An Oxidoreductive Pathway for D-Xylose Assimilation by *Rhodospiridium toruloides*

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Extracts of *Rhodospiridium toruloides* grown aerobically on xylose contained xylitol dehydrogenase and D-xylose reductase activities. Extracts of cells grown on glucose contained one-tenth as much xylose reductase and no detectable xylitol dehydrogenase. The xylitol dehydrogenase was purified to near homogeneity, and is a tetramer of 45 kDa subunits. This labile enzyme could be stabilized by glycerol (25%) and was rapidly inactivated by 10 mM-EDTA. It catalyses the reversible, NAD⁺-dependent oxidation of xylitol to xylulose. Apparent K_m values are 19 mM-xylitol and 0.3 mM-NAD⁺ at 30 °C, pH 8.5. Partially purified preparations of xylose reductase catalysed the NADPH-dependent reduction of D-xylose to xylitol, and were 16 times as active with 33 mM-DL-glyceraldehyde as with 33 mM-D-xylose. Apparently *R. toruloides* grown on xylose has the necessary enzymes to convert xylose to xylulose by the oxidoreductive pathway.

INTRODUCTION

The assimilation of D-xylose by bacteria usually involves its direct isomerization to D-xylulose by xylose isomerase (EC 5.3.1.5). This pathway has also been proposed for two yeasts, *Rhodospiridium toruloides* (Höfer *et al.*, 1971) and *Candida utilis* (Tomoyeda & Horitsu, 1964); more recently, xylose isomerase has been purified from the yeast *Candida boidinii* (Vongsuvanlert & Tani, 1988*b*). However, in eukaryotic micro-organisms, including yeasts, xylose is usually reduced to xylitol by a pyridine nucleotide-linked D-xylose reductase, and the xylitol is then oxidized to xylulose by a xylitol dehydrogenase (Chiang & Knight, 1960; Veiga, 1968*a, b*; Sugai & Veiga, 1981; Smiley & Bolen, 1982; Suihko *et al.*, 1983; Bruinenberg *et al.*, 1984). In *C. utilis*, the enzymes of this oxidoreductive pathway appear to coexist with the isomerase (Horitsu & Tomoeda, 1966; Horitsu *et al.*, 1968).

Here we describe the purification to near homogeneity and characterization of an NAD⁺-linked xylitol dehydrogenase from *R. toruloides*. NADPH-linked xylose reductase activity was also demonstrated. Thus, this red, lipid-forming yeast, which grows readily on D-xylose in aerobic conditions, appears to have the enzyme equipment needed to assimilate xylose by the oxidoreductive pathway.

METHODS

Organism and cultivation conditions. The yeast strain used was *Rhodospiridium toruloides* ATCC 26194 (earlier called *Rhodotorula gracilis* 5/Fres/Harrison). The cultivation medium was 0.67% yeast nitrogen base (Difco) with 4% (w/v) D-xylose, 4% (w/v) D-glucose, 4% (w/v) xylitol or 2% (w/v) ethanol as carbon source. The yeast was grown at 30 °C on a rotary shaker at 200 r.p.m. Inocula for D-xylose and xylitol cultivations were grown on D-xylose and those for D-glucose and ethanol cultivations on D-glucose or ethanol, respectively. The residual sugar transferred to cultivations with the inocula was less than 0.05% (w/v). Growth was estimated with a Klett–Summerson colorimeter (600 nm filter) or a Coulter counter.

Enzyme assays. Xylitol dehydrogenase and xylose reductase were assayed at 30 °C and 340 nm with a recording spectrophotometer. Standard xylitol dehydrogenase assays contained 0.1 M-Tris/HCl, pH 8.5, 33 mM-xylitol and 0.3 mM-NAD⁺. Standard xylose reductase assays contained 0.1 M-potassium phosphate, pH 6.0, 33 mM-xylose and

0.12 mM-NADPH. Assays were usually started by addition of substrate after observing any blank reaction for a few minutes. One unit (U) of each enzyme catalyses the reduction (or oxidation) of 1 μ mol NAD⁺ (or NADPH) min⁻¹ under these standard conditions. Other conditions are described in Results.

