Kinetics and inhibition effects of furfural and hydroxymethyl furfural on enzymes in yeast

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Abstract

Fermentatively produced ethanol from lignocellulose by yeast is a renewable alternative to fossil fuels. However, inhibitors originating from the breakdown process of the raw material decrease the fermentation rate. Previous studies have made known inhibition effects by the furan compounds furfural and hydroxymethyl furfural (HMF). In the current work, the kinetics and inhibition effect of furfural, and in some cases also HMF, on the enzymes alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AIDH), and pyruvate dehydrogenase (PDH) were studied. This was done using in vitro spectro-photometric assays with commercially available pure enzymes. The study showed that furfural strongly inhibited both AlDH and PDH, but the inhibition effects of HMF on AlDH and PDH were not as big. Furthermore, ADH was much less affected than AIDH and PDH by furfural, and was similarly inhibited by both furans. The kinetic analysis suggested that furfural and HMF most likely inhibited ADH and AIDH in a competitive manner, whereas PDH was inhibited in a mixed mode in the presence of furfural. The present results offer an explanation to the strong effects exerted by furans on the yeast metabolism.

1. Introduction

The current extensive use of fossil resources for energy production is well known to significantly influence the environment. Therefore, in order to lower the impact on the environment, and find an economical alternative to fossil fuels, a changeover to energy sources made from renewable resources is an attractive strategy. One renewable resource is lignocellulose, e.g., wood, which is also widely available in Sweden. From wood, ethanol can be fermentatively produced and utilised as an alternative energy source.

The production of ethanol from lignocellulose requires pretreatment steps preceding the fermentation, in which the sugar polymers are made accessible to the fermenting microorganism. This is achieved by hydrolytic conversion of the polymer to its corresponding single units. One of the most commonly applied methods is chemical hydrolysis by dilute acid. In addition to the sugars in the hydrolysate there are compounds formed or released during the hydrolysis, that are toxic to microorganisms, including yeasts. Identified inhibitors are, e.g., acetic acid, formic acid, levulinic acid, furfural, HMF, phenol, and vanillin.

The mechanism of inhibition for the individual toxins on yeast metabolism is in most cases not understood. Furthermore, there is also a possibility for interaction effects between compounds [1]. Furan compounds originate from Maillard reactions of pentoses and hexoses during acid hydrolysis, and is one of the most important inhibitor groups in dilute-acid hydrolysates. Furfural results from the breakdown of pentoses, e.g., xylose, whereas HMF is formed from hexoses, e.g., glucose.

The effect of furfural on cultivation of yeast has been considerable in many studies. Among known effects for batch cultivations are a decrease in the ethanol production rate and specific growth rate. The mode in which furfural inhibit yeast metabolism is not completely known. Though, it has been suggested that furfural inhibits central enzymes in glycolysis, e.g., hexokinase, phosphofructokinase, and triosephosphate dehydrogenase [2]. In addition, enzymes coupled to the citric acid cycle and ethanol formation, e.g., alcohol dehydrogenase and aldehyde dehydrogenase, have also been suggested [3]. Yeast has been reported to take up and transform furfural into furfuryl alcohol and furoic acid under anaerobic conditions [4]. The most probable route of this conversion is that furfural replace acetaldelyde as substrate for alcohol dehydrogenase and aldehyde dehydrogenase.
The objective of the current work was to characterise inhibition effects of the furans furfural and HMF on enzymes in yeast. The experimental method used was in vitro measurements of activity by spectrophotometry. Three enzymes, alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AlDH) and pyruvate dehydrogenase (PDH) were studied.

2. Kinetic analysis

The general mechanism used in the study, including substrate inhibition and product formation from inhibitor is shown in Scheme 1. For each enzyme different pathways were applicable, which was determined by the kinetic analysis of the enzymes with substrate and/or inhibitor.

\[
\begin{align*}
\text{ESS} & \quad K_s \\
S & \quad E+S \\
& \quad K_{cS} + k_{cS} \quad E + P_1 \\
& \quad + I \\
& \quad I \\
\text{EI} + S & \quad K_{cE} \\
& \quad P_2 + E \\
& \quad k'_{cE} \\
& \quad ESI
\end{align*}
\]

Scheme 1.

