Temperature Sensitivity of the Induction of Xylose Reductase in Pachysolen tannophilus

Nancy J. Alexander
Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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Pachysolen tannophilus is a yeast capable of fermenting xylose to ethanol. This organism is of interest due to its potential for converting the xylose found in agricultural and forestry residues to ethanol. Hemicellulosic sugars (i.e., xylose) are relatively abundant in certain plant residues and can be isolated with relative ease (for review, see ref. 3).

The assumed pathway of xylose utilization in P. tannophilus involves NADPH-dependent xylose reductase, converting xylose to xylitol, followed by the conversion of xylitol to xylose by NAD-dependent xylitol dehydrogenase. Both enzymes are induced in the presence of xylose. Under anaerobic conditions, NADH may serve as the cofactor for xylose reductase. The presence and induction of these enzymes has been reported in other fungi, including Candida and Penicillium. However, certain bacteria and yeasts utilize xylose through an isomerization step, converting xylose directly to xylulose.

We have found that when P. tannophilus is grown on xylose at 37°C, xylose reductase activity is greatly reduced from that found in cells grown on xylose at 30°C. Even though xylose reductase activity is reduced, cellular growth and division continues to occur at the higher temperature, suggesting either that xylose reductase activity is not a rate-limiting step in the utilization of xylose or that there is an alternate pathway for xylose utilization in Pachysolen.

METHODS AND MATERIALS

Organisms and Media

Pachysolen tannophilus (NRRL Y-2460) was maintained at 30°C on YEP (1% yeast extract, 2% peptone) and 2% dextrose. Liquid cultures contained YEP with or without 5% carbohydrate (r-glucose or D-xylose) as indicated in the text.

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Growth Determinations

Cells were inoculated into YEP-5% glucose and grown to stationary phase. YEP-5%-xylose cultures were inoculated from the stationary cultures at 10^5 cells/mL and incubated with shaking at 30 and 37°C. Samples taken at different times were counted by hemocytometer, plated for viability, and tested for enzyme activities, ethanol, and carbohydrate concentrations.

Enzyme Analysis

Cells were inoculated into 250 mL (500-mL flask) YEP-5% glucose and grown 48 h to a stationary phase. The culture was divided into equal aliquots, harvested at 5000 rpm in the Beckman JA-14 rotor, washed once in sterile water, resuspended in fresh medium (125 mL in a 250-mL flask of YEP-5% glucose, YEP-5% xylose, or YEP-no carbohydrate), and incubated (200 rpm) at either 30 or 37°C for 48 h. For enzyme analysis, cells were centrifuged as described, washed with sterile water, and resuspended at a density of 10^10 cells/mL in 0.1M potassium phosphate buffer (pH 7.2). Cells (2.0 mL) were put into 20-mm-diameter sterile tubes, glass beads (0.5 mm) added, and the tubes vortexed in short bursts for 5-10 min, or until 90% breakage was observed under the light microscope. The tubes were then centrifuged (4000 rpm, JA-20 rotor) for 5 min and the supernatant reserved on ice for further analyses.

Enzyme activity was determined with a Cary 219 double-beam recording spectrophotometer using a 1-cm light path quartz cuvette. Xylose reductase and xylitol dehydrogenase procedures were modified slightly. For each xylose reductase assay, the 3-mL cuvette contained 1.65 mL H_2O, 300 μL 0.1M β-mercaptoethanol, 150 μL K_2HPO_4 (1.0M, pH 7.2), 100 μL NADPH (1.3mM final concentration, Type X, Sigma), and 100 μL of cell extract. The background activity of the oxidation of NADPH to NADP was measured by following the decrease in absorbance at 340. Xylose reductase activity was then recorded after addition of 300 μL of 0.5M xylose, the substrate for
the enzyme. After subtracting background activity, xylose reductase activity was expressed in nmol NADPH oxidized/min/mg protein based on an extinction coefficient of $6.2 \times 10^3$ for NADPH at 340 nm. Xylitol dehydrogenase was measured in a cuvette containing 1.5 mL H$_2$O, 300 \mu L 0.1M \beta$-mercaptoethanol, 450 \mu L 0.5M Tris (pH 8.6), 100 \mu L NAD (1.26mM final concentration, Grade V, Sigma), and 50 \mu L of cell extract. The background activity of the reduction of NAD to NADH was measured by increase in absorbance at A$_{340}$. After addition of 300 \mu L of 0.5M xylitol, the xylitol dehydrogenase activity was recorded. This activity was expressed (after subtracting background activity) in nmol NAD reduced to NADH/min/mg protein based on an extinction coefficient of $6.22 \times 10^3$ for NAD at A$_{340}$.