Protein assays. Protein was determined by the methods of Warburg & Christian (1941) or Bradford (1976). The Bradford (1976) method was standardized with ovalbumin.

Preparation of cell extracts. Cells were harvested by centrifugation in a cold rotor, and all subsequent steps done at 0 to 8 °C. Cells (about 10 g fresh wt) were washed twice with the appropriate buffer (see below), suspended in the same buffer to a final volume of 20 ml, mixed with 27 ml of 0.5 mm diameter glass beads and shaken five times for 1 min in a Braun MK II homogenizer. The beads were removed with a G1 sinter and the disintegrated yeast centrifuged for 20 min at 28000 g. The resulting supernatant was used for enzyme and metabolite assays (xylitol dehydrogenase and xylose reductase could not be detected in the sediment).

Purification of xylitol dehydrogenase. Yields (U) and specific activities [U (mg protein)⁻¹] are shown in parentheses after each step.

Step 1. *R. toruloides* (9.7 g fresh wt) grown on xylose was suspended in TEMD buffer (25 mM-Tris/HCl, pH 7.5/0.1 mM-EDTA/1 mM-MgCl₂/1 mM-dithiothreitol) containing PMSF (1 mM) and pepstatin A (10 μ g ml⁻¹) and disintegrated as described above. Further PMSF (0.4 mM) and pepstatin A (4 μ g ml⁻¹) were added to the 28000 g supernatant (23 ml, 30 U, 0.04 U mg⁻¹).

Step 2. (NH₄)₂SO₄ (6.2 g) was added to 22 ml of the 28000 g supernatant, and after 20 min the precipitated protein was removed by centrifugation. A further 3.7 g of (NH₄)₂SO₄ was added to the supernatant. After 20 min, the precipitated protein was collected by centrifugation (10 min, 28000 g) and suspended in TEMD containing 25% (v/v) glycerol (TEMD/glycerol). PMSF (1 mM) and pepstatin A (10 μ g ml⁻¹) were added and insoluble material removed by centrifugation (2.1 ml, 22.5 U, 0.26 U mg⁻¹).

Step 3. The supernatant was passed through a 15 × 50 mm column of Sephadex G25 equilibrated with TEMD/glycerol and applied to a 9 × 70 mm column of NAD⁺-(C8)-agarose (Sigma). The column was washed at 15 ml h⁻¹ with, sequentially, 20 ml TEMD/glycerol, 20 ml TEMD/glycerol/0.1 M-KCl, 2 ml TEMD/glycerol and 12 ml TEMD/glycerol/0.5 mM-NAD⁺. The enzyme was then eluted at 7 ml h⁻¹ with TEMD/glycerol/3.4 mM-NAD⁺ (9.3 ml, 11.6 U, 15.4 U mg⁻¹).

Step 4. The eluate was applied to a 7 × 75 mm column of DE52-cellulose (Whatman) equilibrated with TEMD/glycerol. The column was washed at 11 ml h⁻¹ with 50 ml 0.1 M Tris/HCl, pH 7.5/0.1 mM-EDTA/1 mM-MgCl₂/1 mM-dithiothreitol/25% glycerol and the enzyme eluted with 0.3 M-KCl in this buffer (1.4 ml, 2.3 U, 12.2 U mg⁻¹).

Partial purification of xylose reductase. Xylose reductase from cells grown on xylose was studied using enzyme partially purified as follows. The cells (8.1 g) were suspended in TED buffer, pH 7.5, (25 mM-Tris/HCl, pH 7.5/0.1 mM-EDTA/1 mM-dithiothreitol) containing 1 mM-PMSF, and disintegrated and centrifuged as described above. The protein precipitated between 20 and 45 g (NH₄)₂SO₄ per 100 ml of 28000 g supernatant was suspended in 1.7 ml TED (pH 7.5) and passed sequentially through columns of Sephadex G25 (15 × 50 mm) and DE52-cellulose (14 × 135 mm) equilibrated with TED (pH 7.5).

