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Characterization of Xylitol Dehydrogenase from *Debaryomyces hansenii*

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ABSTRACT

The xylitol dehydrogenase (EC 1.1.1.9) from xylose-grown cells of *Debaryomyces hansenii* was partially purified in two chromatographic steps, and characterization studies were carried out in order to investigate the role of the xylitol dehydrogenase-catalyzed step in the regulation of D-xylose metabolism. The enzyme was most active at pH 9.0–9.5, and exhibited a broad polyol specificity. The Michaelis constants for xylitol and NAD^+ were 16.5 and 0.55 m*M*, respectively. Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect the enzyme activity. Conversely, Zn^{2+}, Cd^{2+}, and Co^{2+} strongly inhibited the enzyme activity. It was concluded that NAD^+-xylitol dehydrogenase from *D. hansenii* has similarities with other xylose-fermenting yeasts in respect to optimal pH, substrate specificity, and K_m value for xylitol, and therefore should be named L-iditol:NAD^+-5-oxidoreductase (EC 1.1.1.14). The reason *D. hansenii* is a good xylitol producer is not because of its value of K_m for xylitol, which is low enough to assure its fast oxidation by NAD^+-xylitol dehydrogenase. However, a higher K_m value of xylitol dehydrogenase for NAD^+ compared to the K_m values of other xylose-fermenting yeasts may be responsible for the higher xylitol yields.

Index Entries: Xylitol dehydrogenase; polyol dehydrogenase; *Debaryomyces hansenii;* xylose metabolism; xylitol-producing yeast.

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INTRODUCTION

Xylose-fermenting yeasts are known to produce ethanol (major product) and xylitol (minor product) during the semiaerobic growth on D-xylose-containing materials (e.g., lignocellulosic hydrolysates) (*1–3*). However, depending on the cofactor specificity of the first enzyme of xylose metabolism, xylose reductase (XR, EC 1.1.1.21), some yeasts, such as *Debaryomyces hansenii, Candida guillermondii,* and *Candida parapsilosis,* accumulate xylitol as the major product from the xylose metabolism (*4–6*). These yeasts have been described to contain a specific NADPH-dependent XR that catalyzes the reduction of xylose to xylitol. Xylitol is further oxidized to xylulose in an enzymatic step catalyzed by an NAD^+-linked xylitol dehydrogenase (XDH, EC 1.1.1.9), and the metabolism proceeds through the pentose phosphate pathway. Recently, there has been a great interest to produce xylitol from xylan/xylose by using a biological process instead of the costly and unspecific chemical synthesis currently in use (*5*).

For further metabolic engineering work to improve either xylitol or ethanol-producing bioprocesses it is important to investigate the enzymology of the initial steps of xylose metabolism. A lot of attention has been given to XDH purification and characterization studies for ethanol-forming yeasts, like *Candida shehatae* (*7*), *Pichia stipitis* (*8*), and *Pachysolen tannophilus* (*9*), whereas XDH characterization studies from xylitol-producing yeasts were seldom considered. In the present work, we investigated the characteristics of XDH from *D. hansenii,* a good xylitol-producing yeast (*4*), compared to the characteristics of XDH from good ethanol-forming yeasts.

MATERIALS AND METHODS

Yeast and Culture Medium

The organism used was *D. hansenii,* and it was isolated from sugar cane as described earlier (*4*). Cultures were maintained on agar plates made from D-xylose (Merck, Darmstadt), Bacto-yeast extract (Difco), Bacto-malt extract (Difco), and Bacto-peptone (Difco). The xylose-containing growth medium used was a chemically defined medium according to Du Preez and van der Walt (*10*). D-Xylose was added as carbon source at 50 g/L.

Enzyme Purification

Fifteen grams of wet cells were resuspended in 100 m*M* potassium phosphate buffer (pH 7.2), and disrupted by using the glass beads technique (*11*). After centrifugation at 27,000*g* for 20 min, the supernatant (crude extract) was used for enzyme purification. The crude extract was

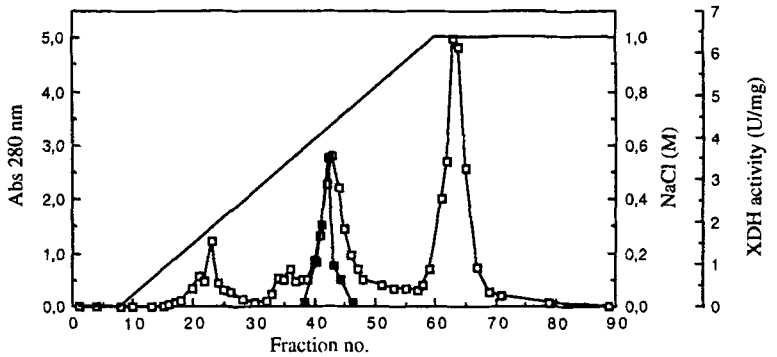

Fig. 1. Anion-exchange chromatography of *D. hansenii* crude extract. The fraction volume was 7.5 mL. Other chromatographic conditions were as described in the Materials and Methods section. Symbols: (□), absorbance of the fractions at 280 nm; (■), XDH activity; (—), salt gradient concentration.

