDH activities could be described well by a competitive inhibition model, whereas the inhibition of PDH was best described as non-competitive. The estimated K_m value of AIDH for furfural was found to be about 5 μ M, which was lower than that for acetaldehyde (10 μ M). For ADH,

however, the estimated K_m value for furfural (1.2 mM) was higher than that for acetaldehyde (0.4 mM). The inhibition of the three enzymes by 5-hydroxymethylfurfural (HMF) was also measured. The inhibition caused by HMF of ADH was very similar to that caused by furfural. However, HMF did not inhibit either AIDH or PDH as severely as furfural. The inhibition effects on the three enzymes could well explain previously reported *in vivo* effects caused by furfural and HMF on the overall metabolism of *Saccharomyces cerevisiae*, suggesting a critical role of these enzymes in the observed inhibition.

Key words: acetaldehyde, ADH, AIDH, HMF, PDH.

INTRODUCTION

Furfural and 5-hydroxymethylfurfural (HMF) are the respective hydrolysis products of pentoses and hexoses [1]. As such, they will be present in the media of several biotechnological processes in which pentoses and/or hexoses are heat-pretreated, or in processes where these sugars are obtained from lignocellulosic materials by hydrolysis. The presence of these compounds in the lignocellulosic hydrolysates to be used for fermentative ethanol production has been of particular interest, since both furfural and HMF have been found to have significant inhibitory effects on the metabolism of the yeast *Saccharomyces cerevisiae* [2,3]. Furthermore, HMF is of importance in the food industry, where it is a product of browning (Maillard) reactions as a result of heating processes [4,5]. Furfural and HMF are usually considered toxic to many different types of organism, such as mammalian cells [6,7], fungi [8], yeast [9] and bacteria [10].

Although the overall inhibitory effects of these furans have been studied by several groups, surprisingly little is known about what enzymes are affected and the mode of inhibition exerted by the compounds. Banerjee et al. [11] investigated the inhibition of furfural (> 1 g/l) on different glycolytic enzymes, such as hexokinase, phosphofructokinase, triosephosphate dehydrogenase, aldolase and alcohol dehydrogenase. They reported the glycolytic dehydrogenases to be the enzymes most sensitive to furfural. However, *in vivo* studies on intact yeast cells indicated that the inhibition effects of furfural and HMF on non-glycolytic enzymes could be even more important than effects on the glycolytic enzymes [9,12]. This was indicated by a very strong effect on specific growth rate, but only a partial inhibition of the glycolytic flux by addition of furfural or HMF (4 g/l) to the medium. In addition, even for respirative growth on ethanol and acetate (i.e. no active glycolysis), furfural was found to be a strong inhibitor

of metabolism in *S. cerevisiae* [13]. The study by Taherzadeh et al. [13] suggested aldehyde dehydrogenase (AIDH) to be an important site of *in vivo* inhibition. HMF is generally reported to have less-inhibitory effects *in vivo* than furfural [12]. Therefore, different mechanisms of inhibition are sometimes suggested [14]. However, actual kinetic studies of the inhibition of relevant enzymes are still lacking.

In the current work the inhibition effects of furfural and HMF on three specific enzymes, all strongly implied to be important for *in vivo* inhibition effects in yeasts, were studied. These enzymes were alcohol dehydrogenase (ADH; EC 1.1.1.1), AIDH (EC 1.2.1.5) and the pyruvate dehydrogenase (PDH) complex. It was found that both AIDH and PDH were strongly inhibited by furfural and somewhat less strongly inhibited by HMF. ADH was less inhibited than AIDH and PDH, but there was no big difference in the inhibition exerted by furfural and HMF. Furthermore, kinetic analysis of the mode of furfural's inhibition of the enzymes was performed. The data were in good agreement with competitive inhibition of ADH and AIDH by furfural, whereas an non-competitive mode of inhibition was suggested for PDH.

MATERIALS AND METHODS

Kinetic analysis

The experimental data were analysed in relation to the inhibition scheme presented in Scheme 1, where the enzyme E is inhibited by inhibitor I during the reaction with substrate S. Product formation from the inhibitor is included in the model, and K_s and K_{ie} are the dissociation constants of the ES and EI complexes, respectively. K_{is} and K_{iu} are the dissociation constants of the ESI complex for the breakdown to EI + S and ES + I, respectively. The obtained differential equation describing the system and the

Abbreviations used: HMF, 5-hydroxymethylfurfural; AIDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; PDH, pyruvate dehydrogenase.

