Characterization of Ethanol Production from Xylose and Xylitol by a Cell-Free \textit{Pachysolen tannophilus} System

JIE XU AND KENNETH B. TAYLOR*

Department of Biochemistry, The Fermentation Facility, The University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 14 July 1992/Accepted 12 October 1992

Whole cells and a cell extract of \textit{Pachysolen tannophilus} converted xylose to xylitol, ethanol, and CO$_2$. The whole-cell system converted xylitol slowly to CO$_2$ and little ethanol was produced, whereas the cell-free system converted xylitol quantitatively to ethanol (1.64 mol of ethanol per mol of xylitol) and CO$_2$. The supernatant solution from high-speed centrifugation (100,000 × g) of the extract converted xylitol to ethanol, but did not metabolize xylitol unless a membrane fraction and oxygen were also present. Fractionation of the crude cell extract by gel filtration resulted in an inactive fraction in which ethanol production from xylitol was fully restored by the addition of NAD$^+$ and ADP. The continued conversion of xyllose to xylitol in the presence of fluorocitrate, which inhibited aconitase, demonstrated that the tricarboxylic acid cycle was not the source of the electrons for the production of xylitol from xylose. Therefore, the source of the electrons is indirectly identified as an oxidative pentose-hexose cycle.

A cell-free system that metabolizes xylose and xylitol has been developed from \textit{P. tannophilus} and partially characterized in our laboratory. The effect of fluorocitrate on xylitol production was determined to identify the electron source.

**MATERIALS AND METHODS**

\textbf{Strain and growth conditions.} \textit{P. tannophilus} (NRRL Y-2460) was from the Northern Regional Research Center. The growth medium (per liter) consisted of 45 g of xylose, 3 g of yeast extract, 5 g of peptone, 7.5 g of NH$_4$Cl, 10 g of KH$_2$PO$_4$, and 0.244 g of MgSO$_4$. The initial pH before sterilization was adjusted to 5.2 with 5 N potassium hydroxide, if necessary. The liquid culture of 800 ml was incubated in a 2-liter Erlenmeyer flask at 31°C with rotary shaking at 200 rpm and an amplitude of 3 cm. The cell growth was monitored by measurement of the turbidity at 650 nm.

\textbf{Preparation of cells.} Cells at late log phase were harvested by centrifugation (1,500 × g, 10 min), washed twice with 1 volume of resuspension buffer (73 mM KH$_2$PO$_4$, 1.0 mM MgSO$_4$, pH 5.2; 4°C), and resuspended to a final cell density of 200 optical density units at 650 nm (approximately 40 g of dry cell weight per liter). The resulting cells were used for the whole-cell experiments.

\textbf{Preparation of cell extract fraction.} Cells were prepared as described above except that resuspension buffer at pH 6.6 was used throughout the entire preparation. The cells were disrupted by a single passage through a French press at 20,000 lb/in$^2$ (4°C). After the intact cells and the cell debris were removed by centrifugation at 1,500 × g for 20 min, the resulting supernatant solution was the cell extract.

The high-speed supernatant fraction was obtained by centrifugation of the cell extract at 100,000 × g for 1 h. The supernatant solution was removed, and the pellet was washed once with phosphate buffer and centrifuged. The corresponding pellet resuspended with a minimum volume of buffer was used as the pellet fraction.

The high-molecular-weight (HMW; molecular weight, >1,000) and the low-molecular weight (LMW; molecular weight, <1,000) fractions were obtained by gel filtration of the cell extract on a PD-10 column (Sephadex G-25 M;
Pharmacia, Uppsala, Sweden). After 2.5 ml of the cell extract was loaded on the column, the first fraction, the HMW fraction, was eluted with 3.5 ml of buffer. The second fraction, the LMW fraction, was eluted with an additional 4.0 ml of buffer. A more complete characterization of these fractions is presented in Results.

