生物工程学报
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Chin J Biotech 2008, July 25; 24(7): 1221-1227
Chinese Journal of Biotechnology ISSN 1000-3061

研究报告

Production and Accumulation of Xylooligosaccharides with Long Chains by Growing Culture and Xylanase of a Mutant Strain of *Bacillus pumilus* X-6-19

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Abstract: *Bacillus pumilus* X-6-9 isolated from soil and subsequently identified, produced xylooligosaccharides with long chains from xylan and accumulated them in the culture. By improving the culture conditions and mutating the bacterium, a 3.2-fold increase in the production of the xylooligosaccharides was established, when compared to the original culture conditions of *B. pumilus* X-6-19. The addition of D-glucose to the culture of the mutant strain U-3 of *B. pumilus* X-6-9 repressed the synthesis of β-xylosidase, but not xylanase. Thus, it was revealed that strain U-3 was a good organism for the production and accumulation of xylooligosaccharides with long chains from xylan by a microbial culture. Xylanase produced by strain U-3 was purified to homogeneity and characterized. The hydrolyzates generated by the purified xylanase contained xylobiose, xylotriose, xylotetraose, and xylopentaose, but not xylose.

Keywords: xylooligosaccharides, mutant strain, xylanase, β-xylosidase

Xylooligosaccharides are used for moisturizing agents for food, sweeteners, specific health foods, etc. In particular, the use of xylooligosaccharides as a specific health food is effective for improving intestinal disorders, because xylooligosaccharides are resistant to digestion in the stomach and intestines and serve as growing factors for beneficial intestinal microflora such bifidobacteria[1–3]. It was reported that xylooligosaccharides with longer chains are more effective for growing bifidobacteria in the intestines and suppressing the growth of the *Bibrio* and *Clostridium* strains[4,5]. The production of xylooligosaccharides has been conducted by the hydrolysis of xylan with microbial enzymes[6–8] and the combination of xylanase with sulfuric acid[5], aqueous ammonia[9], or heat[10] treatment. In addition, the production of xylooligosaccharides using recombinant xylanase was recently reported[11].

On the other hand, the production of xylooligosaccharides from xylan by a microbial culture has several advantages, compared with their production using xylanase, because it is primarily no need to prepare and purify the enzyme. Although some purified xylanases may essentially act on xylan and its derivatives, microorganisms could use not only xylans but also another substances as carbon and energy sources and are expected to accumulate oligosaccharides from xylans. These processes for the production of xylooligosaccharides seem to be simple and energy saving, if an excellent microorganism is obtained. However, there have been no reports about the production of xylooligosaccharides by a microbial culture.

Thus we attempted to isolate the microorganisms that accumulate xylooligosaccharides with longer chains of xylotriose and over from xylan in the culture. We also examined the promotion of the xylooligosaccharide production using improved culture conditions and the mutation of the isolate, and analyzed the enzyme systems responsible for the xylan degradation.

1 Materials and Methods

1.1 Materials

Xylan (oat spelt) was purchased from Sigma, St. Louis, MO, USA; xylose, xylooligosaccharide mixtures, *N*-

Received: September 29, 2007; **Accepted:** March 13, 2008
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methyl-*N'*-nitro-*N*-nitrosoguanidine, and active carbon were from Wako Pure Chemicals, Osaka, Japan; *p*-nitrophenyl-β-D-xylopyranoside was from Nacalai Tesque, Kyoto, Japan; CM52 cellulose was from Whatman Chemical Separation, Clifton, NJ, USA; the TLC aluminum sheet (Silica Gel 60) was from Merck, Darmstadt, Germany; Bio Gel P-2 (Extra Fine) was from Bio-Rad, Richmond, VA, USA; and CM-Toyopearl 650S was from Tosoh Corporation, Tokyo, Japan.