Alternative coenzyme assays were carried out under the same conditions except NADH was substituted for NADPH in the reductase assay and NADP for NAD in the dehydrogenase assay.

Xylose isomerase activity was tested by measuring the amount of xylulose present after a timed reaction. Several methods were combined to produce the following xylose isomerase reaction mixture: 10 \mu L xylose (10mM-100mM final concentration), 50 \mu L 0.1M Tris (pH 7.5), 10 \mu L 0.1M MnCl$_2$ (or 10 \mu L 0.1M MgCl$_2$ to test the effect of Mn$^{2+}$), 20 \mu L H$_2$O, and 10 \mu L enzyme (ca. 10 mg protein/mL). Control reactions contained 10 \mu L of a 5-min preboiled enzyme. The reaction mixture was incubated at 30, 37, or 4°C for 15 min, 30 min, 1 h, or 48 h. The reaction was stopped by putting the samples on ice or by adding 900 \mu L of 0.1N HCl. A freshly prepared 1.5% cysteine-HCl solution (100 \mu L) was added. Addition of 100 \mu L of 0.12% carbazole (in 95% ethanol) solution was immediately followed by 3 mL 70% H$_2$SO$_4$. The samples, after 1 h, were read at A$_{540}$ and A$_{750}$.

Enzyme Reactions for HPLC Analysis

The procedure for in vitro reactions of Bolen and Detroy was followed. Briefly, cell supernatants were prepared as described above and dialyzed overnight against 0.01M potassium phosphate (pH 7.2), 0.001M \beta$-mercaptoethanol at 5°C. The in vitro reaction mixture contained 40 \mu L of 0.5M D-xylose, 40 \mu L of 0.25M NADPH (50mM final concentration), and 120 \mu L of cell extract. After addition of the last component, a 60-\mu L sample (to represent 0 h) was boiled and then frozen at $-20°C$ until HPLC analysis. The remaining 140 \mu L of reaction mix was incubated for 48 h on ice or for 4 h at room temperature. All samples at the end of the reaction were boiled for 5 min to stop enzyme reaction and then frozen until HPLC analysis. The control reaction mixes substituted 40 \mu L water for the xylose, 40 \mu L 0.25M NADP, 40 \mu L 0.5M xylitol, and cell extract preboiled for 5 min before addition to the reaction mixture.

Ethanol and Carbohydrate Determination

Cell-free samples were assayed for ethanol in a Packard 428 gas chromatograph equipped with a 6-ft Porapak Q column FID detector and operated isothermally at 170°C. Carbohydrate was determined with a Waters HPLC equipped with a refractive index indicator and a Biorad HPX-87C ion exclusion column. The solvent was 0.01N sulfuric acid and the column was kept at ambient temperature. The running speed was 0.6 mL/min.

Polyacrylamide Gel Electrophoresis (PAGE) and Protein Determination

Protein determination was done following the procedure of Read and Northcote on the supernatant of cells prepared in the procedure described above. An equal amount of protein from each sample (ca. 60 \mu g) was loaded onto a 12.5% poly/bis acrylamide gel and run under denaturing conditions for 8–10 h, constant 30 mA current, following the procedure of Laemmli. The gels were stained with Coomassie blue R-250 (Serva Co.) and destained following standard procedures.

RESULTS

Enzyme activities of cells grown at 30 and 37°C are presented in Table I. Although cells grown on xylose at 30°C have a high xylose reductase activity, those grown at 37°C have little measurable activity. It is important to note that glucose-grown cells at 37°C also have a small measurable amount of xylose reductase.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Temperature (°C)</th>
<th>Xylose reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Xylitol dehydrogenase (nmol NAD reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>30</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Glucose</td>
<td>37</td>
<td>6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Xylose</td>
<td>30</td>
<td>84</td>
<td>228</td>
</tr>
<tr>
<td>Xylose</td>
<td>37</td>
<td>5</td>
<td>165</td>
</tr>
</tbody>
</table>
Even though xylose reductase activity was greatly reduced in 37°C xylose-grown cells, the NAD-linked xylitol dehydrogenase activity was only slightly reduced. Cells held in xylose at 37°C did not increase NADPH-xylose reductase activity with time as cultures harvested at 72, 96, and 120 h had an activity of 4–8 nmol/min/mg protein.

