Evidence that the gene *YLR070c* of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase

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Abstract The open reading frame *YLR070c* of *Saccharomyces cerevisiae* has high sequence similarity to *S. cerevisiae* sorbitol dehydrogenase and to xylitol dehydrogenase of *Pichia stipitis*. Overexpression of this open reading frame in *S. cerevisiae* resulted in xylitol dehydrogenase activity. The enzyme is specific for NADH. The following Michaelis constants were estimated: D-xylulose, 1.1 mM; NADH, 240 μM (at pH 7.0); xylitol, 25 mM; NAD, 100 μM (at pH 9.0). Xylitol dehydrogenase activity with the same kinetic properties can also be induced by xylose in wild type *S. cerevisiae* cells.

Key words: Xylitol dehydrogenase; Xylose metabolism; D-Xylulose; Enzyme kinetics; *Saccharomyces cerevisiae*

1. Introduction

Saccharomyces cerevisiae is an efficient ethanol producer but cannot ferment xylose which would be relevant in the context of ethanol production from lignocellulosic materials. Although *S. cerevisiae* is not able to ferment or grow on xylose unlike many other yeasts, it is reported to metabolise it, though with a very slow rate [1]. Yeasts that metabolise xylose generally use a pathway of three cytosolic enzymes: xylose reductase, xylitol dehydrogenase, and xylulokinase to convert xylose to xylulose 5-phosphate which is then metabolised through the pentose phosphate pathway [2]. Xylose uptake in *S. cerevisiae* is thought to occur through some of the glucose transporters [3,4]. Evidence for activities of the xylose pathway enzymes xylose reductase, xylitol dehydrogenase and xylulokinase has been reported in *S. cerevisiae*.

Kuhn et al. [5] purified an aldo-keto reductase from *S. cerevisiae*. This enzyme showed highest activity with *p*-nitrobenzaldehyde, but also high activity with D- and L-glyceraldehyde and D-xylose and lower activity with L-arabinose. It was found to be specific for NADPH. The enzyme was reported to be constitutively expressed with a specific activity of 5 mU (0.3 nkat) per mg of protein [3]. The N-terminal amino acid sequence was found to be SSLVTLNNGLKMP. Our searches against the yeast genome [6] showed that this sequence corresponds to the open reading frame *YHR104w* which is thus likely to encode xylose reductase.

S. cerevisiae is able to grow on xylulose and has xylulokinase activity. The *S. cerevisiae* gene encoding xylulokinase was cloned [7], overexpressed [8] and sequenced [9]. The reported sequence is almost identical to that of the yeast genome open reading frame *YGR194c* which was recently renamed *XKS1* [22].

The literature concerning xylitol dehydrogenase in *S. cerevisiae* is contradictory. Batt et al. [10] reported xylitol dehydrogenase activity in *S. cerevisiae* whereas van Zyl et al. [3] could not detect such activity. In order to clarify the existence of xylitol dehydrogenase in *S. cerevisiae* we have carried out enzyme activity measurements of *S. cerevisiae* in different culture conditions and searched for putative xylitol dehydrogenase encoding open reading frames in the *S. cerevisiae* genome.

2. Materials and methods

2.1. Strains, plasmids and recombinant DNA techniques

The yeast strain W303-1B [11] was used for cloning of the open reading frame *YLR070c*. This open reading frame and the xylitol dehydrogenase gene (*XYL2*) from *Pichia stipitis* [18] were also overexpressed in this strain. The yeast strain ENY.WA-1A [12] was used for the xylitol dehydrogenase activity measurements. *Escherichia coli* DH5α strain was used as a host in the bacterial cloning steps.

Plasmid pMA91 [13] containing the PGK promoter and terminator was used for expression of the open reading frame *YLR070c* and the *P. stipitis XYL2*. Vector pRS423 [14] was used for expression of *P. stipitis XYL2* in strain W303-1B.

Standard recombinant DNA methods were used [15]. Yeast transformation was done as described by Gietz et al. [16] and Hill et al. [17].

2.2. Cloning of the xylitol dehydrogenase gene from P. stipitis

The *XYL2* gene from *P. stipitis* [18] was cloned into the *Bgl*II site of the pMA91 vector between the PGK promoter and terminator. The *XYL2* gene was released from this vector as a *Hin*dIII cassette and cloned into the *Eco*RV site of yeast expression vector pRS423 after filling in the termini of the fragments.