¹ To whom correspondence should be addressed (e-mail Gunnar.Liden@chemeng.lth.se).

Scheme 1 Schematic representation of reversible inhibition

S, substrate; E, enzyme; I, inhibitor; P, product. The constants K_x denote dissociation constants for the respective reactions; for further details, see the text.

mixed non-competitive/competitive model is summarized in eqn (1):

$$\frac{d([P_1] + [P_2])}{dt} = \frac{\frac{V_1[S]}{K_m} + \frac{V_2[I]}{K_{ic}}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_{ic}} + \frac{[S][I]}{K_m K_{iu}}} \quad (1)$$

Depending on the values of the dissociation constants, this general model can be simplified. For a very large value of K_{iu} , purely competitive inhibition is obtained and eqn (1) is simplified to eqn (2):

$$\frac{d([P_1] + [P_2])}{dt} = \frac{\frac{V_1[S]}{K_m} + \frac{V_2[I]}{K_{ic}}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_{ic}}} \quad (2)$$

Alternatively, equal values of K_{iu} and K_{ic} results in non-competitive inhibition (eqn 3):

$$\frac{d([P_1] + [P_2])}{dt} = \frac{\frac{V_1[S]}{K_m} + \frac{V_2[I]}{K_{ic}}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_{ic}} + \frac{[S][I]}{K_m K_{ic}}} \quad (3)$$

Finally, purely non-competitive inhibition is obtained when the value of the dissociation constant K_{ic} is very large (eqn 4):

$$\frac{d([P_1] + [P_2])}{dt} = \frac{\frac{V_1[S]}{K_m}}{1 + \frac{[S]}{K_m} + \frac{[S][I]}{K_m K_{iu}}} \quad (4)$$

Furfural's inhibition of all the three dehydrogenases was modelled based on the general model shown in eqn (1). The general model was subsequently simplified based on significance tests of $1/K_x$, where K_x denotes the dissociation constant for the tested reaction.

Chemicals

ADH and ALDH were obtained from Boehringer Mannheim enzymic bioanalysis/food analysis kits (catalogue nos. E0668613 and E0176290; R-Biopharm, Darmstadt, Germany). These

enzymes were purified from baker's yeast. PDH from porcine heart was purchased from Sigma (St. Louis, MO, U.S.A.). The coenzymes NAD⁺ (98 % purity), NADH, CoA (from yeast, 93 % purity), MgCl₂ (98 % purity) and thiamine diphosphate (98 % purity) were all obtained from Sigma. Substrates and inhibitors were acetaldehyde from Merck, and pyruvate, furfural and HMF from Sigma (all > 99 % purity). Phosphate and diphosphate buffers were prepared from potassium salts of analytical grade purchased from Sigma.

Enzyme assays

Activity measurements for ADH, ALDH and PDH were made in the presence of furfural in the range 0–4 g/l and HMF in the range 0–4 g/l, corresponding to 0–41.6 mM furfural and 0–31.7 mM HMF, respectively. Enzyme assays were carried out at a constant temperature (30 °C) and the rates of the reactions were monitored by either depletion or formation of NADH at 340 nm. The relevant reactions are given by eqns (5–7).

Measurements were made using a Unicam Helios Alpha UV-visible spectrometer (Thermo Spectronic, Cambridge, U.K.), equipped with a cell programmer and connected to a water-bath temperature-control unit. Commercially available software (Vision; Thermo Spectronic) was used for data acquisition. Disposable macro cuvettes (Plastibrand, Wertheim, Germany) were used as reaction vessels and a maximum of four samples was processed concurrently in the cell programmer.

Buffer and coenzymes were pipetted into a cuvette and the sample solution was added. Substrate and/or inhibitor were incubated together with the buffer system for 5 min to equilibrate the temperature. Finally, the enzyme solution was added to start the reaction. During the incubation and reaction periods, the cuvettes were sealed with Parafilm to avoid evaporation of volatile compounds.

