A New Method for Preparation of Xylobiose, Eliminating Xylose from Enzymatic Xylan Hydrolyzate by Yeast[†]

Isao KUSAKABE, Tsuneo YASUI and Tatsuyoshi KOBAYASHI

Laboratory of Wood Hydrolysis and Fermentation, Faculty of Agriculture,
Tokyo University of Education, Komaba-cho, Meguro-ku, Tokyo

Received October 24, 1974

Establishment of a new method for preparation of xylobiose was investigated.

When hardwood xylan was hydrolyzed by the xylanase system of *Streptomyces* sp. E-86, the hydrolyzate mainly consisted of xylose and xylobiose in an approximate ratio of 1:1.

Yeast strains selected in this paper, effectively and selectively metabolized all of xylose without any degradation of xylobiose.

By successive treatments of xylan with *Streptomyces* xylanase system and xylose-metabolizable yeast, 70.4 g of crystalline xylobiose was obtained from 172 g of xylan.

Xylobiose^{1,2)} has been obtained from the partial hydrolyzates of various hemicellulose (for example, from corncobs and rice straw). However, this ordinary preparation method has not been applicable for yielding substantial quantity of xylobiose, because of low yield and complexity in operation.

In this paper, we deal with an establishment of a novel and effective method for the preparation of xylobiose from xylan.

MATERIALS AND METHODS

Microorganisms. Strain E-86 of *Streptomyces* was used throughout this work, which was isolated from soil and maintained on Bennet's agar slant at room temperature. The strain formed a large clear zone when grown on the xylan agar plate.

Yeast strains used were originated in the collection of IFO (Institute for Fermentation, Osaka, Japan), IAM (Institute of Applied Microbiology, University of Tokyo, Japan), AHU (Faculty of Agriculture, Hokkaido University, Japan), NI (Nagao Institute, Tokyo, Japan) and our laboratory (K-number), and maintained on the malt yeast-extract agar slants at room temperature.

Xylan. A commercial hardwood xylan was supplied by the Sanyô-Kokusaku Pulp Company, which was

a by-product from mercerization process of rayon or staple fiber manufacturing and actually insoluble in water.

Xylobiose. Xylobiose was prepared from the hydrolysis products of the xylan by the *Streptomyces* xylanase,³⁾ using the charcoal column chromatography,⁴⁾ followed by concentration and crystallization.

Determination of sugar. The amount of reducing sugar was determined by Somogyi's method⁵⁾ and Willstätter-Schudel's method⁶⁾ throughout this work and represented as mg of xylose. This value was designated as direct reducing sugar. The amount of total reducing sugar was determined by the same method after hydrolysis with 4% H₂SO₄ at 100°C for 2~2.5 hr and represented as mg of xylose.

Preparation of enzyme solution. The culture filtrate of *Streptomyces* sp. E-86 in a xylan-containing medium was used as enzyme solution (see result).

Determination of xylanase activity. The xylanase activity was measured as follows. The reaction mixture containing 70.3 mg (equivalent to 50 mg of anhydroxylose) of crude xylan, 4 ml of McIlvaine buffer solution (pH 5.70) and 5 ml of water in a L-form tube was preincubated at 55°C for 15 min. Reaction was started by addition of 1 ml of the enzyme solution (1/10 dilution) to the reaction mixture, and incubation was continued at 55°C on a Monod skaker (Ohtake Seisakusho, Japan). After incubation for 30 min, 1 ml of the mixture was drawn and added to 5 ml of Somogyi's reagent in a test tube. The test tube was kept in a boiling water bath for 15 min, then, the reducing sugar was determined as xylose. The xylanase activity was expressed as "mg of xylose/10 ml of reaction mixture/

[†] Studies on Xylanase System of *Streptomyces*. Part II.

This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, held in Nagoya, April 2, 1968.