**Analytical methods.** Carbon dioxide was analyzed with a Perkin-Elmer vapor fractometer model-154 equipped with a thermal conductivity detector. The column (CTR I; Alltech Associates, Inc., Deerfield, Ill.) was operated at 80°C, and the flow rate of the carrier gas, helium, was 300 ml/min. Ethanol, xylose, and xylitol were analyzed with a Hewlett-Packard 7590A gas chromatograph equipped with a flame ionization detector. The flow rate of the carrier gas, nitrogen, was 20 ml/min. For ethanol determination, the injector and the column (10% C1500 on 80/100 Chromosorb WHP; Alltech Associates, Inc.) were maintained at 90°C. Xylose and xylitol were converted to trimethylsilyl derivatives (33) before gas chromatography analysis. The injector and the column (3% OV-17 on 100/120 Supelcoport; Supelco, Inc., Bellefonte, Pa.) were maintained at 160°C.

The ratio of the area of the peak (Hewlett-Packard HP3936A integrator) of each chemical species to that of an internal standard was calculated. The amount of each chemical species was determined by interpolation of the ratio in a calibration curve. The amounts of the products formed and the substrate consumed were calculated as the net change of each chemical species during the incubation.

The protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as a standard, and the A 495 was measured.

**Enzyme determination.** Aconitase and NADP-linked isocitrate dehydrogenase were assayed as described previously (14). The assay mixture (1.5 ml) contained 76 mM triethanolamine (pH 7.3), 50 μM MnCl2, 330 μM NADP+, and either 133 μM sodium citrate for the aconitase or 250 μM sodium isocitrate for the isocitrate dehydrogenase assay. A sample (25 μl) of a cell-free fraction was added, and the A 450 was monitored.

The reaction mixture (1.38 ml) for the membrane-associated succinate dehydrogenase assay (4) contained 54 mM Tris-HCl (pH 7.5) and 2 mM CaCl2. A sample (100 μl) of a cell-free fraction was added, and the oxygen uptake was monitored with a Yellow Springs oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). After equilibration for several minutes to get a stable baseline, sodium succinate was added to a final concentration of 14.5 mM and the oxygen uptake was monitored.

The reaction mixture (1.62 ml) for the soluble succinate dehydrogenase assay (18) contained 86 mM phosphate buffer (pH 7.7), 0.37 mM potassium ferricyanide, and 12 mM sodium succinate. A sample (100 μl) of a cell-free fraction was added, and the A 420 was measured.

The reaction mixture (1.52 ml) for the xylene reductase assay (1) contained 60 mM phosphate buffer (pH 7.2), 12 mM β-mercaptoethanol, 0.15 mM NADPH, and 8 μl of sample. Xylose was added to a final concentration of 60 mM, and the A 340 was measured.

The reaction mixture (1.73 ml) for the xylene dehydrogenase assay (1) contained 87 mM Tris-HCl buffer (pH 8.6), 12 mM β-mercaptoethanol, 0.15 mM NADP+, and 4 μl of sample. Xylool was added to a final concentration of 58 mM, and the A 340 was measured. The enzymatic activity was calculated from the initial reaction rate.

**Carbon balance experiments.** Carbon balance experiments were performed to determine the quantitative relationship between substrate consumption and product formation. A typical reaction mixture contained 9.0 ml of either the cell suspension or the cell extract and 1.0 ml of either xylose or xylitol to give a final concentration of 66.7 or 50 mM, respectively. The reaction mixture was incubated for 2 h at 31°C in a 72-ml glass bottle sealed with a stopper under either air or N2. At both the beginning and the end of the incubation, 100 μl of the gas phase was withdrawn and analyzed for CO2, and a sample (100 μl) of the liquid phase was analyzed for ethanol after the addition of 100 μl of 0.4% (vol/vol) n-butanol (internal standard). For the determination of xylose and xylitol, a sample (50 μl) of the liquid phase was lyophilized along with 106 μg of D-sorbitol (internal standard) and the trimethylsilyl derivatives were prepared. In the whole-cell experiment, a sample (50 μl) of the cell suspension was mixed with the internal standard and extracted with 2 N perchloric acid. The resulting supernatant solution, after removal of the cell debris and proteins by centrifugation (1,500 × g, 10 min), was neutralized with 2 N KOH to precipitate potassium perchlorate. After centrifugation, the supernatant solution was lyophilized and the trimethylsilyl derivatives were prepared.