3. Material and methods

All enzymes used in this study were commercially available and primarily of yeast origin. Alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.5) were obtained from Boehringer Mannheim enzymatic bioanalysis/food analysis kits (Cat. No. E0668613 and E0176290, R-Biopharm, Darmstadt, Germany). The source of these enzymes was yeast, and the estimated specific activity for ADH and AlDH, were 400 and 35 U/mg protein respectively. Pyruvate dehydrogenase 1 U/mg (from porcine heart) was purchased from Sigma (St. Louis, MO, USA). The coenzymes NAD\(^+\) (98% purity), NADH, coenzyme A (from yeast, 93% purity), and thiamine diphosphate (98% purity) were all obtained from Sigma. Substrates/inhibitors were acetaldehyde from MERCK, pyruvate, furfural, and 5-hydroxymethyl furfural from Sigma, all of 99% purity. Phosphate and diphosphate buffers were prepared from potassium salts purchased from Sigma.

Experiments for activity measurements and the determination of kinetic parameters were performed in a Hélios Alpha UV-Visible Spectrometer from Unicam, equipped with a cell programmer connected by a tubing kit to a thermostatted water bath (HAAKE circulator C10, bath P5). All assays were conducted at constant temperature and the progress curves were recorded with the controlling software VISION from Unicam. Disposable macro cuvettes (Plastibrand, Germany) were used as reaction vessels and a maximum of 4 samples were processed concurrently in the cell programmer.

Enzyme assays were carried out at 30 °C and the rate of the reactions were monitored by either depletion or formation of NADH at 340 nm (\(\varepsilon_{340, \text{NADH}} = 6.3 \text{ l·mmol}^{-1}·\text{cm}^{-1}\)). The measurements were based on the following reactions:

\[
\begin{align*}
\text{Acetaldehyde + NADH + H}^+ & \xrightarrow{\text{ADH}} \text{Ethanol + NAD}^+ \\
\text{Furfural + NADH + H}^+ & \xrightarrow{\text{ADH}} \text{Furfuryl alcohol + NAD}^+ \\
\text{Acetaldehyde + NAD}^+ & \xrightarrow{\text{AlDH}} \text{Acetic acid + NADH + H}^+ \\
\text{Furfural + NAD}^+ & \xrightarrow{\text{AlDH}} \text{Furoic acid + NADH + H}^+ \\
\text{Pyruvate + CoA + NAD}^+ & \xrightarrow{\text{PDH}} \text{AcetylCoA + NADH + H}^+ + \text{CO}_2
\end{align*}
\]

The following assumptions were made for the kinetic analysis. 1) The enzyme concentration was negligible compared to substrate and inhibitor concentrations. 2) Either rapid equilibrium or steady state conditions were fulfilled. 3) Initial rates were measured, i.e., no significant substrate depletion or product formation took place during the observation time. The initial rate data were obtained from the first linear phase of the progress curve where assumption 3 was acceptable. The span of this period varied from one experiment to another, but the enzyme concentration was chosen to usually obtain a linear phase of 3-5 min for sufficient resolution.

In the normal experimental procedure, 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 reach temperature equilibrium. 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. In a typical experiment, each cuvette was...
filled with 2.4 ml buffer solution containing coenzymes, 0.3 ml sample solution (varied concentrations of acetaldehyde, furfural, and HMF), and 0.05 ml enzyme solution. Unless stated otherwise, the reaction medium contained 0.1 mM potassium phosphate buffer (pH 9.0) and 0.1 mM NADH with ADH, 0.1 mM potassium phosphate buffer (pH 9.0) and 0.4 mM NAD⁺ with AlDH, and 25 mM potassium phosphate buffer (pH 7.0), 0.2 mM thiamine diphosphate (ThDP) and 0.13 mM coenzyme A (CoA) with PDH.