This procedure was modified in order to compare the enzyme from cells grown on xylose, glucose or ethanol. The cells were broken as described above and the 28000 g supernatants dialysed overnight against TED (pH 7.5) to remove compounds that interfere with the assay. The dialysed supernatants from about 3.5 g of cells were again centrifuged at 28000 g, passed through Sephadex G25 equilibrated with TED (pH 8.5) and loaded onto a DE52-cellulose column (14 × 135 mm) equilibrated with TED (pH 8.5). At this pH the enzyme bound to the column and was eluted at 24 ml h⁻¹ with a linear gradient of 0 to 0.5 M-KCl in 120 ml TED (pH 8.5).

Native M_r determinations. Purified xylitol dehydrogenase (200 μ l) was run at 5 °C and 15 ml h⁻¹ through a Superose 12 HR 10/30 FPLC column (Pharmacia) equilibrated with TEMD/glycerol containing 0.1 M-KCl. The enzyme in 0.7 ml fractions was assayed. The column was calibrated in the same buffer with ferritin (440 kDa), lactate dehydrogenase (144 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), myoglobin (17.2 kDa) and cytochrome c (11.7 kDa).

Electrophoresis. SDS-PAGE was done by the method of Laemmli (1970) with a linear gradient from 13% to 7% (w/v) acrylamide in the separator gel and a 3.7% (w/v) stacker gel. Discontinuous electrophoresis of native samples was done, at 8 °C, in the same system except that 2-mercaptoethanol and SDS were replaced by 20% (v/v) glycerol. Continuous native electrophoresis was done in the same way except that samples and stacker gels both contained 75 mM-Tris/HCl, pH 8.8/20% glycerol (the separator gel buffer was 375 mM-Tris/HCl, pH 8.8).

Xylitol dehydrogenase activity was located by incubating the gels for 60 min at 25 °C in 100 ml 0.1 M-Tris/HCl, pH 8.5, containing 50 mg NAD⁺, 10 mg nitrobluetetrazolium, 2 mg phenazinemetosulphate and 60 mM-xylitol. Protein bands were located by fixing the gels with 50% (w/v) trichloroacetic acid and staining with Coomassie blue.

Analysis of sugars and sugar alcohols. The 28000 g supernatants of cells broken in 0.1 M-potassium phosphate/5% glutathione, pH 7.0, were brought to 5% (w/v) trichloroacetic acid and centrifuged for 15 min at 28000 g. The

supernatants were neutralized with KOH, deionized by shaking with Amberlite MB-3 ion exchanger, filtered, and concentrated *in vacuo* at 40 °C.

Growth media were filtered through 0.2 µm Millex filters (Millipore) to remove cells, and deionized with Amberlite MB-3.

The product of the xylitol dehydrogenase reaction was formed by adding 15 µl (23 mU) of purified (step 4) xylitol dehydrogenase to 3 ml 2.9 mM-LiNAD/KOH, pH 8.6, containing 33 mM-xylitol. Buffer and glycerol were omitted because they interfered with HPLC analyses. Over 6 h incubation at 30 °C a further 3.1 mM-LiNAD was added as solid. The pH fell to 6.9.

The product of the xylose reductase reaction was formed by adding 0.3 ml (152 mU) xylose reductase (DE-52 eluate at pH 7.5, specific activity 70 mU mg⁻¹) to 2.7 ml 0.1 M-potassium phosphate, pH 6.0, containing 1.0 µmol NADPH and 33 mM xylose. Over 5 h incubation at 30 °C a further 7 µmol NADPH was added (final volume 3.5 ml).

Blank reaction mixtures lacking enzyme or substrate were handled in a similar way.

All samples were then filtered through SepPak C18-cartridges (Waters). HPLC analysis at 85 °C was done (cell extracts, growth media and reaction mixtures) on a 300 × 1.8 mm Resolution Carbohydrate Ca²⁺ column developed with water at 0.5 ml min⁻¹ or (cell extracts only) on a 150 × 4.6 mm Nucleosil 5 NH₂ column developed with acetonitrile/water (82.5:17.5, v/v) at 0.8 ml min⁻¹. Eluted compounds were detected refractometrically and identified by comparison with standard sugars and sugar alcohols. Except for D-xylulose, these standards were from Alltech.