### RESULTS

**Metabolism of xylose and xylitol by whole cells and cell extract.** Xylose was metabolized aerobically and anaerobically by both the whole cells and the cell extract (Table 1). The major products were xylitol, CO2, and ethanol. Xylose utilization and product formation by the whole-cell system were two- to threefold higher than those by the cell-free system. The presence of oxygen enhanced somewhat the production of CO2 and ethanol and decreased the accumulation of xylitol by both systems.

Xylitol was utilized only in the presence of oxygen. In the whole-cell system, xylitol was metabolized slowly to CO2 and to some unidentified products, since 30% of carbon input was not recovered (Table 1). No detectable ethanol was made. It was unlikely that a significant part of the unrecovered carbon became biomass since the experiment was conducted in the absence of a nitrogen source. In contrast to the whole-cell system, the cell extract converted xylitol to ethanol and CO2 with good efficiency (theoretical yield, 1.67 mol of ethanol per mol of xylitol). The amount of xylitol

<table>
<thead>
<tr>
<th>System</th>
<th>Substrate utilized (mmol)</th>
<th>Product formed (mmol)</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylose</td>
<td>Xylitol</td>
<td>Xylitol</td>
</tr>
<tr>
<td>Aerobic</td>
<td>Whole cell</td>
<td>0.62</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td>0.27</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Whole cell</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td>0.44</td>
<td>0.72</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Whole cell</td>
<td>0.62</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Whole cell</td>
<td>&lt;0.04</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td>&lt;0.01</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

* ND, not determined; since little substrate was utilized, the recovery was not calculated.
TABLE 2. Determination of enzymatic activities for various cell-free fractions

<table>
<thead>
<tr>
<th>Extract fraction</th>
<th>Activity (U/mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICDH</td>
</tr>
<tr>
<td>Cell extract</td>
<td>120</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>350</td>
</tr>
<tr>
<td>100,000 × g pellet</td>
<td>0</td>
</tr>
<tr>
<td>HMW</td>
<td>100</td>
</tr>
</tbody>
</table>

* One unit is defined as 1 nmol of product per min. ICDH, isocitrate dehydrogenase; SDH1, soluble succinate dehydrogenase; SDH2, succinate dehydrogenase activity associated with the membrane and measured by oxygen uptake; XR, xylitol reductase; XDH, xylitol dehydrogenase.

utilized by the cell-free system was about threefold higher than that used by the whole-cell system. The recovery of carbon and the yield of products based on xylitol and xylitol were also calculated (Table 1).

Characterization of cell-free fractions. The cell-free fractions were characterized by measurement of the activities of marker enzymes for mitochondria (9, 24): aconitase, isocitrate dehydrogenase, and succinate dehydrogenase, as well as xylitol reductase and xylitol dehydrogenase (Table 2). Aconitase and isocitrate dehydrogenase activities were detected in the crude cell extract, 100,000 × g supernatant solution, and HMW fractions. Xylitol reductase and xylitol dehydrogenase activities were also detected in the same fractions. The activities of the above enzymes were not detected in the pellet fraction. The activity of succinate dehydrogenase measured by the ferricyanide method was detected in the same fractions and also in the pellet fraction. However, succinate dehydrogenase activity associated with oxygen uptake was not detected in the 100,000 × g fraction.

Requirements for ethanol production from xylitol and xylitol by various cell-free fractions. The cell extract was fractionated by gel filtration and differential centrifugation. Various combinations of the resulting fractions were tested for ethanol production. Although neither the HMW nor the LMW fraction alone was active in ethanol production from xylitol (Table 3), the combination of the two resulted in activity. Furthermore, neither NAD+ nor ADP alone was able to restore ethanol production in the presence of the HMW fraction, but the activity was restored when NAD+ and ADP were both present. These results demonstrate that

