Oxidation and Reduction of D-Xylose by Cell-Free Extract of *Pichia quercuum*

TOSHIYUKI SUZUKI and HIROSHI ONISHI  
Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan

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The fermentation mechanism of the simultaneous production of D-xyloonic acid and xylitol from D-xylose by *Pichia quercuum* was studied by using a cell-free enzyme preparation. Nicotinamide adenine dinucleotide phosphate (NADP)-dependent D-xylose dehydrogenase activity and NADP-dependent D-xylose reductase activity were detected, and the oxido-reduction reaction of D-xylose was able to couple through regeneration of NADP and NADPH to produce D-xyloonic acid and xylitol.

In a previous paper (8), the authors reported that *Pichia quercuum* produced D-xyloonic acid and xylitol by aerobic dissimulation of D-xylose with a good yield of 40% of the sugar used. A large amount of both products of oxidation and reduction of D-xylose were simultaneously accumulated in the fermented broth. The present study was undertaken to understand the mechanism of this fermentation. This note demonstrates the oxidation and reduction activities of D-xylose and the coupled reaction by the cell-free enzyme preparation of *P. quercuum*.

*P. quercuum* IFO 0949, which was originally isolated and named as *P. quercibus* by Phaff and Knapp (6), was used. The yeast culture, on malt extract-agar slants, was inoculated into 500-ml shake flasks, each containing 50 ml of a pH 5.0 medium having the following composition: D-xylose, 10%; D-glucose, 1%; KH₂PO₄, 0.1%; MgSO₄·7H₂O, 0.05%; CaCl₂·2H₂O, 0.01%; NaCl, 0.01%; vitamin-free Casamino Acids (Difco), 0.4%; and yeast extract (Difco), 0.1%. The flasks were shaken on a reciprocal shaker (7.5-cm stroke, 140 cycles/min) at 30 C for 9 days. After harvesting by centrifugation, the yeast cells were washed three times with cold, 0.03 M phosphate buffer (pH 7.0) and stored at -20 C. The frozen cells (about 8 g wet weight) were suspended in 25 ml of 0.03 M phosphate buffer, pH 7.0, containing 1 mM mercaptoethanol and disintegrated with a Vibrogen cell mill (Edmund Bühler Co.) for 15 min at 13 C. The cellular debris was removed by centrifugation at 20,000 × g for 30 min at 4 C to provide the crude extract. This extract was further purified by centrifugation at 100,000 × g for 2 h at 4 C and subsequent dialysis of the supernatant fluid obtained for 24 h at 4 C against a large volume of 0.03 M phosphate buffer, pH 7.0, containing 1 mM mercaptoethanol. The enzyme preparation thus obtained was designated as the dialyzed enzyme.

Oxidation of D-xylose was examined by the following three procedures. (i) Assay mixtures (2.0 ml) contained 70 mM phosphate buffer (pH 7.0), 100 mM D-xylose, 0.3 mM nicotinamide adenine dinucleotide (NAD), or nicotinamide adenine dinucleotide phosphate (NADP) and enzyme solution. The reaction was initiated by the addition of coenzyme, and the change of absorbance at 340 nm was observed at room temperature. (ii) Reaction mixtures (2.0 ml) contained 70 mM phosphate buffer (pH 7.0), 100 mM D-xylose, 0.1 mM 2,6-dichlorophenolindophenol (3), and enzyme solution. The reaction was started by the addition of enzyme solution, and the change of absorbance at 600 nm was observed at room temperature. (iii) Reaction mixtures (2.5 ml) contained 27 mM D-xylose, 2.0 ml of chromagen-peroxidase mixture (pH 7.0) (2), and enzyme solution. The reaction mixture was incubated at 30 C, and the absorbance at 420 nm was measured. The reduction of D-xylose was examined by the same method as above (i), except that 0.13 mM reduced form NAD (NADH) or NADP (NADPH) was used instead of NAD or NADP. In all experiments, control readings were made without added D-xylose and were designated as endogenous activity.

Protein was determined by the method of Lowry et al. (5). Ascending paper chromatography was performed on Whatman no. 1 filter paper by using a solvent system of ethyl ace-
tate-pyridine-water (10:4:3, vol/vol/vol). Polyhydric compounds were detected by the KIO₄-tetrahase method (12), and lactone was detected by the hydroxylamine method (1). Barium D-xylonate was prepared as described previously (8), and D-xylololactone was prepared by the method of Weimberg (11).