30 min." The linear relationship between the amount of enzyme and the increase of reducing power of the reaction mixture was not observed, therefore, the activity was calculated from a standard curve previously prepared.⁷¹ The activity showing 8.7 mg was defined as 10 units.⁷¹

Extent of hydrolysis of xylan and average degree of polymerization of liquefied xylan. The reaction mixture was centrifuged to remove remained xylan, and the supernatant solution obtained (liquefied xylan solution) was then hydrolyzed in 4% H₂SO₄ at 100°C for 2~2.5 hr. The reducing sugar in the hydrolyzate of the supernatant was estimated as xylose, and the amount was designated as total liquefied xylan. The extent of hydrolysis of xylan was obtained as a ratio of the total liquefied xylan to the original xylan (total xylose). Average degree of polymerization (D.P.) of liquefied xylan was obtained as a ratio of the total reducing sugar to the direct reducing sugar.

Paper chromatography. An appropriate amount (about 0.01 ml containing 30~150 µg of xylose) of sugar solution was spotted on a Tōyō filter paper No. 51 (400 mm × 400 mm) and chromatographed at 25°C for 20~25 hr with a solvent system of *n*-butanol:pyridine: water (6: 4: 3 v/v). After removal of solvent, spot of reducing sugar was developed by spraying *p*-anisidine-HCl solution in *n*-butanol and heating at 130~140°C for 10 min.

RESULTS

Production of xylanase system from Streptomyces sp. E-86

The medium (pH 5.3) for cultivation of *Streptomyces* sp. E-86 was composed of xylan 2%, peptone (Daigoeiyō Kagaku, Japan) 1.4%, yeast extract (Daigoeiyō Kagaku, Japan) 0.1%, corn steep liquor 0.5%, KH₂PO₄ 1.0%, MgSO₄·7H₂O 0.05% and a small amount of Tōshiba Silicone as defoamer. Twelve liters of the medium was placed in a 20-liter Jar fermentor (Waldhof type) and sterilized by thermal supply through a heating coil. After cooling, an amount of 200 ml of seed culture, which had been grown in the same medium in shake flasks at 35°C for about one day on a reciprocal shaker, was inoculated to the Jar fermentor. The cultivation was carried out at 35~37.5°C with an air supply at a flow rate of 7 liters per min and with an agitation at a speed rate of 540 rpm. Figure 1

FIG. 1. Time Course of Xylanase Production.

The experimental conditions are described in the text. ○—○, direct reducing sugar; ●—●, xylanase activity; △—△, mycelia weight; ×—×, pH.

shows the time course of the cultivation. The xylanase activity in the culture filtrate reached a maximum at 24~28-hr cultivation. After 28 hr, the mycelium was filtered off through Buchner funnel, and the culture filtrate was used as the xylanase solution without any treatment. The enzyme solution showed an activity of 115 units/ml.

Thermal stability of the xylanase system

The enzyme solution (at pH 5.7) was treated in water baths at intervals of 5°C from 40°C to 70°C, then, quickly cooled in ice water. The residual activities were assayed. As shown in Fig. 2, the enzyme solution retained about

FIG. 2. Thermal Stability of Xylanase System.

The experiment was performed as described in the text. ○—○, 40°C; ●—●, 45°C; ○—○, 50°C; ●—●, 55°C; ×—×, 60°C; ●—●, 65°C; △—△, 70°C.

100% of the activity after the treatment at 40°C to 45°C for 7 hr, and 23% of the activity was lost by the treatment at 50°C for 30 min. Heating at 70°C for 30 min showed a complete loss of the activity.

Effect of pH and temperature on the direct reducing sugar forming-activity

At 55°C, the optimum pH for the xylanase activity was around 5.7 (Fig. 3).

At pH 5.7, the xylanase activity was effected by temperature as shown in Fig. 4-1. The higher the temperature is, the greater the rate of the activity becomes, in early stage of the reaction, such as 30-min incubation. After 24-hr incubation, the highest value of the direct reducing sugar was observed at 55°C. Furthermore, Fig. 4-2 shows that the hydrolyzate obtained at 35~60°C was mainly composed of xylose and xylobiose, but a significant amount of xylotriose was formed during the hydrolysis at 65~70°C. These chromatographic results correspond to D. P. values (presented in parenthesis of Fig. 4-1) of hydrolysis products.