<table>
<thead>
<tr>
<th>Cell-free fraction</th>
<th>Protein concn (mg/ml)</th>
<th>Added component(s) (conc)</th>
<th>EtOH made (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW</td>
<td>6.6</td>
<td>LMW (0.75 ml)</td>
<td>19</td>
</tr>
<tr>
<td>LMW</td>
<td>0.1</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>HMW</td>
<td>13.2</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>HMW</td>
<td>13.2</td>
<td>NAD+ (50 μM)</td>
<td>0</td>
</tr>
<tr>
<td>HMW</td>
<td>13.2</td>
<td>ADP (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>HMW</td>
<td>13.2</td>
<td>NAD+ (50 μM), ADP (100 μM)</td>
<td>42</td>
</tr>
<tr>
<td>100,000 × g supernatant soln</td>
<td>11.7</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>100,000 × g supernatant soln</td>
<td>11.7</td>
<td>Pellet (100 μl)</td>
<td>61</td>
</tr>
</tbody>
</table>

* The volume of the reaction mixture was 1.75 ml containing 71 mM xylitol. Only ethanol was measured.

TABLE 4. Effect of fluorocitrate on xylitol production from xylitol

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Fluorocitrate (μM)</th>
<th>Xylitol utilized (mmol)</th>
<th>Product Formed (mmol)</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0.415</td>
<td>0.247</td>
<td>0.156</td>
</tr>
<tr>
<td>B</td>
<td>200</td>
<td>0.399</td>
<td>0.270</td>
<td>0.142</td>
</tr>
</tbody>
</table>

* The reaction mixture contained 8.8 ml of the 100,000 × g supernatant fraction, 1.0 ml of xylitol (667 mM), and 0.2 ml of either H2O (incubation A) or fluorocitrate (10 mM); (incubation B).

NAD+ and ADP are the only two dissociable LMW species required for ethanol production from xylitol.

The high-speed supernatant solution was unable to convert xylitol to ethanol in the presence of oxygen unless the membrane fraction was also present (Table 3). This reflects the requirement for electron transport proteins and terminal oxygenase, which are characteristics of the membrane pellet fraction.

On the other hand, ethanol production from xylitol took place with the high-speed supernatant solution alone (Table 4). Although the recoveries of carbon were fairly good, the high-speed supernatant fraction was less efficient in converting xylitol to ethanol than the crude cell extract. The pellet fraction enhanced ethanol production, but it was not required.

Effect of fluorocitrate on xylitol production. The results in Fig. 1 demonstrate aconitase activity in the 100,000 × g supernatant solution of P. tannophilus as well as its inhibition by fluorocitrate. The selectivity of the inhibitor for aconitase in the assay was demonstrated by the observation that the addition of isocitrate to the reaction system restored the production of NADPH. The crude cell extract gave similar results (not shown). The concentration of fluorocitrate required for the complete inhibition was in the micromolar range at a low protein concentration (0.1 mg/ml). However, since the xylitol utilization experiment was carried out with a more concentrated cell-free fraction, it was necessary to investigate the relationship between the protein concentration and the concentration of fluorocitrate for

FIG. 1. Inhibition of aconitase activity by fluorocitrate. The initial reaction mixture was the same as described for the aconitase assay and contained buffer, NADP+, Mn2+, 100,000 × g supernatant fraction, and (A) citrate (positive control), (B) nothing (negative control), or (C) citrate and fluorocitrate (5 μM). Arrows indicate the additional injections of citrate, fluorocitrate, and isocitrate into cuvettes. Abs., absorbance.
complete inhibition. It was found that a higher concentration (100 μM) of inhibitor was required to block the activity of the tricarboxylic acid (TCA) cycle completely at the higher protein concentration.

The effect of fluorocitrate on xylitol production was determined by incubation of the 100,000 × g supernatant fraction (6.4 mg of protein per ml) in the presence of xylose (63 mM) and fluorocitrate (200 μM). The results (Table 4) show no significant differences in the utilization of xylose or in the production of xylitol, ethanol, or CO₂ in the presence and absence of fluorocitrate.

**DISCUSSION**

The enzyme determinations in the various cell fractions indicate that mitochondria were broken during sample preparation and their contents were released as soluble enzymes. However, oxygen uptake is associated only with the mitochondrial membrane. Although Baker’s yeast contains both NAD- and NADP-linked isocitrate dehydrogenases (19), only NADP-linked isocitrate dehydrogenase activity was detected in P. tannophilus. The NADP-linked isocitrate dehydrogenase was suggested to be one of the possible electron sources for xylitol production in Candida utilis (7).