Unless otherwise stated, the ADH assay mixture contained 0.1 mM potassium diphosphate buffer (pH 9.0), 0.1 mM NADH and 0.14 mM acetaldehyde. The reaction was started by addition of ADH (8.0 units/l). The mixture for the ALDH assays contained 0.1 mM potassium diphosphate buffer (pH 9.0), 0.4 mM NAD⁺ and 0.14 mM acetaldehyde, and the reaction was initiated by addition of ALDH (1.4 units/l). The PDH assay mixture contained 25 mM potassium phosphate buffer (pH 7.0), 0.4 mM NAD⁺, 0.2 mM thiamine diphosphate, 1.0 mM MgCl₂, 0.13 mM CoA and 0.1 mM pyruvic acid. The reaction was initiated by addition of PDH (6.2 units/l). Rate data were taken from the first linear phase (3–5 min).

Data analysis

Kinetic parameters were estimated using non-linear regression analysis. The sub-routine NLINFIT from the Statistics Toolbox (MathWorks) was used together with MATLAB for performing Gauss–Newton non-linear least-squares parameter fitting. For experiments in which both acetaldehyde and furfural were present in the reaction medium, the combined rate of acetaldehyde and furfural conversion was measured and the appropriate equations for that case were used.

RESULTS

Activity measurements of ADH, AIDH and PDH

The activities of the enzymes ADH, AIDH and PDH were all clearly affected by the presence of furfural (Figure 1a). Most apparent was the severe inhibition of AIDH and PDH. The activities of these enzymes decreased by 80 % in the presence of only 1.3 mM (0.12 g/l) furfural, and by more than 90 % in the presence of 10.4 mM (1.0 g/l) furfural. However, furfural did not affect ADH to the same extent; 90 % of its activity was maintained in the presence of 1.3 mM furfural, and as much as 40 % of the original activity was maintained in the presence of 41.6 mM furfural. The corresponding activities of AIDH and PDH in the latter case were only 5 % and 0.2 %, respectively.

The relative activities of the dehydrogenases in the presence of different concentrations of HMF were measured in the same way as for furfural. The decrease in ADH activity caused by HMF was comparable with that caused by furfural. In contrast, the activities of PDH and AIDH were clearly less affected by HMF (Figure 1b).

Enzyme kinetics

The kinetics of the three enzymes were first evaluated for the natural substrate, in order to allow for subsequent modelling of inhibition effects.

ADH

The measured rate of acetaldehyde reduction catalysed by ADH at different substrate concentrations is shown in Figure 2(a). The kinetics of this reaction can be described well by a Michaelis–Menten model (eqn 8), as shown by the model prediction in Figure 2(a).

$$V = V_{\max} \frac{[S]}{K_m + [S]} \quad (8)$$

The estimated parameter values of $V_{\max}$ and K_m were 0.0217 $\mu\text{mol}/\text{min}$ and 0.372 mM, respectively, and the proportion of variance (R^2) explained by the model was 0.993, indicating a good model fit (Table 1).

Furfural may be reduced to furfuryl alcohol by ADH, according to eqn (9):

The rate of this reaction was measured at different furfural concentrations (Figure 2b). Due to the much lower specific rate of reaction, 80 units/l ADH was used in the assay. Also in this case a Michaelis–Menten model (eqn 8) was found to adequately describe the kinetics. The estimated $V_{\max}$ and K_m values were 0.0442 $\mu\text{mol}/\text{min}$ and 1.83 mM, respectively.

The simultaneous reduction of acetaldehyde and furfural by NADH with ADH, yielding ethanol and furfuryl alcohol, respectively, was investigated at different concentrations of both furfural (0–21 mM) and acetaldehyde (0.18–5.6 mM; Figure 2c). The results were first modelled by the general inhibition model (eqn 1). The standard variation of K_{iu} was the same order of magnitude as the parameter value. The hypothesis test $1/K_{\text{iu}} > 0$ was also found not to be significant. Therefore, parameter estimation for a purely competitive model (eqn 2) was performed. The variation explained by the purely competitive model was almost the same as that explained by the general inhibition model, despite the use of fewer parameters in the former. All parameters in the purely competitive model were, furthermore, significant (Table 1). The model predictions for the purely competitive model are shown in Figure 2(c). The estimated K_m

Figure 1 Activity measurements on the enzymes ADH (■), AIDH (●) and PDH (□)

(a) Activity in the presence of furfural; (b) activity in the presence of HMF.

values indicate that the affinity of ADH for acetaldehyde is approx. 3-fold higher than that for furfural.