Preliminary experiments with the crude extract showed that no oxidation activity of D-xylose could be detected when assayed by the three methods. Because oxidation activity was not found, even for long incubations of 1 to 4 h by method (iii) with the chromagen-peroxidase mixture, the crude extract did not contain D-xylose oxidase activity. In this case, the oxidation reaction would be catalyzed by dehydrogenase. Because the crude extract gave high, endogenous activities which reduced 2,6-dichlorophenolindophenol or NADP and oxidized NADPH or NADH, further purification of the crude enzyme preparation was necessary for observation of dehydrogenase activities for D-xylose oxidation. On the other hand, the reduction reaction of D-xylose by the crude extract was distinctly observed; NADPH was D-xylose dependent, but NADH was not (Table 1).

The dialyzed enzyme preparation contained only negligible endogenous activity and showed that the oxidation activity of D-xylose required NADP as coenzyme but not NAD (Table 1). 2,6-Dichlorophenolindophenol could not serve as a hydrogen acceptor for the oxidation of D-xylose. Apparent low-oxidation activity of D-xylose in the presence of NADP would be due to interference of high-oxidation activity of NADPH (Table 1). We suggest that the oxidation of D-xylose couples to the reduction of the sugar through regeneration of the same coenzyme and that the dialyzed enzyme contains

<table>
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<tr>
<th>Enzyme prep</th>
<th>Oxidation of D-xylose*</th>
<th>Reduction of D-xylose*</th>
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<tr>
<td></td>
<td>NADP</td>
<td>NAD</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>Without d-xylene</td>
<td>d-Xylose</td>
</tr>
<tr>
<td>Crude extract</td>
<td>0</td>
<td>93.0</td>
</tr>
<tr>
<td>Dialyzed enzyme</td>
<td>1.2</td>
<td>0.1</td>
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* Assay mixtures (2.0 ml) contained 70 mM phosphate buffer (pH 7.0), 100 mM D-xylose, 0.3 mM NADP or NAD, or 0.13 mM NADPH or NADH and enzyme solution. The change of absorbance at 340 nm was observed at room temperature. The activity is expressed as nanomoles of NADPH or NADH formed or NADPH or NADH consumed per minute per milligram of protein.

The value was subtracted from endogenous activity.

The value shows endogenous activity.

The coupled reaction of oxidation and reduction of D-xylose with the dialyzed enzyme was confirmed as follows: the reaction mixtures (5.0 ml) composed of 67 mM D-xylose, 40 mM phosphate buffer (pH 7.0), 2.8 mM NADP or NADPH, and 3.5 ml of the dialyzed enzyme was incubated for 20 h at 25 C, and reaction products were examined by paper chromatography. The paper chromatogram gave two spots of polyhydric products with $R_f$ values of 0.27 and 0.03. The $R_f$ value of 0.27 corresponds to that of authentic xylitol, and the product of $R_f$ 0.03 seems to be aldonic acid. A portion of the reaction mixtures was lactonized with 1 N HCl at 100 C for 2 h, and the lactonized product was identified as D-xylonolactone by co-chromatography with authentic D-xylonolactone. In this experiment, neither NAD nor NADH could replace NADP or NADPH. The results showed that both D-xyonic acid and xylitol were formed from D-xylose with the dialyzed enzyme preparation in the presence of either NADP or NADPH, and that this oxido-reduction reaction of D-xylose could couple through regeneration of

- **TABLE 1. The oxidation and reduction of D-xylose by the crude, cell-free extract and the dialyzed enzyme preparation from Pichia quercuum**

<table>
<thead>
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The value shows endogenous activity.
NADP and NADPH. Although D-xylonolactone was not detected as a product in the reacted mixture by us, the primary product of D-xylose dehydrogenase-catalyzed reaction would be D-xylonolactone which was hydrolyzed enzymatically or nonenzymatically to D-xylic acid. A presumed schema of the coupled reaction is illustrated in Fig. 1.

The simultaneous formation of D-xylic acid and xylitol by the intact calf lens was observed by van Heyningen and showed that different pyridine nucleotides (NAD and NADPH) were required for oxidation and reduction of D-xylose, respectively (9, 10). On the contrary, the same pyridine nucleotides were required for the simultaneous formation of D-xylic acid and xylitol by P. quercuum. Very little has been known about enzymology of alose oxidation by yeasts (4), and D-xylose reductase of P. quercuum resembles the Candida utilis polyl dehydrogenase (7) in coenzyme specificity. Detailed studies on purification and properties of the enzymes of D-xylose oxidation and reduction are now going on.

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LITERATURE CITED