Materials. D-Xylulose was from Sigma (product X-4625). For growth media, D-glucose, D-xylose and xylitol were Art 8337, Art 8692 and Art 8666, respectively, from Merck. Standard proteins, ancillary enzymes and coenzymes were from Sigma or Boehringer. Other reagents were analytical grade or from sources stated in the text.

RESULTS

Xylitol as a carbon source and metabolite of xylose

Fig. 1 shows that *R. toruloides* grows as rapidly on 4% xylitol as on 4% xylose, and that during the phase of rapid growth the two carbon sources were used at similar rates (0.34 g xylose and 0.29 g xylitol l⁻¹ h⁻¹). In both cases, rapid growth ceased before the carbon source was consumed and at similar cell densities (0.9 × 10⁸ cells ml⁻¹ for xylose and 1.3 × 10⁸ cells ml⁻¹ for xylitol). A striking difference between the cultivations was the longer lag phase before rapid growth on xylose, although the inocula for the cultivations were identical samples of cells grown on xylose.

Extracts of cells grown to late exponential phase on xylose contained xylose (23 mM), xylitol (14 mM) and xylulose (6 mM). Concentrations were calculated assuming 0.5 ml cytosol per g fresh wt of cells. No attempt was made to stop metabolism rapidly. The results show that xylose appears in the cells and is converted to xylitol as well as xylulose. Arabitol (0.25%) and xylitol (0.05%) were found in the growth media after 102 h cultivation on xylose.

Changes in oxidoreductase activities with carbon source

The amounts of xylitol dehydrogenase and xylose reductase in cells grown to late exponential phase on different carbon sources are shown in Table 1. The activities of both enzymes were much higher with xylose as carbon source. Similar results were obtained with cell extracts concentrated in a Minicon concentrator, which removed molecules smaller than 15 kDa. During growth on xylose, the specific activity of xylitol dehydrogenase was highest in early exponential phase and decreased by about 40% by late exponential phase (not shown). In contrast, the specific activity of mannitol dehydrogenase (assayed by replacing the xylitol in the standard assay with 33 mM-mannitol) increased about 2-fold between early and late exponential phase.

Physical properties of xylitol dehydrogenase

Enzyme purified through the (NH₄)₂SO₄ fractionation and dissolved in TED (pH 7.5) lost 80% of its activity in 24 h at 5 °C. It could not be stabilized by 0.18 M-NaCl or KCl, 0.1 M-potassium phosphate, pH 6.0, or 0.1 M-Tris/HCl, pH 8.1. Addition of 40% (w/v) glycerol preserved 90% of the activity for 24 h. MgCl₂ (9 mM) did not affect the stability, but 9 mM-EDTA caused 95% inactivation within 30 min at 0 °C. Adding 3 mM-EDTA to assay mixtures

Fig. 1. Growth (open symbols) and consumption of xylose or xylitol (closed symbols) by *R. toruloides* grown on YNB/xylose ($\triangle$, $\blacktriangle$) or YNB/xylitol ($\circ$, $\bullet$). Growth was followed with a Klett–Summerson colorimeter and xylose and xylitol determined by HPLC as described in Methods.

Table 1. *Xylose reductase and xylitol dehydrogenase from cells grown on different carbon sources*

R. toruloides grown on 4% (w/v) xylose, 4% (w/v) glucose or 2% (w/v) ethanol to about 5×10^7 cells ml^{-1} was harvested and cell extracts made in TED (pH 7.5) as described in Methods. Enzymes were assayed in the dialysed 28000 g supernatant.

Carbon source	Xylose reductase [U (g fresh wt cells) $^{-1}$]	Xylitol dehydrogenase [U (g fresh wt cells) $^{-1}$]
Xylose	0.96	1.9
Glucose	0.095	<0.06
Ethanol	0.27	<0.04

inhibited the enzyme by more than 30% within 2 min. Mannitol dehydrogenase in the same samples was unaffected by EDTA and was stable in the absence of glycerol. Together with the opposite changes in specific activities of xylitol and mannitol dehydrogenases during growth on xylose (see above), this suggests that the major xylitol dehydrogenase of *R. toruloides* is distinct from its major mannitol dehydrogenase, and may be a metalloprotein.