There are significant differences between the whole-cell and the cell-free systems in the metabolism of xylitol. The overall rate of xylitol utilization and the yield of ethanol are higher for the cell extract than for the whole cell. Schwester et al. (28) reported that P. tannophilus could not reassimilate xylitol aerobically since the concentration of xylitol in the medium remained constant after all xylose had been consumed. Maleszka and Schneider (21) examined 15 xylose-fermenting yeast strains and found that no detectable amount of ethanol was produced during the 7-day incubation period, although there was limited growth (optical density at 600 nm, >1.0). Studies by Neirinck et al. (23) indicated that xylitol could be used as a carbon source to support cell growth when present in high concentration, 50 to 60 g/liter, but the authors also asserted that the history of the inoculum was important. However, under the most favorable conditions, the shortest doubling time was 0.7 day, and ethanol was detectable only when a large inoculum was used. All reports on xylitol metabolism reflect the conclusion that xylitol is not a good carbon source for either growth or ethanol production.

The hypothesis of xylitol as an obligatory intermediate of the metabolic pathway for xylose would ordinarily require xylitol to be utilized just as well as xylose by xylose-fermenting yeasts. Electron acceptors have been shown by a number of researchers to be associated with the disappearance of xylitol and the formation of ethanol (2, 20, 34). The oxygen requirement (Table 1) for xylitol utilization indicates that it acts as a sink for excess electrons generated from xylitol. It seems that oxygen and artificial electron acceptors can both serve as the electron sink.

However, the carbon balance experiments (Table 1) indicate that the presence of oxygen was sufficient for xylitol metabolism by the cell extract but not sufficient for metabolism by the whole cells. This difference can be explained by the hypothesis of limited transport. Although Taylor et al. (34) demonstrated the production of ethanol from xylitol by the whole cells in the presence of acetone, which suggests the existence of adequate transport for xylitol, acetone may play a dual role as a membrane-permeabilizing reagent as well as the electron sink (2).

The results of carbon balance experiments (Table 1) show that the yield of ethanol from xylose is limited by the amount of xylitol produced. The carbon recovery of more than 80% in these experiments suggests that xylitol, ethanol, and CO₂ are the only major products. These results are consistent with the 13C-nuclear magnetic resonance findings (34).

Since the membrane organelles have been removed from the 100,000 × g supernatant fraction, the metabolism of xylose to ethanol by this fraction demonstrates that the conversion can be isoelectronic, as expected. However, the fact that the conversion is less efficient than that by the crude extract demonstrates that the stimulation by oxygen is minimized. In addition, the carbon balances show that more CO₂ is produced than can be accounted for by the production of ethanol. The excess CO₂ could result from the generation of electrons for xylitol production.

Although the TCA cycle was suggested to be the electron source for xylitol formation from xylose in Candida shehatae (34), the source in P. tannophilus has not been identified. The absence of other oxidized products requires that the electrons must result from one or more complete oxidation pathways. There are two possible pathways that meet this requirement: the TCA cycle and the oxidative pentose-hexose cycle. In the latter cycle pentose phosphate is converted to hexose phosphate by the action of transketolase and transaldolase. The hexose phosphate formed can be either used for ethanol production or converted back into pentose phosphate by oxidative decarboxylation (34) with the generation of reduced nucleotides. The relative contribution of each source might be determined by the measurement of xylitol production when either of them is inhibited.

Although no satisfactory inhibitors for the pentose-hexose cycle have been identified, the TCA cycle can be inhibited by fluorocitrate, a potent inhibitor ofaconitase (8, 22, 25, 26). Therefore, the presence of fluorocitrate would prevent the generation of electrons from the TCA cycle and the accumulation of xylitol should decrease, if the TCA cycle provides electrons for xylitol formation. The experimental results show no significant difference in the production of xylitol and suggest that in P. tannophilus the entire TCA cycle is an insignificant source of electrons for xylitol production in the cell extract. The present results are consistent with the hypothesis that the pentose-hexose cycle described above provides the electrons for xylitol formation. Because of the nucleotide imbalance, this source of electrons becomes significant in the metabolism of xylose. Although it is possible that the TCA cycle could contribute a significant quantity of electrons in whole cells, it is certainly not the only source.

**REFERENCES**