first adsorbed on a column (2.6 × 40 cm) packed with Q-Sepharose Fast Flow (Pharmacia) previously equilibrated with 20 m*M* Tris/HCl buffer, pH 7.2, containing 5 m*M* $MgCl_2$ and 5 m*M* mercaptoethanol as protein stabilizers. The XDH enzyme was eluted in a linear 0–1*M* NaCl gradient at a flow rate of 2 mL/min (Fig. 1). The XDH active fractions were combined and concentrated with an Amicon microultrafiltration system (membrane cutoff, 10,000 Dalton). The concentrated enzyme solution was loaded in a gel-filtration Sephacryl S-300 column (1.6 × 100 cm) equilibrated with the same buffer as in the previous run. The pooled active fractions were used for XDH characterization.

Enzyme Assay

XDH activity was measured spectrophotometrically. The standard reaction mixture contained (in a 1-mL) glycine/NaOH buffer, 60 m*M* (pH 9.5), NAD^+ 2 m*M*, and enzyme. The assay was started by xylitol addition (50 m*M*). The enzyme activity was followed by measuring the increase in the optical density at 340 nm. Protein was determined by the Lowry method (*12*), and bovine serum albumin was used as standard protein. To follow protein elution from chromatographic columns, protein was determined by the absorption at 280 nm.

One International Unit (IU) was defined as the amount of enzyme that catalyzes the reduction of 1 μmol NAD^+/min under the assay conditions.

Optimum pH Determination

Fifty-millimolar buffer solutions ranging from pH 5.6–10.0 were used to assay the XDH activity. Citrate-phosphate buffer was used for pH 5.6–6.6. Buffers for pH 7.0–8.0, 7.0–8.8, and 9.4–10 were sodium phosphate, Tris-HCl, and glycine-NaOH, respectively.

Kinetic Determinations

The XDH activity was successively measured as indicated in the standard assay by varying the xylitol (or NAD^+) concentration while the enzyme concentration was constant. The kinetic of the enzyme was studied by varying one substrate and keeping the other substrate constant. The data were fitted to the Michaelis-Menten equation using the program Enzfiter (Elsevier Biosoft, Amsterdam). The apparent kinetic constants (K_m and V_{max}) were determined from Lineweaver-Burk plots. All experiments were repeated three times.

Substrate Specificity

The standard assay was used to measure the substrate specificity. Fifty-microliter aliquots of enzyme were used in the assay mixtures before the reaction was started by adding the appropriate substrate (50 m*M*) instead of xylitol.

Influence of Metal Ions

The enzyme assay for NAD^+-xylitol dehydrogenase was carried out in the presence of several metal ions. Reaction mixtures were made up to 10 m*M* calcium, magnesium, manganese, and zinc chloride, cadmium, and copper sulfates. Fifty-microliter aliquots of enzyme were incubated in the assay mixture containing one metal ion for 2 min before the reaction was started by adding xylitol.

Carbonyl and Sulfhydryl Protecting or Oxidizing Agents

Various compounds were investigated regarding their inhibitory effects on the enzyme activity. The reactions were carried out in the standard reaction mixture containing 100 µL of one of the following compounds: phenylhydrazine, sodium azide, mercurium chloride, silver nitrate, reduced gluthatione, EDTA, and dithiothreitol. The reaction was started with xylitol.

Nucleotides and Artificial Electron Acceptors

Standard enzyme assays for XDH were carried out in the absence (control) and presence of 1 m*M* ATP or 1 m*M* GTP. Additional assays were carried out by replacing 2 m*M* NAD^+ by 2 m*M* potassium ferricyanide or 0.2 m*M* 2,6-dichlorophenolindophenol.

RESULTS

The NAD^+ XDH from *D. hansenii* was partially purified in two chromatographic steps. The enzyme preparation did not show contamination of

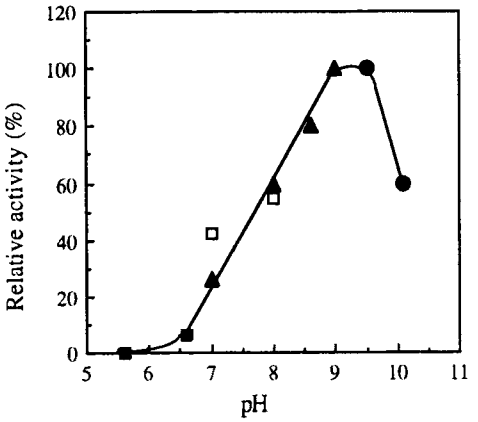

Fig. 2. Effect of pH on the NAD$^+$-XDH activity in xylose-grown *D. hansenii*. The ionic strength for all buffers was 50 m*M*. (■), citrate-phosphate; (□), sodium phosphate; (●), glycine-NaOH; (▲), Tris-HCl. (100% of enzyme activity = 0.322 U/mg protein).

of other common dehydrogenases when assayed with glycerol, ethanol, acetate, formate, or succinate.