During purification, the highest specific activity (15.4 U mg^{-1}) of xylitol dehydrogenase was reached at step 3, and represents a 380-fold purification with a 38% yield. Only 20% of the activity was recovered through the DE-52 column of step 4, and the specific activity fell to 12.2 U mg^{-1} . This product, which was used in subsequent studies, may contain some inactivated xylitol dehydrogenase protein.

SDS-PAGE showed a main protein band at $45 \pm 1 \text{ kDa}$ and a minor band at 59 kDa (Fig. 2). Enzyme activity comigrated with the major protein band in both continuous (Fig. 3) and discontinuous (not shown) native electrophoresis systems. In gel-filtration on Superose 12 in TEMD/glycerol/0.1 M-KCl, more than 90% of the applied activity was recovered in a symmetrical peak corresponding to a native molecular mass of $200 \pm 35 \text{ kDa}$. Probably, the native enzyme is a tetramer.

Catalytic properties of xylitol dehydrogenase

At 33 mM-xylitol, 0.3 mM- NAD^+ , purified xylitol dehydrogenase had a pH optimum between 7.5 and 8.5 in Tris/HCl (not shown).

No significant activity was observed when NAD^+ in the standard assay was replaced by 0.3 mM-NADP. With different sugar alcohols in the standard assay the following relative rates were observed: at 33 mM; xylitol, 100; D-sorbitol, 62; ribitol, 47; L-arabitol, 11; D-arabitol, ≤ 1 ;

Fig. 2

Fig. 2. SDS-PAGE of xylitol dehydrogenase (10 U mg^{-1}). Lanes B ($0.8 \mu\text{g}$) and C ($2.8 \mu\text{g}$) xylitol dehydrogenase. Lane A, molecular mass markers [from top to bottom: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; lactate dehydrogenase, 36 kDa; triose phosphate isomerase, 26.5 kDa; myoglobin, 17.2 kDa; and cytochrome c, 11.7 kDa. (The latter two are coincident.)].

Fig. 3

Fig. 3. Continuous native gel-electrophoresis of xylitol dehydrogenase. Lanes C, D, E and F, $1.7 \mu\text{g}$ xylitol dehydrogenase (10 U mg^{-1}). Lanes A, B, and G, myoglobin ($2 \mu\text{g}$), lactate dehydrogenase ($1 \mu\text{g}$) and bovine serum albumin ($1 \mu\text{g}$). Lanes A to D were stained for protein with Coomassie blue and lanes E to G for xylitol dehydrogenase activity with nitroblue tetrazolium, as described in Methods.

D-mannitol, ≤ 1 ; and erythritol, ≤ 1 . Glycerol at 3.3 M gave $\leq 5\%$ of the rate with 33 mM -xylitol. DL-Dithiothreitol at 1 mM caused 85% inhibition. This may be related to the sugar alcohol structure of dithiothreitol, because 1.4 mM -2-mercaptoethanol did not inhibit.

Purified enzyme lost about 50% of its activity when preincubated for 3 min at 30°C in standard assay mixtures lacking xylitol or NAD^+ , and progress curves were very non-linear even when reactions were started with enzyme. Addition of 25% glycerol to standard assay mixtures decreased the initial rate by about 20% but caused the progress curves to be linear for at least 2 min . Affinities for xylitol and NAD^+ were therefore measured in the presence of 25% glycerol. Hofstee plots were linear for xylitol with apparent Michaelis constants of 19 mM at 0.3 or 1.0 mM - NAD^+ (Fig. 4*b*). At 33 mM -xylitol, data between 3.7 and 0.1 mM - NAD^+ could be fitted satisfactorily by an apparent Michaelis constant of 0.33 mM but the Hofstee plot was detectably convex towards the origin (Fig. 4*a*).

