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

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The fermentation mechanism of the simultaneous production of D-xyronic 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-xyronic acid and xylitol.

In a previous paper (8), the authors reported that *Pichia quercuum* produced D-xyronic acid and xylitol by aerobic dissimilation 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. quercibius* 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, 1.5%; 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). Pol-
yhydric compounds were detected by the KIO4-
tetrahydrate method (12), and lactone was de-
tected by the hydroxylamine method (1). Bar-
ium d-xyonate was prepared as described previ-
ously (8), and D-xylonolactone was pre-
pared by the method of Weimberg (11).

Preliminary experiments with the crude ex-
tract 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 oxida-
tion reaction would be catalyzed by dehy-
rogenase. Because the crude extract gave high, 
endogenous activities which reduced 2,6-
dichlorophenolindophenol or NADP and ox-
ized 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 reduc-
tion 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 oxida-
tion of D-xylose couples to the reduction of the 
sugar through regeneration of the same coen-
zyme and that the dialyzed enzyme contains

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<th>Enzyme prep</th>
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<tr>
<td></td>
<td>NADP</td>
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<td>d-Xylose</td>
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<td>Crude extract</td>
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<td>Dialyzed enzyme</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.

Fig. 1. A presumable schema of oxidation and 
reduction of D-xylose by Pichia quercuum.

both D-xylose dehydrogenase and D-xylose redu-
tase activities.

The coupled reaction of oxidation and reduc-
tion 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 chromatogra-
phy. The paper chromatogram gave two spots of 
polyhydric products with Rf values of 0.27 and 
0.03. The Rf value of 0.27 corresponds to that of 
authentic xylitol, and the product of Rf 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-chromato-
graphy with authentic D-xylonolactone. In this 
experiment, neither NAD nor NADH could 
replace NADP or NADPH. The results showed 
that both D-xyronic acid and xylitol were formed 
from D-xylose with the dialyzed enzyme prepa-
ration in the presence of either NADP or 
NADPH, and that this oxido-reduction reaction of 
D-xylose could couple through regeneration of
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