AIDH

Preliminary experiments showed that neither the oxidation of acetaldehyde (eqn 5) nor the oxidation of furfural by NAD^+ (eqn 10) using AIDH, could be well described by a simple Michaelis–Menten model:

This was most probably due to the substrate inhibition, which is known to occur at high acetaldehyde concentrations (Scheme 2 [15]).

From measured reaction rates using either acetaldehyde or furfural as substrate, the values of the parameter K_{si} were found to be 1.0 and 22 mM for acetaldehyde and furfural, respectively (Figures 3a and 3b). The values suggest that acetaldehyde's substrate self-inhibition of AIDH is much stronger than that of furfural. In order to avoid substrate inhibition, the concentration of acetaldehyde was chosen to be below 0.1 mM and that of furfural was kept below 0.3 mM in subsequent experiments. In this concentration range, the enzyme's behaviour was well described by Michaelis–Menten kinetics for both substrates (Figures 3c and 3d). The obtained values of K_m were 10.1 and 4.89 μM for acetaldehyde and furfural, respectively.

The measured conversion rates for simultaneous addition of acetaldehyde (9.15–68.6 μM) and furfural (6.36–25.4 μM) are presented in Figure 3(e). In this assay 0.74 units/l AIDH was used. Statistical analysis of the model resulted its simplification to

Figure 2 Measurements of the initial conversion rate obtained for ADH versus (a) acetaldehyde, (b) furfural and (c) acetaldehyde in the presence of furfural. In (c), furfural concentrations were: ○, 20.8 mM; ●, 5.2 mM; □, 0.52 mM; ■, 0 mM. ΔAbs, change in absorbance at 340 nm.

Table 1 Kinetic parameters for ADH determined from initial rate measurements

Acetaldehyde, furfural or both acetaldehyde and furfural were used as substrates. Only parameter estimates for the best inhibition model are shown.

Model	Substrate	Parameter name	Estimated value	<i>R</i> ²
Michaelis–Menten	Acetaldehyde	<i>V</i> (μmol/min)	0.0217 ± 0.00048	0.993
		<i>K_m</i> (mM)	0.372 ± 0.035	
Michaelis–Menten	Furfural	<i>V</i> (μmol/min)	0.00441 ± 0.0016	0.9958
		<i>K_m</i> (mM)	1.83 ± 0.84	
Competitive inhibition	Acetaldehyde + furfural	<i>V</i> ₁ (μmol/min)	0.0234 ± 0.00061	0.9748
		<i>V</i> ₂ (μmol/min)	0.00655 ± 0.00061	
		<i>K_m</i> (mM)	0.358 ± 0.036	
		<i>K_i</i> (mM)	1.18 ± 0.26

Scheme 2 Schematic representation of substrate inhibition

S, substrate; E, enzyme; P, product. The constants *K_x* denote dissociation constants for the respective reactions; for further details, see the text.

a purely competitive one (Table 2). Interestingly, and contrary to the case for ADH, the affinity of AIDH was clearly higher for furfural than for acetaldehyde. The *V_{max}* value was, however, approx. 10-fold lower for furfural.

PDH

A major difference between PDH and the enzymes discussed above, ADH and AIDH, is that furfural is not reduced by PDH. Therefore, only one conversion term needs to be considered in eqn (1). Similarly to the previous cases, the kinetics of pyruvate conversion were first considered separately and then in the

Figure 3 Measurements of the initial conversion rate obtained for AIDH

Reactions were carried out in the presence of (a) acetaldehyde and (b) furfural. Lower concentrations of (c) acetaldehyde and (d) furfural were then used to avoid substrate inhibition. (e) Reactions were performed with acetaldehyde in the presence or absence of furfural: $\circ$, 25.4 μM ; $\bullet$, 12.7 μM ; $\square$, 6.4 μM ; $\blacksquare$, 0 mM. ΔAbs , change in absorbance at 340 nm.

presence of furfural. The conversion rate of pyruvate is shown in Figure 4(a). The results were described well by a simple Michaelis–Menten-type equation, with a K_m value of 18.7 μM . The conversion rates in the presence of both pyruvate (5–50 μM) and furfural (0–0.325 mM) are presented in Figure 4(b). In this case a non-competitive model was found to be most adequate (Table 3). The K_m value for pyruvate was higher than for furfural; however, both values were low, indicating a strong affinity.