Fig. 4. Apparent Michaelis constants of xylitol dehydrogenase. Complete reaction mixtures (1 ml) contained 60 mM-Tris/HCl, pH 8.5, 25% (v/v) glycerol and substrates. In (a) reactions were started by addition of 30 μl xylitol (○) or 10 μl enzyme (□) to give, in both cases, final concentrations of 0.1 to 3.7 mM- NAD^+ and 33 mM-xylitol. In (b) reactions were started with xylitol (○) or enzyme (□) to give final concentrations of 2.5 to 100 mM-xylitol and either 0.3 mM (○) or 1.0 mM (□) NAD^+ . Lines are drawn for V and K_m values computed by the direct linear plot method of Eisenthal & Cornish-Bowden (1974).

Over a 6 h period of incubation of purified enzyme with NAD^+ and xylitol, as described in Methods, 0.26 mM- NAD^+ was reduced (according to A_{340} measurements) and 0.20 mM-xylulose and 0.01 mM-xylose were formed (according to HPLC analysis).

In 0.1 M-potassium phosphate, pH 7.0, or 0.1 M-Tris/HCl, pH 8.5, the purified xylitol dehydrogenase catalysed the oxidation of 0.27 mM- NADH in the presence of xylulose (about 30 mM) but not in the presence of xylose (33 mM), with a rate at pH 8.5 [$21 \mu\text{mol min}^{-1}$ (mg enzyme) $^{-1}$] greater than that [$13 \mu\text{mol min}^{-1}$ (mg enzyme) $^{-1}$] for the reduction of 0.27 mM- NAD^+ by 33 mM-xylitol at pH 8.5. We conclude the enzyme catalyses the reversible, NADH -linked reduction of xylitol to xylulose.

Properties of xylose reductase

During DE52-cellulose chromatography at pH 8.5, as described in Methods, the xylose reductase activities from cells grown on xylose or glucose were recovered as single peaks at the same KCl concentration (0.1 M) with specific activities of 145 and 44 mU mg $^{-1}$, respectively. Enzyme partially purified from xylose-grown cells by chromatography at pH 7.5 (see Methods), was relatively stable (90% remained after 20 h at 5 °C in TED buffer) and was unaffected by 9 mM-EDTA. No significant activity was detected when NADPH was replaced by 0.12 mM- NADH at either pH 6 or pH 8.5. When D-xylose in the standard assay was replaced by other aldoses at 33 mM the relative activities (D-xylose = 100) were: DL-glyceraldehyde, 1600; L-arabinose, 140; DL-arabinose, 86; D-ribose, 70; and D-glucose, 28. At 0.3 mM- NADPH , an apparent Michaelis constant of about 0.2 M-xylose was deduced from measurements between 40 and 200 mM-xylose (not shown).

During a 5 h incubation of partially purified enzyme with xylose and NADPH as described in Methods, 2.3 mM- NADPH was oxidized (A_{340} measurements) and 1.6 mM-xylitol appeared (HPLC analyses). However, no reduction of NADP^+ by the enzyme preparation (7.8 mU ml $^{-1}$) was detected during incubations with 33 mM-xylitol (at pH 6.0, 7.0 or 8.1), glycerol (at pH 6.0, 7.0, 8.1 or 9.0) or sorbitol (at pH 6.0 or 8.5). We conclude that the preparation contained an NADPH -linked D-xylose reductase that produces xylitol, and is effectively irreversible at these pH values.

DISCUSSION

R. toruloides grows readily on xylose under aerobic conditions and has been reported (Höfer *et al.*, 1971) to contain xylose isomerase (EC 5.3.1.5). Xylose isomerase is rare in eukaryotic micro-organisms, and efforts in this laboratory (P. Kristo, I. Suomalainen and M. Korhola, unpublished work) to demonstrate it in xylose-grown *R. toruloides* under a variety of conditions (including Mg^{2+} , Co^{2+} or Mn^{2+} as activating cation, borate or other buffers, and use of fresh cell extracts or acetone powders prepared according to Höfer *et al.* (1971) have not succeeded. Possibly this represents a strain difference or some unexpected enzyme characteristic. Höfer *et al.* (1971) also reported that *R. toruloides* grown on D-xylose did not respond to xylitol, whereas we found that it grew readily on xylitol (Fig. 1). We therefore tested whether *R. toruloides* contains the necessary enzymes for oxidoreductive conversion of D-xylose to D-xylulose via xylitol, and found that NADPH-linked xylose reductase and NAD-linked xylitol dehydrogenase were present in cells grown on xylose.