The purified enzyme was NAD$^+$-dependent, since only a residual NADP$^+$-dependent activity (1–2% of NAD$^+$-dependent activity) was detected.

pH Optimum

A pH range between 5.5 and 10 was used to study the effect of pH in the purified XDH activity (Fig. 2). The enzyme activity increased as pH was increased from 6.5 to 9.0 and thereafter decreased. The enzyme showed an optimal pH activity at 9.0–9.5.

Kinetic Constants

The Lineweaver-Burk plots of the partially purified XDH with respect to xylitol (Fig. 3) and to NAD$^+$ (Fig. 4) are shown. K_m and V_{max} were obtained and compared with the kinetic constants for other xylose-fermenting yeasts (Table 1).

Substrate Specificity

Several polyols were used as alternative substrates for the purified NAD$^+$-XDH. The data obtained for *D. hansenii* were compared with other XDHs from xylose-assimilating fungi (Table 2). Particularly important for hemicellulosic hydrolysate studies, the purified enzyme did not show activity for L-arabitol. However, in crude extracts, dehydrogenase activity was found when either xylitol or L-arabitol was used as substrate.

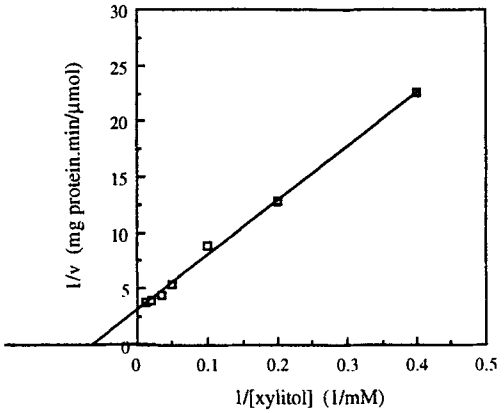

Fig. 3. Lineweaver-Burk plot of the activity of *D. hansenii* XDH by varying the concentration of xylitol.

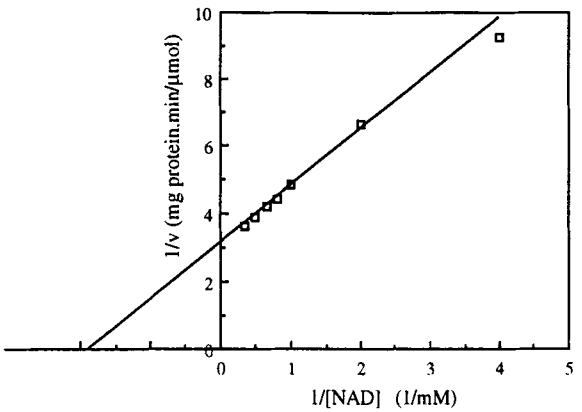

Fig. 4. Lineweaver-Burk plot of the activity of *D. hansenii* XDH by varying the concentration of NAD^+.

Table 1
Apparent Michaelis Constants
for *D. hansenii* and Other Xylose-Fermenting Yeasts

	K_m constant, m*M*	
Yeast	Xylitol	NAD^+
D. hansenii[a]	16.4	0.55
C. shehatae[b]	18.5	0.24
P. stipitis[c]	26.0	0.16
P. tannophilus[d]	70.0	0.01

[a]This work.
[b]Yang and Jeffries (*7*).
[c]Rizzi et al. (*8*).
[d]Ditzelmüller et al. (*20*).

Table 2
Substrate Specificity of NAD$^+$-XDH
from *D. hansenii* and Other Xylose-Assimilating Fungi

	Relative activity, %				
Substrate	*D. hansenii*[a]	*C. shehatae*[b]	*P. stipitis*[c]	*P. tannophilus*[d]	*Aspergillus pullulans*[e]
Xylitol	100	100	100	100	100
Sorbitol	65	45	48	35	—
Ribitol	61	—	69	—	—
Mannitol	—	1	0	0	—
D-Arabitol	—	0	0	0	—
L-Arabitol	0	—	0	—	—
Glicerol	0	0	0	0	0

[a]This work.
[b]Yang and Jeffries (*7*).
[c]Rizzi et al. (*8*).
[d]Ditzelmüller et al. (*20*).
[e]Machová (*22*).