DISCUSSION

The three enzymes studied were selected for their potential importance with respect to inhibition effects exerted primarily by furfural on yeast metabolism. The concentrations of furfural

were chosen to be representative of conditions likely to occur during fermentation of lignocellulosic hydrolysates [9,13,16]. In addition, the highest concentration of acetaldehyde in the present work was 0.5 g/l, which is comparable with the reported highest intracellular concentration of acetaldehyde in yeast, 0.35 g/l [17]. The source of PDH in the present study was porcine heart. Most kinetic and regulatory properties of PDH have been reported to be similar for mammalian and yeast PDHs, although the K_m for pyruvate at the optimum pH has been reported to be significantly higher for yeast PDH [18].

Overall, furfural was found to drastically decrease the activities of AIDH and PDH, whereas ADH was inhibited to a much smaller extent (Figure 1a). Measured K_m values indicate a higher affinity of AIDH for furfural than for its natural substrate, acetaldehyde (Table 2). However, ADH has an almost three

Table 2 Kinetic parameters for AIDH determined from initial rate measurements

Acetaldehyde, furfural or both acetaldehyde and furfural were used as substrates. Only parameter estimates for the best inhibition model are shown.

Model	Substrate	Parameter name	Estimated value	R^2
Michaelis–Menten	Acetaldehyde	V ($\mu\text{mol}/\text{min}$)	0.0104 ± 0.00054	0.9824
		K_m (μM)	10.1 ± 1.4	
Michaelis–Menten	Furfural	V ($\mu\text{mol}/\text{min}$)	0.00101 ± 0.000021	0.9919
		K_m (μM)	4.89 ± 0.49	
Substrate inhibition	Acetaldehyde	V ($\mu\text{mol}/\text{min}$)	0.00283 ± 0.00021	0.9114
		K_m (μM)	9.7 ± 2.4	
		K_{si} (mM)	0.998 ± 0.26	
Substrate inhibition	Furfural	V ($\mu\text{mol}/\text{min}$)	0.00322 ± 0.000093	0.959
		K_m (μM)	7.2 ± 0.93	
		K_{si} (mM)	22.1 ± 6.5	
Competitive inhibition	Acetaldehyde + furfural	V_1 ($\mu\text{mol}/\text{min}$)	0.00475 ± 0.00020	0.9853
		V_2 ($\mu\text{mol}/\text{min}$)	0.000657 ± 0.00024	
		K_m (μM)	10.8 ± 1.4	
		K_{ic} (μM)	6.72 ± 1.6

Figure 4 Measurements of the initial conversion rate obtained for PDH versus (a) pyruvate and (b) pyruvate in the presence of furfural

In (b), concentrations of furfural were: ●, 325 μM ; □, 163 μM ; ■, 81.3 μM ; ○, 0 μM . ΔAbs , change in absorbance at 340 nm.

times higher affinity for acetaldehyde than for furfural. On the other hand, the V_{max} values for both ADH and AIDH were clearly lower for furfural than for acetaldehyde. Therefore, it can be concluded that AIDH binds furfural preferably over acetaldehyde, but that the separation of furfural from the enzyme is much slower than for acetaldehyde.

Table 3 Kinetic parameters for PDH determined from initial rate measurements

Pyruvic acid was used as substrate, and furfural was present as an inhibitor. Only parameter estimates for the best inhibition model are shown.

Model	Substrate	Parameter name	Estimated value	R^2
Michaelis–Menten	Pyruvic acid	V ($\mu\text{mol}/\text{min}$)	0.00991 ± 0.00012	0.9996
		K_m (μM)	18.6 ± 0.75	
Non-competitive inhibition	Pyruvic acid	V ($\mu\text{mol}/\text{min}$)	0.00886 ± 0.00034	0.993
		K_m (μM)	18.5 ± 1.6	
		K_{iu} (μM)	65.7 ± 4.4	