Liquefaction of xylan by the xylanase system under optimum conditions

The time course of the liquefaction under the optimum condition (pH 5.7 and 55°C), is shown in Fig. 5-1. A few ml of the reaction mixture was occasionally withdrawn from the vessel and was centrifuged as soon as possible at 5°C. Direct and total reducing sugars in the supernatant were estimated by Somogyi's and Willstätter-Schudel's methods. After 8-hr incubation, the xylan was liquefied almost completely, and D. P. of the hydrolysis products (liquefied xylan) decreased to 1.50. However, as shown in paper chromatograms (Fig. 5-2), a considerable amount of higher oligomers than dimer remained in the hydrolysis products. After incubation for 20~24 hr, the final products were found to be consisted of xylose and xylobiose in approximately equal amounts, without contamination with detectable amount of other xylooligomers. D. P. value of the products was decreased to 1.35.

Exclusive elimination of xylose from xylose-xylobiose mixture by the activity of yeast

The yeasts, which are able to metabolize

FIG. 3. Effect of pH on the Direct Reducing Sugar-forming Activity of the Xylanase System.

The composition of the reaction mixture was 1 g of xylan, 3 ml of water and 4 ml of McIlvaine buffer solution of various pH values. After preincubation, 2 ml of the enzyme solution (115 U/ml) was added to the reaction mixture and incubated at 55°C continually. An aliquot of reaction mixture was removed in 0.5, 1, 2, 3, 5 and 7 hr and the direct reducing sugar was determined as xylose.

●—●, pH 3.62; △—△, pH 4.30; ○—○, pH 5.07; ⊙—⊙, pH 5.70; ⊖—⊖, pH 6.05; ×—×, pH 6.80.

FIG. 4-1. Effect of Temperature on the Direct Reducing Sugar-forming Activity of the Xylanase System.

The composition of the reaction mixture: 2.0 g of xylan, 8 ml of McIlvaine buffer solution (pH 5.70), 6 ml of water and 4 ml of the enzyme solution (115 U/ml). The reaction mixture was incubated at the various temperature under pH value of 5.70.

●—●, 35°C; ⊖—⊖, 40°C; △—△, 45°C; ○—○, 50°C; ⊙—⊙, 55°C; ×—×, 60°C; ●—●, 65°C; ---, 70°C.

FIG. 4-2. Paper Chromatograms of Xylan Hydrolyzates by the Xylanase System from *Streptomyces* sp. E-86.

The experimental condition and samples were the same as in Fig. 4-1 (reacted for 24 hr).

X₁: authentic xylose.

FIG. 5-1. Liquefaction of Xylan by Xylanase System.

The reaction mixture was composed of 117 g of xylan (86 g as anhydroxylose), 100 ml of tap water and 1200 ml of the enzyme solution (115 U/ml). The mixture was incubated at 55°C in a 1500 ml glass beaker with agitation of 200 rpm. Throughout saccharification, pH was maintained at 5.7 by addition of NaOH solution.

○—○, total reducing sugar or liquefied xylan;
●—●, direct reducing sugar.

Average degree of polymerization:

●—●, Willstätter-Schugel's method.
×—×, Somogyi's method.

exclusively xylose and to leave xylobiose without any loss, were selected from the collection in our laboratory. These are *Candida parapsilosis* var. *komabaensis* K-75, *Candida pulcherrima* NI 7477, *Pichia halophila*, *Geotrichum* species and others. When the cells of the first four strains were grown in medium containing xylose, all strains consumed completely

FIG. 5-2. Paper Chromatograms of Xylan Hydrolyzates by the Xylanase System.

The samples were same ones in Fig. 5-1.

X₁, authentic xylose; X₂, authentic xylobiose.

FIG. 6. Consumption of Xylose by Several Yeast.