2.3. Cloning of the xylitol dehydrogenase gene from S. cerevisiae

The open reading frame *YLR070c* was amplified by PCR from genomic DNA of *S. cerevisiae* W303-1B. The following oligonucleotides were used: 5′-GAACAGGATCCAGCATGACTGACTTAACTA-3′ and 5′-GTATTGGATCCCTTGGATGCCAAAAGTTA-3′, with *Bam*HI restriction sites at the 5′ ends. The PCR reaction conditions were: 94°C 3 min, 30 cycles of: 94°C 1 min, 45°C 30 s, 72°C 2 min and 72°C 10 min for final extension. The PCR product was digested with *Bam*HI, purified from an agarose gel and ligated into the *Bgl*II site of the pMA91 vector. The resulting clone was transformed into the yeast strain W303-1B. Enzyme activity of yeast transformants was measured from crude extracts as described below.

2.4. Xylitol dehydrogenase activity assay

The *S. cerevisiae* strain ENY.WA-1A was grown in shake flasks on a medium containing 1% yeast extract, 2% peptone, and carbon sources as specified. An overnight preculture was 100-fold diluted into the culture medium and grown for various times. Cells were collected by centrifugation and washed twice with a buffer containing 100 mM sodium phosphate pH 7.0. Cells were resuspended at a concentration of 500 mg/ml wet weight, corresponding approximately to 75 mg/ml dry weight, in the same buffer. 1 ml of this suspension was vortexed with 1 g glass beads (0.5 mm diameter) for 15 min at 4°C, centrifuged in an Eppendorf centrifuge (13 000 rpm) and the supernatant was

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assayed. Xylitol dehydrogenase activity was assayed in a medium containing 50 mM PIPES KOH pH 7.0, 0.2 mM NADH. The reaction was started by addition of D-xylulose to a final concentration of 10 mM unless otherwise specified. The protein concentration of the extract was estimated using the Bio-Rad protein assay using IgG as a standard.

The K_m of D-xylulose and NADH were measured as described above; for D-xylulose at an NADH concentration of 0.2 mM, and for NADH at a D-xylulose concentration of 10 mM. The K_m for xylitol and NAD were measured in a buffer containing 200 mM Tris-HCl, pH 9.0. Yeast extract and NAD were added and the baseline recorded. The reaction was started by addition of xylitol. The K_m for xylitol was measured at an NAD concentration of 4 mM, and for NAD at a xylitol concentration of 100 mM. All enzyme assays were performed in a COBAS Mira automated analyser (Roche) at 30°C.

3. Results and discussion

No xylitol dehydrogenase activity was found in *S. cerevisiae* cells grown on glucose as the sole carbon source. Also during growth on 100 g/l xylose and 20 g/l glucose, xylitol dehydrogenase activity could not be detected when glucose was still present in the medium (not shown). Only after the transition from using glucose to using ethanol as a carbon source was xylitol dehydrogenase activity detected. It increased from 0.09 nkat/mg of protein after 16 h growth to 0.61 nkat/mg after 72 h as indicated in Fig. 1. Growth on ethanol without added xylose did not lead to xylitol dehydrogenase activity (data not shown).

It was also tested if the xylose concentration of the culture medium affected the xylitol dehydrogenase activity in the yeast extract. We used xylose concentrations between 0 and 100 g/l. Cells were grown on 20 g/l glucose, 0–100 g/l xylose for 72 h. Results are shown in Fig. 2. At 20 g/l of xylose the activity was 0.05 nkat/mg of protein, at 100 g/l xylose the activity was highest, 0.61 nkat/mg of protein. Higher xylose concentrations were not tested.

The yeast extract with the highest xylitol dehydrogenase activity was used for a kinetic characterisation of the enzyme activity. We estimated the K_m for xylulose to be about 1 mM. The enzyme was about 5–10-fold more active with xylulose than with ribulose when the sugar concentrations were 1 mM. No activity was detected when NADH was replaced by 0.2 mM NADPH.

An unspecific sorbitol dehydrogenase, which also has xylitol dehydrogenase activity, is induced in *S. cerevisiae* by sorbitol [19]. One could argue that the same enzyme is induced by xylose. We tested the yeast cell extracts described above for sorbitol dehydrogenase activity at neutral pH using D-fructose as a substrate and found no activity, i.e. less than 0.5% of the xylitol dehydrogenase activity. Thus we conclude that the xylitol dehydrogenase activity with the above-mentioned properties is different from the sorbitol-induced sorbitol dehydrogenase.

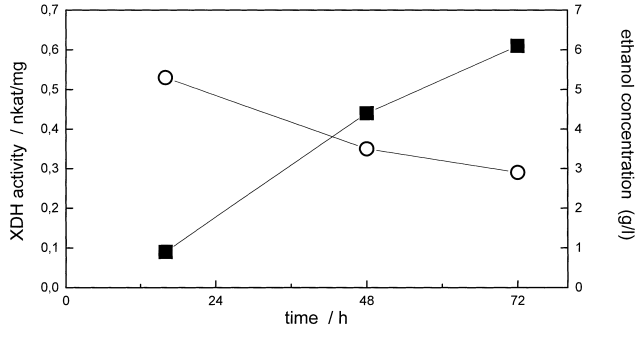

Fig. 1. Xylitol dehydrogenase activity in yeast extracts after different growth times. Cells were grown in shake flasks with 20 g/l D-glucose as a carbon source in the presence of 100 g/l D-xylose.