To determine whether the lack of xylose reductase activity affects the growth of cells on xylose at 37°C, growth was followed with time. Regardless of temperature, growth on xylose was slower than that on glucose [Figs. 1(A) and 1(B)]. However, the final cell density reached was comparable in glucose- and xylose-grown cells at their respective temperatures. At its fastest, the doubling time of the 37°C xylose culture (4.7 h) was about twice the rate of the 30°C xylose culture (2.4 h). Ethanol was produced by the 37°C xylose-grown cells, although peak production was less and at a later time than that of the 30°C xylose culture [Fig. 1(B)]. A rich medium was used for the growth analyses, and quite possibly some of the growth on xylose at 37°C could be due to other carbon sources found in the YEP. However, at 37°C, little growth was seen in cultures with no carbohydrate added [Fig. 1(C)] and no alcohol was detected.

If NADPH-linked xylose reductase is active in 37°C xylose-grown cells, although at very reduced levels (Table I), then xylitol may be detectable as an intermediate product. If xylose isomerase is responsible for conversion of xylose to xylulose, then no xylitol should be detected. When the in vitro reaction mixture of 30°C xylose-grown cells was analyzed with an HPLC system, a peak having the same retention time as xylitol appeared in the 48-h reaction mixture. However, this same peak appeared in the extract from 37°C (Fig. 2) and 30°C glucose-grown cells. This peak did not appear in the reaction mixture containing preboiled extract from 30°C xylose-grown cells, indicating that the presence of the peak was the result of an enzymatic reaction and not due to a natural breakdown of the in vitro reaction mixture. NADPH, NADP, and xylulose-5-P all have the same retention time, using this column, and the increase in this peak from 0 to 48 h may be due to the increase in xylulose-5-P present in the mixture. No xylulose accumulated in the mixture as no xylulose peak was detected under any of the reaction conditions at 48 h.

The presence of xylitol was also detected in the supernatant (extracellular) of 37 and 30°C cultures held in xylose that were prepared for enzyme analysis (Fig. 3). Cultures maintained at 37°C consumed xylose more slowly than did 30°C cultures, but eventually all the xylose disappeared from the medium. Increasing amounts of ethanol were found in the supernatant with time.

Visualization of the proteins xylose reductase and xylitol dehydrogenase by PAGE is enhanced by the inducibility of these proteins. Possibly, xylose-grown cultures at 37°C may not produce the protein xylose reductase, or may produce a form of the protein that is enzymatically reduced in activity. A protein having the molecular weight of xylose reductase (MW of ca. 3.7 × 10^4, Bolen, personal communication) is found in both glucose-grown (30 and 37°C) and xylose-grown (30 and 37°C) cells (Fig. 4). Xylitol dehydrogenase...
Figure 2. HPLC chromatograms of in vitro reaction mixtures. Y-2460 was grown in 5% xylose at 30°C for (A) 0 h, (B) 48 h; grown in 5% xylose at 37°C for (C) 0 h, (D) 48 h; grown in 5% glucose at 37°C for (E) 0 h and (F) 48 h. Control reactions are also shown (G). Peak a is NADPH, b is xylulose-5-P04, c is xylose, d is xylulose, e is xylitol, and f is NADP.

(MW of ca. $4.1 \times 10^4$) is present in xylose-grown cells (30 and 37°C) but not in glucose-grown cells (Fig. 4).

DISCUSSION

P. tannophilus, using xylose as a carbohydrate source, is capable of growth and alcohol production at both 30 and 37°C. However, the low levels of activity of xylose reductase in 37°C grown cells leads to the question of what is supporting growth at the higher temperature. There are two major alternatives: 1) the utilization of xylose at 37°C to produce a growth rate of ca. one-half that at 30°C can be accomplished by low levels of xylose reductase and normal levels of xylitol dehydrogenase; 2) an alternative pathway for the utilization of xylose exists. Possible alternative pathways include a xylose isomerase taking xylose directly to xylulose or an enzyme other than xylose reductase taking xylose to xylitol.