All models and kinetic parameters in the study were calculated by using nonlinear least-squares data fitting by the Gauss-Newton method. This was done in the software MATLAB by the routine Nlinfit. Statistical analysis was calculated with the software NLREG. The significance of parameters, and thus model discrimination was made by inspection of $R^2$ and t-values with corresponding Prob(t). The relevant significance test to be made is $P(1/K > 0)$. However, it can be shown that it is in fact sufficient to see if $K$ is significantly $> 0$.

In experiments when both acetaldehyde and furfural were present in the reaction medium, the measured reaction rate was the combined rate of acetaldehyde and furfural conversion due to the common cofactor NADH. In order to obtain the rate of conversion of acetaldehyde, modification of the measured rate was made according to the following equation:

$$v_i = \frac{v_{\text{max}}_1 [S_i]}{K_m^1 + \frac{v_{\text{max}}_2 [S_2]}{K_m^2}} v_{\text{meas}}$$

where acetaldehyde and furfural are substrate 1 ($S_i$) and substrate 2 ($S_2$), respectively.

### 4. Results

As a first indication of inhibition of the different enzymes, a series of experiments with furfural and HMF in the range of 0-4 g/l was carried out. The initial rates of the reactions 1, 3, and 5, catalysed by ADH, AlDH, and PDH respectively were studied. The result of these experiments, presented in Fig. 1, shows a clear decrease of the initial velocity in the presence of furfural or HMF, for all the investigated enzymes. Most apparent was the drastic inhibition of AlDH and PDH by furfural, which resulted in a more than 90 % reduction of the activity at furfural concentrations above 1 g/l. Neither furfural nor HMF affected ADH to the same extent. The overall inhibition effect caused by HMF (Fig. 1B) on the enzymes was less, but still significant. AlDH was slightly more affected than ADH, whereas PDH, at concentrations above 2 g/l lost practically all its activity.
Table 1
Kinetic parameters

<table>
<thead>
<tr>
<th>Substrate/inhibitor</th>
<th>Alcohol dehydrogenase</th>
<th>Aldehyde dehydrogenase</th>
<th>Pyruvate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$v_{max}$ (mmol/min g)</td>
<td>$K_M$ (µM)</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.37</td>
<td>0.39</td>
<td>10</td>
</tr>
<tr>
<td>Furfural</td>
<td>1.8</td>
<td>81</td>
<td>1.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Alcohol dehydrogenase**

The reduction of acetaldehyde and furfural by NADH with ADH, yielding ethanol and furfuryl alcohol, respectively, was first investigated at different substrate concentrations. This showed that the dependence of initial rates with substrate concentrations agreed with the assumed Michaelis-Menten relationship. In the double reciprocal plot in Fig. 2, the lines intersect with very good precision at the 1/V axis. This suggests a competitive behaviour of the inhibitor. In addition, the $K_M$ and $K_i$ values are very much the same for furfural, which is expected for a competitive inhibitor (Table 1).

**Aldehyde dehydrogenase**

Preliminary experiments showed that the oxidation of acetaldehyde and furfural by NAD$^+$ with AIDH, producing acetic acid, and furoic acid respectively, could not be well described by the tested models. The reason for this was found to be substrate inhibition by high concentrations of acetaldehyde [5]. Substrate inhibition was also found for furfural. However, the inhibition effect was not as strong as for acetaldehyde. The inhibition effect exerted by acetaldehyde was subsequently accounted for in the kinetic analysis. To avoid substrate inhibition, the concentration of acetaldehyde was chosen below 0.1 mM in subsequent experiments.