Determination of relatively small activities of these enzymes in undialysed cell extracts is complicated by blank and competing reactions caused by the presence of endogenous substrates of these and other dehydrogenases. The results with dialysed extracts (Table 1) probably underestimate xylitol dehydrogenase, which is not a stable enzyme, but allow comparisons to be made between cells grown on different carbon sources. The higher levels of both enzymes found in cells grown on xylose suggests that they are importantly involved in xylose utilization. Xylitol dehydrogenase could not be demonstrated in cells grown on glucose or ethanol, suggesting that its *de novo* synthesis may be induced by xylose, as has been shown for *Pullularia pullulans* (Sugai & Veiga, 1988). The level of xylose reductase in ethanol-grown cells was about 30% of that in xylose-grown cells, and decreased further (to about 10%) in glucose-grown cells (Table 1). Bicho *et al.* (1988) have shown that glucose prevents the appearance of xylose reductase in *Pachysolen tannophilus* and *Pichia stipitis* when these yeasts are grown on mixtures of xylose and glucose, and suggested that this may be due to catabolite repression by glucose.

The total amounts of xylose reductase and xylitol dehydrogenase found in *R. toruloides* grown aerobically on xylose are less than those reported for some xylose-fermenting yeast. Thus, Bolen *et al.* (1986) reported 47 mU NADPH-linked xylose reductase (mg protein)⁻¹ (at 50 mM-xylose, pH 7.4, 30 °C) and 140 mU NAD⁺-linked xylitol dehydrogenase (mg protein)⁻¹ (at 50 mM-xylitol, pH 8.6, 30 °C) in cell extracts of *P. tannophilus* grown on xylose. Under our assay conditions we find about 10 mU xylose reductase (mg protein)⁻¹ and 40 mU xylitol dehydrogenase (mg protein)⁻¹ in extracts of *R. toruloides* grown on xylose. The question that arises is whether these amounts can account for the observed rate of xylose utilization.

During the last 20 h of the cultivation on xylose shown in Fig. 1, xylose was consumed at 0.34 g l⁻¹ h⁻¹, which is equivalent to about 4 µmol min⁻¹ (g fresh cells)⁻¹ (the final cell mass was 3.4 g dry wt l⁻¹). This is four times and twice, respectively, the standard activities of xylose reductase and xylitol dehydrogenase shown in Table 1. For xylitol dehydrogenase, which is unstable, activities up to at least 3 U (g fresh wt)⁻¹ in xylose-grown cells have been observed during purification from undialysed cell extracts (see Methods). Xylose reductase has a K_m of about 0.2 M-xylose, so that if the xylose concentration in the cells approaches that in the medium (at 80 h, 2.8% $\equiv$ 0.18 M), the activity of xylose reductase would reach 3.2 U (g fresh cells)⁻¹. Nevertheless, the known cellular contents of xylose reductase and xylitol dehydrogenase are, at best, only just adequate to explain the observed rates of xylose utilization. These enzymes must limit the rate of growth on xylose, unless there is an alternative pathway. *C. utilis* appears to contain xylose isomerase as well as the oxidoreductive pathway, but, as mentioned above, work in this laboratory has not been able to demonstrate xylose isomerase in *R. toruloides*. Furthermore, the original work (Höfer *et al.*, 1971) describing this enzyme in *R. toruloides* gave no indication of how much was present.

The xylitol dehydrogenase purified from *R. toruloides* appears to be a tetramer of 45 kDa subunits, similar to the enzyme from *P. tannophilus* (Bolen *et al.*, 1986). Its rapid inactivation by exposure to EDTA suggests it may contain essential metal. The tetrameric 'long chain' alcohol/polyol dehydrogenases (Jörnvall *et al.*, 1987), which include liver sorbitol dehydro-