To detect a xylose-specific isomerase, the presence of xylulose was also measured. If 37°C xylose-grown cells have an increased isomerase activity, xylulose, as measured by the cysteine-carbazole method and by HPLC analysis, may be detected. However, xylulose was not detected in either the supernatants of xylose-grown, xylose-held cells or in in vitro reaction mixtures.
Figure 4. SDS-PAGE of proteins extracted from *P. tannophilus*. Lanes 1 and 6 show molecular weight markers (6.8 × 10^6; 4.5 × 10^6; 3.2 × 10^6); lanes 2–5 show Y-2460 grown on 5% glucose at (lane 2) 30°C, (lane 3) 37°C, 5% xylose at (lane 4) 37°C, and (lane 5) 30°C. The top arrow is xylitol dehydrogenase (lanes 4 and 5); the bottom arrow is xylose reductase (lane 5). Lanes 2 and 3 have no xylitol dehydrogenase activity; lanes 2, 3, and 4 have no xylose reductase activity.

The supernatants might not contain xylulose if it is immediately phosphorylated and therefore does not accumulate. However, small visible quantities of xylulose may be produced in *in vitro* reactions due to the immediate sequestering of enzymatic activities with the addition of acid. The negative results found (cysteine–carbazole results not shown), suggest that an isomerase is probably not contributing substantially to the conversion of xylose in either 30 or 37°C grown cells.

The presence of xylitol in supernatants of 37°C xylose-grown cells and in *in vitro* reaction mixtures also suggests that an isomerase is not the main method for xylose conversion in *P. tannophilus*. The low levels of xylose reductase activity measured in 37°C xylose-grown cells may be sufficient to produce the growth observed at this temperature. It is interesting to note that, even with reduced xylose reductase activity, xylitol is still excreted from the cells. Xylitol may have an efficient excretion system, or xylitol dehydrogenase may be a rate-limiting step and xylitol builds up as an intermediate product, or a combination of both is occurring in xylose-grown cells. Presumably, xylitol dehydrogenase is in itself not the rate-limiting factor, as it is abundant in both 30 and 37°C xylose-grown cells (protein gels visualization) and is very active when supplied with sufficient NAD (spectrophotometric assay). Our data, as well as others, support the hypothesis that *in vivo*, NAD is limiting the conversion of xylose. Bruinenberg et al. have proposed that xylose reductase and xylitol dehydrogenase in *P. tannophilus*, as well as in other yeasts, exhibit a specificity for dual coenzymes, in that the reductase can function with either NADH or NADPH and the dehydrogenase with NAD or NADP, although at reduced levels with the alternative coenzyme.

Both 37 and 30°C xylose-grown cells in this study had low levels of NADH xylose reductase activity (5–10 nmol NADH oxidized/min/mg protein). No activity (less than 2 nmol NADP reduced/min/mg protein) was detected in cells under either temperature condition when xylitol dehydrogenase activity was assayed using the alternative coenzyme NADP. However, an intensive study of these alternative coenzymes was not undertaken to try and identify the optimum reaction conditions. Detection of the oxidation of NADH may be difficult spectrophotometrically, due to the immediate reduction by the xylitol dehydrogenase of any NAD formed. These results do not rule out an alternative pathway or cofactor specificity for xylose conversion in *P. tannophilus*, but do support, coupled with other observations, that NADPH-linked xylose reductase and NAD-linked xylitol dehydrogenase are the primary enzymes involved in xylose conversion and that the dehydrogenase is a rate-limiting step.

The presence in 37°C xylose-grown cells of a protein of the molecular weight of xylose reductase, as seen by polyacrylamide gels, suggests that 37°C xylose-grown cells produce an enzymatically altered protein. The growth of *P. tannophilus* on xylose at 37°C, although at a rate of one-half that when grown at 30°C, is most likely the result of reduced NADPH-linked xylose reductase and normal NAD-linked xylitol dehydrogenase activities. The conversion of xylose to ethanol at the elevated temperatures presumably is carried out by small quantities of active xylose reductase and not by alternative enzymes.

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References