This conclusion suggests that the presence of furfural (e.g. 1 g/l) in a yeast culture could effectively stop both pathways leading to acetyl-CoA from pyruvate. That is, both the direct reaction from pyruvate to acetyl-CoA via PDH, and the PDH-bypass route involving pyruvate decarboxylase, AIDH and acetyl-CoA synthetase [19]. However, the flux from pyruvate to ethanol may still proceed at an appreciable rate (Scheme 3). This is in good agreement with reports on the effects of furfural on yeast metabolism. Growth has been found to be more sensitive to furfural than ethanol production (e.g. [20]). Addition of furfural (4 g/l) to an exponentially growing batch culture of *S. cerevisiae* caused a decrease in the specific growth rate by 93 % and a decrease in the specific ethanol production rate by 68 % [9]. Furthermore, for purely respirative growth on ethanol, addition of furfural (4 g/l) completely inhibited cell growth and caused a decrease in the ethanol-consumption rate by 56 % [13]. As a comparison, the activity of PDH, AIDH and ADH decreased by 99.8 %, 95 % and 60 %, respectively, in the presence of 4 g/l furfural (Figure 1a). Interestingly, addition of acetate after furfural addition largely restored the production of CO_2 [13]. The tricarboxylic acid cycle is the main source of CO_2 production during respirative growth, and the above results support the suspicion that AIDH is more sensitive than the other enzymes in the tricarboxylic acid cycle. Furthermore, acetaldehyde has been found to give inhibition effects on yeast growth at a concentration of 0.07 g/l [21]. This could again be connected to inhibition effects on AIDH (Figure 2a).

Furfural is not the only aldehyde known to inhibit the studied dehydrogenases. PDH has been reported previously to be inhibited by acetaldehyde [22]. Addition of 1 and 5 mM acet-

Scheme 3 Simplified scheme showing major pathways in the metabolism of the yeast *S. cerevisiae* and possible sites of interaction with furfural

TCA cycle, tricarboxylic acid cycle.

aldehyde decreased the specific activity of PDH by 89 % and 95 %, respectively. The PDH activity also decreased by 98 %, 85 % and 85 % in the presence of 5 mM benzaldehyde, propionaldehyde and butyraldehyde, respectively. Steinman and Jakoby [15] compared the conversion of a large number of different aldehydes, including furfural, using AIDH. They reported the relative velocity and K_m value of the enzyme using furfural as a substrate to be 8 % and 55 %, respectively, of the corresponding values using acetaldehyde. This is in good agreement with our results, which gave 10 % of the maximum velocity and a K_m value of 48 % of the corresponding values for acetaldehyde.

The relative maximum velocities of conversion of formaldehyde, propionaldehyde, *n*-butyraldehyde, isobutyraldehyde, D,L-glyceraldehyde, glutaraldehyde, glycoaldehyde, benzaldehyde and *m*- and *p*-nitrobenzaldehyde were between the values of acetaldehyde and furfural; the exception was 2,4-dihydroxybenzaldehyde, which had a slightly lower value than that of furfural. On the other hand, the K_m value for furfural was not the lowest found. For human liver AIDH, it has been reported that the K_m of aldehydes can be related to the length and size of the molecules, i.e. bigger molecules result in lower K_m values [23].

Yeast ADH has a rather low specificity, and catalyses the conversion of several substrates [24,25]. The catalytic activity tends to decrease with increasing size of the aldehyde substrate. Reversibility of the inactivation is also an issue when the enzyme faces some substrates such as *o*-phthalaldehyde [26].

During continuous cultivation of *S. cerevisiae*, only low furfural concentrations are found in the medium. Taherzadeh and co-workers [16] reported a furfural concentration of 0.05 g/l using a feed medium containing 5.8 g/l furfural in a continuous cultivation at dilution rate of 0.1 h⁻¹. Washout occurred at higher concentrations of furfural in the feed. A specific growth rate of 0.1 h⁻¹ corresponds to 22 % of the maximum specific growth rate. In the present study it was found that a furfural

concentration of 0.05 g/l gave 24 % remaining AIDH activity, whereas as much as 91 % remaining ADH activity was found.

The inhibition effects of HMF on activities of the three dehydrogenases were also measured (Figure 1b). The activity of ADH decreased in a very similar way for the two furans. However, the decrease in activity of both AIDH and PDH was considerably less than that for furfural. At an HMF concentration of 10 mM, for example, 50 % AIDH activity was maintained, whereas only 10 % activity was maintained at the same furfural concentration. The same applied, qualitatively, for PDH. The weaker inhibition effects of HMF on these enzymes was well in line with its observed smaller *in vivo* effects on yeast metabolism [12].

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