genase, typically have zinc at their active sites. Like the xylitol dehydrogenases from the yeasts *Candida albicans* (Veiga *et al.*, 1968*b*) and *P. tannophilus* (Morimoto *et al.*, 1986), the enzyme from *R. toruloides* oxidized D-sorbitol and ribitol at comparable but somewhat smaller rates than xylitol and had little or no activity towards D-arabitol, D-mannitol and erythritol. These enzymes show little or no activity with NADP, and have alkaline pH optima for xylitol oxidation. The K_m for xylitol of the *P. tannophilus* enzyme is reported to be 11 mM at pH 9.5 (Morimoto *et al.*, 1986) and 70 mM at pH 7.0 (Ditzelmüller *et al.*, 1984*b*). We do not know whether the K_m for xylitol of the *R. toruloides* enzyme (19 mM at pH 8.5; Fig. 4*b*) is markedly dependent on pH. If, like most pyridine-nucleotide-linked dehydrogenases, xylitol dehydrogenase operates by a ternary complex mechanism, then an increase in the apparent K_m for xylitol is expected at sufficiently small NAD⁺ concentrations. Within experimental error, no change was detected between 1 mM and 0.3 mM-NAD (Fig. 4*b*), suggesting that even at as little as 20 µM-NAD the apparent K_m will still be below 40 mM-xylitol (the value calculated by assuming a 5% increase in K_m between 1 mM and 0.3 mM-NAD). Thus, at least at pH 8.5, the apparent K_m for xylitol is not much greater than the probable concentration of xylitol *in vivo*, which appears to reach 14 mM during growth on xylose (see Results). The Hofstee plot for NAD⁺ in Fig. 4(*a*) is slightly convex towards the origin. K_m values of 0.45 and 0.22 mM-NAD⁺, respectively, can be obtained (not shown) from the data above and below 0.4 mM-NAD⁺. A much greater dependency of the K_m for NAD on NAD concentration has been reported for the xylitol dehydrogenase from *P. pullulans*, with limiting K_m values for NAD⁺ of 1.1 mM and 0.14 mM (Sugai & Veiga, 1981). Although mild negative cooperativity cannot be excluded, a more likely explanation is that the purified enzyme is a mixture of slightly different molecules, possibly, for example, with different zinc contents.

Enzymes with xylitol dehydrogenase activity but different patterns of substrate specificity have been reported in two other yeasts. *Pullularia pullulans* contains an enzyme that is inactive with D-sorbitol (of eight polyols tested, only xylitol and ribitol were oxidized; Sugai & Veiga, 1981), whereas *Candida boidinii* contains an enzyme which is 7- to 18-fold more active with D-mannitol, D-sorbitol or ribitol than with xylitol (Vongsuvanlert & Tani, 1988*a*). The D-mannitol dehydrogenase of *R. toruloides* is distinct from the major xylitol dehydrogenase (see Results), and since it is present in glucose-grown cells (not shown), extracts of which cannot oxidize xylitol, its activity towards xylitol must be small.

The NADPH-linked xylose reductase found in *R. toruloides* was much more active with DL-glyceraldehyde than with xylose. The preparation studied was not pure, but the ratio of activities towards DL-glyceraldehyde and xylose (16 at 33 mM substrate) did not change during the partial purification. Several yeasts, including *Rhodotorula* sp. (Watson *et al.*, 1969), *P. stipitis* (Verduyn *et al.*, 1985) and *P. tannophilus* (Ditzelmüller *et al.*, 1984*a*) contain NADPH-linked aldose reductases with a similar preference for glyceraldehyde to xylose. These enzymes catalyse the reverse reaction only slowly and at high pH (Sheys & Doughty, 1971; Verduyn *et al.*, 1985), and with our preparation the reverse reaction could not be detected. It seems to be characteristic of yeasts that can use xylose with limited oxygen supply that they contain (also) NADH-linked xylose reductase, apparently to maintain the redox balance (Bruinenberg *et al.*, 1984). *R. toruloides* cannot grow anaerobically, and we could detect no NADH-linked xylose reductase.

In conclusion, we have shown that xylose-grown *R. toruloides* contains both xylose reductase and xylitol dehydrogenase and grows as rapidly on xylitol as xylose, and with a shorter lag phase. Conversion of xylose to xylulose by these enzymes is likely to play a major role in the aerobic utilization of xylose by this yeast.

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