The medium was composed of xylose 2.5~6.5%, peptone 0.4%, yeast extract 0.1%, KH_2PO_4 0.1% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%. Each 100 ml of the medium was placed into 500 ml of shake flasks and sterilized. Two ml of seed culture grown on the same medium was inoculated to it and cultivated at 30°C on reciprocal shaker. A: *Geotrichum* 58, B: *Geotrichum* 61, C: *Candida pulcherrima* NI 7477, D: *Candida parapsilosis* var. *komabaensis* K-75.

xylose within 6 days (Fig. 6). Among the yeasts tested, *C. parapsilosis* and *C. pulcherrima* could eliminate xylose in rather high concentra-

FIG. 7. Non-assimilability of Xylobiose by the Yeasts Tested.

The medium was composed of xylobiose 4%, peptone 0.4%, yeast extract 0.1%, KH_2PO_4 0.1% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%. Experimental condition was the same as shown in Fig. 6.

A: *Geotrichum* 58, B: *Geotrichum* 61, C: *Candida pulcherrima* NI 7477, D: *Candida parapsilosis* var. *komabaensis* K-75.

FIG. 8. Elimination of Xylose from Mixture of Xylose and Xylobiose by Several Xylose-assimilating Yeasts.

The medium consisted of xylose 3.2%, xylobiose 3.9% and other nutrients as shown in Fig. 6. Experimental condition was as shown in Fig. 6 also.

A: *Geotrichum* 58, B: *Geotrichum* 61, C: *Candida pulcherrima* NI 7477, D: *Candida parapsilosis* var. *komabaensis* K-75.

tions at a high speed. As seen in Fig. 7, all the yeasts tested did not assimilate xylobiose at

all under the condition described in Fig. 6. When these strains were grown in a medium composed of 3.2% of xylose, 3.9% of xylobiose and nutritional supplements, they could degrade completely xylose after 2 days without any loss of xylobiose (Fig. 8). For long period of incubation, the concentration of total sugar (xylobiose) and D. P. of sugar in the culture filtrates remained constant.

For the practical purpose, the elimination of xylose from the enzymatic hydrolyzate of xylan was examined with 2 yeast strains, *C. parapsilosis* and *C. pulcherrima*. Time course of elimination of xylose is summarized in Figs. 9-1 and 9-2. When both organisms were cultured in the hydrolyzate-media containing 7.4% of total reducing sugar (D. P. 1.35), the concentration of the total reducing sugar was decreased to about 4% after 2 days, and remained constant through further incuba-

FIG. 9-1. Elimination of Xylose from Enzymatic Hydrolyzate of Xylan by the Xylose-utilizable Yeasts.

The medium was composed of peptone 0.4 g, yeast extract 0.1 g, KH_2PO_4 0.1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g in 100 ml of enzymatic hydrolyzate (pH 5.7) in 500 ml of shake flasks. Seed cultures were inoculated to the medium and cultivation was performed at 30°C on a reciprocal shaker.

A: *Candida pulcherrima* NI 7477,

B: *Candida parapsilosis* var. *komabaensis* K-75.

○—○, direct reducing sugar (as xylose); ●—●, total reducing sugar (as xylose); —●—●, average degree of polymerization.

FIG. 9-2. Elimination of Xylose from Hydrolyzate by Several Xylose-assimilating Yeasts.

Samples were derived from the culture broths shown in Fig. 9-1. A: *Candida pulcherrima* NI 7477, B: *Candida parapsilosis* var. *komabaensis* K-75.

tion. The activities of both strains were almost the same.

Preparation of xylobiose

Actually, xylan (equivalent to 172 g of anhy-

droxylose) was hydrolyzed by 2400 g of the solution of *Streptomyces* xylanase according to the procedure described above. After the removal of insoluble fractions by centrifugation from the hydrolyzate, 2619 g of clear solution containing 200 g of total xylose was obtained. The solution was supplemented with nutrients, sterilized, inoculated with a seed culture of the yeasts, *C. parapsilosis*, and aerobically incubated at 30°C for 2.5 days. After the incubation, the yeast cells were discarded by centrifugation, thus, 2400 g of the supernatant solution containing 100.7 g of total xylose was obtained. The solution was decolorized with carbon, followed by deionization by treatment with the columns of cation (IR-200) and anion (IRA-68) exchange resins. The treated solution was concentrated under reduced pressure. Xylobiose (70.4 g) was crystallized from the concentrated solution at room temperature. The flow sheet for preparation of xylobiose is summarized in Fig. 10-1.