**Pyruvate dehydrogenase**

PDH was not as thoroughly studied as ADH and AIDH. A kinetic study of PDH was carried out, although including less data points. Oxidation of pyruvate to acetyl-CoA by NAD$^+$ with CoA and PDH at different pyruvate concentration was first studied. This reaction

![Fig. 3. Inhibition of AIDH by furfural. The reciprocal initial rate plotted vs. the reciprocal of the acetaldehyde concentrations at different furfural concentrations. Each line was obtained by non-linear regression of the untransformed Michaelis-Menten relationship. The furfural concentrations (mM) were: 0 (●), 0.52 (○), 5.2 (●), 20.8 (○), and the NAD$^+$ concentration used was 0.4 mM.](image)

![Fig. 4. Inhibition of PDH by furfural. The reciprocal initial rate plotted vs. the reciprocal of the pyruvate concentrations at different furfural concentrations. Each line was obtained by non-linear regression of the untransformed Michaelis-Menten relationship. The furfural concentrations (µM) were: 0 (●), 6.62 (○), 66.2 (●), and the NAD$^+$ and CoA concentration used were 0.4 and 0.13 mM, respectively.](image)
obeyed Michaelis-Menten kinetics. The velocity of conversion of pyruvate to acetyl-CoA was monitored at fixed furfural concentrations 0-4 g/l with different pyruvate concentrations. The result presented in Fig. 4, where the lines intersect left of the 1/V axis, indicated mixed inhibition by furfural.

5. Discussion

In the present work it has been shown that the enzymes ADH, AIDH, and PDH, were all affected by the presence of furfural and HMF. It was found that the effect could be well described by competitive inhibition for ADH and AIDH, and mixed inhibition for PDH. The degree of inhibition by furfural was very high on AIDH and PDH, whereas ADH functioned well at high furfural concentrations. In comparison with furfural, HMF did not have the same strong effect on AIDH and PDH. However ADH was affected in practically the same way for both furans. The competitive mode of inhibition of ADH and AIDH by furfural was expected, as the enzymes were able to convert furfural to furfuryl alcohol and furoic acid, respectively.

The mode, in which HMF caused inhibition of the enzymes, is merely a case of speculation. However, the previous reports of conversion of HMF in the same way as furfural indicate a similar mode of inhibition [6]. Though, the affinity of HMF for AIDH and PDH, and the maximum rate of conversion would presumably be lower, considering the observed slower rate of conversion and less inhibitory effect.

The obtained Michaelis constants of AIDH for acetaldehyde and furfural [5], and that of ADH for acetaldehyde [7] is similar to previous reported for yeast. Both ADH and AIDH catalyse the conversion furfural at a slower rate, compared to their natural substrate acetaldehyde, which also is anticipated. The conversion rate of acetaldehyde is about 5 and 10 times faster for ADH and AIDH, respectively. For PDH and pyruvate, the case is quite different, as furfural is not a possible substrate. Furthermore, the source of the PDH enzyme in this study was porcine heart. The obtained Michaelis constant for pyruvate is comparable to other studies conducted with this enzyme source [8], but it differs strikingly from the yeast PDH complex. A $K_{mi}$ approximately 10 times the $K_{mi}$ of porcine PDH in this study has been reported for yeast PDH [9].

Previous studies of fermenting S. cerevisiae have shown that no, or very little cell growth occurs when furfural is present in the cultivation broth. However, ethanol production is held at a fairly high level despite the presence of furfural. Accumulation of pyruvate and acetaldehyde has also been seen. These effects can be the total consequence of different actions by furfural on the studied enzymes, which practically stops both of the shuttles from pyruvate leading to acetyl-CoA and TCA cycle, but not to ethanol.

6. Conclusion

Furfural and HMF may severely affect the fermentation rate in ethanol production, and inhibition at the enzymatic level is considered one reasonable explanation for this occurrence. In this study the extent of inhibition on AIDH and PDH was strong, whereas the effect on ADH was not as big. HMF was less inhibitory to AIDH and PDH in comparison with furfural, while the effect on ADH was similar to that of furfural. Furfural could serve as substrate for ADH and AIDH, and hence inhibited AIDH and ADH in a competitive manner. PDH was inhibited in a mixed manner by the presence of furfural. These results agree well with previous reports from in vivo studies of fermenting S. cerevisiae and support the hypothesis of inhibition by furfural and HMF at the enzymatic level.

References