Metal Ions

The enzyme assay for NAD$^+$-XDH was carried out in the presence of several metal ions. The enzyme activity was not affected by calcium, magnesium, and manganese chlorides (10 m*M* each). Conversely, zinc chloride strongly inhibited XDH, decreasing by 50% its activity at a concentration of 0.75 m*M*. Other metal compounds (cadmium and copper sulfates) fully inhibited the XDH activity at concentrations as low as 0.05 m*M*.

Carbonyl and Sulfhydryl Protecting or Oxidizing Agents

Sodium azide and phenylhydrazine (20 m*M*) did not affect the XDH activity. Conversely, 50% of the activity was lost in the presence of $AgNO_3$ (6 m*M*), reduced gluthatione (13 m*M*), EDTA (14 m*M*), and DTT (0.2 m*M*). $HgCl_2$ fully inhibited the enzyme activity at 50 μM concentration.

Nucleotides and Artificial Electron Acceptors

A 15% decrease of NAD$^+$-XDH activity when either ATP (1 m*M*) or GTP (1 m*M*) was added to the enzyme assay was observed. When potassium ferricyanide (2 m*M*) or 2,6-dichlorophenolindophenol (0.2 m*M*) replaced NAD$^+$ as coenzyme, enzyme activity was not detected.

DISCUSSION

The regulation of D-xylose metabolism by yeasts had been studied in the last decade (*1,13*). For xylose-fermenting yeasts, like *C. shehatae* or *P.*

stipitis, exhibiting dual cofactor (NADH and NADPH) specifity linked to XR activity, the production of NADH in the XDH-catalyzed reaction is balanced by the oxidation of NADH in the first step of xylose metabolism. This close redox balance was particularly significant when oxygen became severely restricted (semiaerobic conditions) preventing xylitol accumulation. The xylose transport has been claimed to be the rate-limiting step for xylose fermentation by these yeasts (*14,15*).

On the other hand, for yeasts exhibiting very low or nil NADH-dependent XR activity, like *Candida utilis* (*16*) or *D. hansenii* (*17*), the oxidation of xylitol to D-xylulose has been described to be the rate-limiting step of xylose metabolism both under semiaerobic and anaerobic conditions (*8*). Hence, the rate of the NADH reoxidation has a modulator role of the overall xylose metabolism in order to get an intracellular redox balance. Physiologically, only low-ratio NADH/NADPH-XR yeasts have been reported to produce xylitol in high yields from xylose metabolism. With this in mind, we compared the enzymatic properties of the xylitol-oxidizing enzyme from *D. hansenii* and the enzymatic properties already published of XDH from xylose-fermenting yeasts.

The highest enzymatic activity under standard conditions was observed in the pH range between 9.0 and 9.5. Similar pH optimal activities were found for XDH from *P. stipitis* (*8*), *Pachysolen tannophilus* (*18*), *C. shehatae* (*7*), and *C. utilis* (*19*).

The kinetic constants of XDH from *D. hansenii* suggest an explanation for the extensive xylitol production in this yeast. Although the K_m for xylitol is on the same order of magnitude compared with the K_m for other xylose-fermenting yeasts (Table 1), the two- to fivefold higher K_m for NAD^+ observed in *D. hansenii* suggests that any NAD^+ shortage will greatly reduce the xylitol oxidation by XDH leading to xylitol accumulation.

Literature reports (*7,20*) have suggested that the higher ethanol yields and consequently low xylitol production rates for *P. tannophilus* and *C. shehatae* were owing to the low affinity of XDH for xylitol. The similar K_m magnitude found for the xylitol-producing *D. hansenii* (this work) and for the ethanol-producing *C. shehatae* (*7*) do not support such a hypothesis.

The inhibition by EDTA probably means that double-charged cations would be essential for the enzymatic activity. However, the addition of Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect the enzyme activity. Conversely, Zn^{2+}, Cd^{2+}, and Co^{2+} strongly inhibited the enzyme activity. The total inhibition of the enzyme activity for low added concentration of $HgCl_2$ suggests that at least one thiol group must be present in the protein redox center. The purified NAD^+-XDH enzyme showed similar substrate specificity when compared to other xylose-fermenting yeasts. Therefore, the enzyme from *D. hansenii* should be named L-iditol:NAD^+-5-oxidoreductase (EC 1.1.1.14) according to McCorkindale and Edson (*21*).

The absence of dehydrogenase activity in the purified enzyme toward L-arabitol (Table 2) along with the detectable activity on crude extracts indicate that a constitutive NAD^+-dependent arabitol dehydrogenase

might be present in *D. hansenii*. This finding has industrial importance for biological utilization of hemicellulosic hydrolysates, since any integrated process for lignocellulosic residue upgrading as predicted on the use of all sugars present in the hydrolysates.

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