Properties of xylobiose prepared and its acetyl derivative

Crude crystal of xylobiose obtained was twice recrystallized from about 85% aqueous ethanol. The recrystallized xylobiose was paper-chromatographically pure, and 2.0 of D. P. which was determined by Willstätter-Schudel's method. The xylobiose was acetylated with anhydrous sodium acetate and acetic anhydride mixture according to the method of Whistler *et al.*,⁸⁾ and its acetyl derivative was recrystallized from ethanol. Their properties are summarized in Table I, in comparison with the data of Whistler *et al.*⁸⁾

TABLE I. SOME PROPERTIES OF XYLOBIOSE OBTAINED AND ITS HEXAACETATE

Arrows indicate mutarotatory property.

	Xylobiose		Xylobiose hexaacetate	
	Obtained here (Recrystallized)	From data of Whistler ^{1,2)}	Obtained here (Recrystallized)	From data of Whistler ⁸⁾
$[\alpha]_D^{25}$	-34.5 → -25.0 (c=2.0 in water)	-32.0 → -25.5 (c=1 in water)	-73.8 (c=1.0 in CHCl ₃)	-74.47 (c=0.9 in CHCl ₃)
Melting point (°C)	187~189	186~187	154.4~155.1	154~155

FIG. 10-1. Procedure and Mass Balance Sheet of Preparation of Xylobiose from Xylan Hydrolyzate Using *Candida parapsilosis* var. *komabaensis* K-75.

A*, B*, C* refer to ones in Fig. 10-2.

FIG. 10-2. Paper Chromatograms of Sugar Samples in Various Stages of Xylobiose Preparation.

X₁, authentic xylose; X₂, authentic xylobiose; A*, xylan hydrolyzate by the xylanase system of *Streptomyces* sp. E-86; B*, A* after treatment with *Candida parapsilosis* var. *komabaensis* K-75; C*, crude crystal of xylobiose; D*, xylobiose recrystallized from 85% ethanol. A*, B*, C*, in this figure correspond to A*, B*, C*, in Fig. 10-1.

DISCUSSION

Hydrolyzate of hardwood xylan by the *Streptomyces* xylanase system was composed of xylose and xylobiose in an approximate ratio of 1:1 with small amount of contaminants of other oligosacchrides. Xylanase system producing exclusively xylobiose from xylan have never been known. Hardwood xylan contains a small amount of glucuronide residue as a minor component in addition to a large amount of xylose residue (unpublished data),

therefore, a small of a glucuronide-containing oligosaccharides should be present in the hydrolyzate. However, these minor components did not interfere the preparation of crystalline xylobiose. For separation of sugars the chromatographical technique is very effective, therefore, microbiological method including the selective fermentation with certain yeast has little application. However, in the case of simple sugar composition, especially composed of only two sugars, the selective fermentation method may be very convenient for obtaining a special sugar in a relatively large quantity. In our case, selected yeasts had a satisfactory nature, that is, they could assimilate only xylose, but they had no assimilability against xylobiose at all. We could successfully adopt the characteristic nature of the *Streptomyces* xylanase system and the speciality in sugar assimilability of yeasts in this procedure.

REFERENCES

- 1) R. L. Whistler and C. C. Tu, *J. Am. Chem. Soc.*, **73**, 1389 (1951).
- 2) R. L. Whistler and C. C. Tu, *ibid.*, **74**, 3609 (1952).
- 3) I. Kusakabe, T. Yasui and T. Kobayashi, *Nippon Nôgeikagaku Kaishi*, **43**, 145 (1969).
- 4) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **72**, 677 (1950).
- 5) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).
- 6) R. Willstätter and G. Schudel, *Ber.*, **51**, 780 (1918).
- 7) I. Kusakabe, T. Yasui and T. Kobayashi, *J. Ferment. Technol.*, **53**, 135 (1975).
- 8) R. L. Whistler and C. C. Tu, *J. Am. Chem. Soc.*, **74**, 4334 (1952).