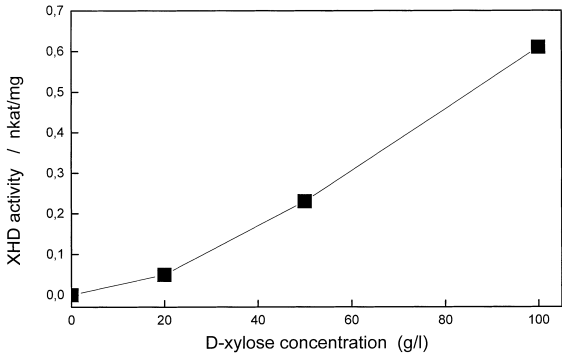

Fig. 2. Xylitol dehydrogenase activity in yeast cell extracts grown in the presence of various D-xylose concentrations. Cells were grown as in Fig. 1 for 72 h in the presence of 20 g/l glucose and the D-xylose concentrations indicated.

The *S. cerevisiae* genome database [6] has three open reading frames which show high sequence similarity (encoding about 50% identical amino acids) to the *P. stipitis* xylitol dehydrogenase gene [18]. One of these is the sorbitol-induced sorbitol dehydrogenase [19] and another one has the same sequence except for one amino acid. We decided to clone the third open reading frame, *YLR070c*, encoding 44% identical amino acids with the *P. stipitis* xylitol dehydrogenase, and test whether it encodes a xylitol dehydrogenase. A vector with the open reading frame under the constitutive PGK promoter was constructed and transformed to *S. cerevisiae* cells.

Extracts of the transformed cells grown on glucose in the absence of xylose (to avoid background xylitol dehydrogenase activity) contained 20–50 nkat of xylitol dehydrogenase activity per mg of protein. With this high enzyme activity the kinetic properties of the enzyme were now easier to obtain (Fig. 3A–D). The enzyme is specific for NADH, no activity with NADPH was found. The K_m for NADH was found to be 55 ± 15 μM.

The K_m for xylulose was 1.1 ± 0.4 mM, close to that shown above for the xylitol dehydrogenase activity of untransformed cells grown in the presence of xylose. The activity with D-ribulose was 10 times lower than with D-xylulose at sugar concentrations of 1 mM. The K_m for ribulose was estimated to be around 10 mM. No sorbitol dehydrogenase activity was observed, i.e. no activity was detected with fructose as a substrate. In the reverse reaction at pH 9.0 the K_m for NAD was 240 ± 40 μM and the K_m for xylitol was 25 ± 3 mM.

Our results confirm the claim of Batt et al. [10] that *S. cerevisiae* can naturally produce xylitol dehydrogenase. However, we found that xylitol dehydrogenase activity is present only when cells are grown in the presence of xylose but not during growth on glucose. This may reflect regulation on the expression level of xylitol dehydrogenase. Our results could also explain those of van Zyl et al. [3]. They could not demonstrate xylitol dehydrogenase activity in *S. cerevisiae* probably because they did not grow the yeast in the presence of xylose.

Xylitol dehydrogenase in other yeasts such as *Debaryomy-*

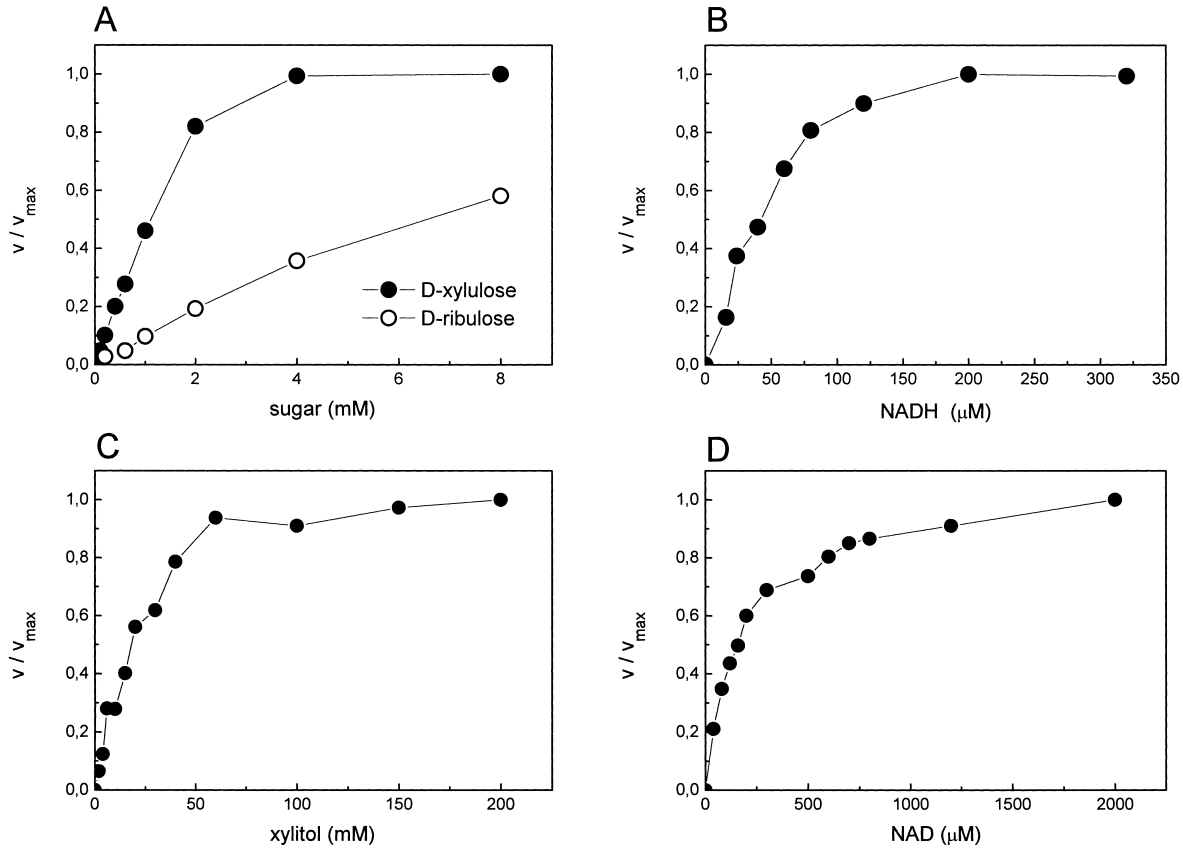

Fig. 3. Enzyme activities at different substrate concentrations (A) for D-xylulose, NADH is 200 μM, (B) NADH, D-xylulose is 10 mM, (C) xylitol, NAD is 4 mM and (D) NAD, xylitol is 100 mM.

ces hansenii, *Candida shehatae*, *P. stipitis* and *P. tannophilus* (for review see [20]) are reported to have also sorbitol dehydrogenase activity, which we did not find for the *S. cerevisiae* xylitol dehydrogenase. This would point to a unique property of the *S. cerevisiae* enzyme. In the earlier reports, the activities were measured with sorbitol as substrate at pH 9. Consequently we also analysed the sorbitol dehydrogenase activity of a *S. cerevisiae* strain overexpressing the *P. stipitis* xylitol dehydrogenase (see Section 2) and did not find any activity using our assay conditions, i.e. with fructose as substrate at neutral pH, we did find, however, high activity with xylulose. We therefore suggest that alkaline pH might change the enzyme properties.

Overexpression of the open reading frame *YLR070c* leads to xylitol dehydrogenase activity in *S. cerevisiae* indicating that this open reading frame encodes the *S. cerevisiae* xylitol dehydrogenase. We therefore suggest naming this gene *XYL2*. The *S. cerevisiae* xylitol dehydrogenase has similar kinetic properties as the xylitol dehydrogenase from *P. stipitis* [21] (K_m xylitol 26 mM, K_m NAD 160 μM) but with the difference that the *S. cerevisiae* enzyme is more specific for xylulose. The activity of the *P. stipitis* enzyme towards D-ribulose is 65% of that towards xylulose, whereas the *S. cerevisiae* xylitol dehydrogenase has a ribulose/xylulose activity ratio of 10 at 1 mM sugar concentration. There is some evidence that the enzyme encoded by YLR070c is identical to the enzyme present in cells grown in the presence of xylose. Both enzymes are specific for NADH. Both enzymes can utilise xylulose and ribulose but have a much higher affinity towards xylulose. Both enzymes have no sorbitol dehydrogenase activity. The natural promoter of *YLR070c* is possibly xylose-induced and glucose-repressed but the role of other activating mechanisms or post-translational modifications of the enzyme cannot be excluded.

In this communication we could demonstrate that *S. cerevisiae* has the genetic prerequisites for xylose metabolism. The organism's inability to grow on xylose or to ferment xylose to ethanol is not because it lacks the genes of the pathway. It is probably sufficient to overexpress the enzymes of this pathway to enable xylose utilisation. Several groups have demonstrated xylose utilisation and even fermentation with *S. cerevisiae* when overexpressing xylose reductase and xylitol dehydrogenase genes from *P. stipitis*, and additionally xylulokinase from *S. cerevisiae* [4,9,18,23–27]. The same should now be possible using only the endogenous genes. We conclude that *S. cerevisiae* has a pathway for xylose fermentation, but this pathway is insufficient or partially silent under the conditions tested.

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