1.2 Culture media

For the basal medium, two solutions were separately prepared. Solution A contained xylan, 1 g; yeast extract, 0.05 g; NH_4Cl, 0.2 g; KH_2PO_4, 0.4 g; NaCl, 0.2 g; and deionized water, 86 mL. The pH of solution A was adjusted to pH 7.0 with 0.1 mol/L NaOH. Solution B contained $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot 4 \sim 5H_2O$, 5 mg; and deionized water, 16 mL. Solutions A and B were separately autoclaved. The two solutions were then mixed at room temperature. The agar basal medium was prepared by adding 1.5%(*W*/*V*) agar to the basal medium.

1.3 Microorganisms

Strain X-6-19 isolated from soil and identified as *Bacillus pumilus* and its mutant, *B. pumilus* X-6-19 strain U-3, were used throughout this study.

1.4 DNA manipulations and sequencing

The total DNA of *B. pumilus* X-6-19 was prepared as previously reported[12]. The 16 S rRNA gene of the strain, corresponding to a region between positions 8 and 1542 in the gene of *Escherichia coli*, was amplified by the method reported by Edwards *et al*[13,14] and sequenced with a Shimadzu DSQ-2000L DNA sequencer (Shimadzu, Kyoto) after the sequencing reactions with the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences, Piscataway, NJ).

1.5 Mutation

Cells of *B. pumilus* X-6-19 were irradiated with UV light, or were treated with *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine. The mutants were selected, which made a large transparent zone around their colonies on the agar basal medium containing 0.25%(*W*/*V*) each of xylan, D-glucose, and sodium acetate trihydrate.

1.6 Analysis of oligosaccharides by column chromatography

Xylooligosaccharides produced in the culture of *B. pumilus* X-6-19 were analyzed by column chromatography, in addition to TLC. The culture fluid (100 mL) incubated in the basal medium for 24 h with shaking was centrifuged at 12 000×g and 4°C for 10 min. The supernatant was then evaporated to dryness. The residues were suspended in 200 mL of 80%(*V*/*V*) ethanol. The suspension was stirred at 70°C for 1 h and then the precipitates were removed by centrifugation at 12 000×g and 10°C for 10 min. The supernatant was evaporated to dryness. The residues were dissolved in 10 mL of deionized water. The solution was applied to a column (2 cm×15 cm) of active carbon. The column was washed with 150 mL of deionized water. The xylooligo-saccharides were eluted with a linear gradient (0%~80%, *V*/*V*) of 300 mL of ethanol; the xylooligosaccharides (reducing sugars) in the fractions were estimated by the method of Somogyi-Nelson using D-xylose as the marker. All the fractions containing the xylooligosaccharides were pooled and then the pooled solution was evaporated to dryness. The residues dissolved in 10 mL of 10%(*V*/*V*) ethanol were applied to a column (3.2 cm×62 cm) of Bio Gel P-2 (Extra Fine) equilibrated with 10%(*V*/*V*) ethanol. The xylooligosaccharides were eluted with 500 mL of 10%(*V*/*V*) ethanol; the xylooligosaccharides in the fractions were estimated as above and analyzed on a TLC plate.

1.7 Cultural conditions for xylooligosaccharide production

To the basal medium, ten carbon sources [1%(*W*/*V*) each at the final concentrations] including sugars, sugar alcohols, and organic acids and six nitrogen sources [0.2%(*W*/*V*) each] were added. Incubation with *B. pumilus* X-6-19 was carried out at 30°C with shaking. At an appropriate interval, 0.5 mL of the culture was harvested. The sample was then centrifuged at 12 000×g for 10 min. Reducing sugars in the supernatant were then estimated.

1.8 Production of enzymes

B. pumilus X-6-19 strain U-3 was incubated in 500 mL of basal medium containing 1%(*W*/*V*) sodium acetate trihydrate in the presence or absence of 1%(*W*/*V*) D-glucose at 30°C with shaking. At an appropriate interval, 50 mL of the culture was harvested. The sample was then centrifuged at 12 000×g for 10 min. The xylanase and β-xylosidase activities in the supernatant and precipitated fractions were assayed. The cell extracts from the precipitated fraction were prepared by disruption with a Kubota 201 mol/L ultrasonic oscillator (Kubota Shoji, Tokyo) at 180 W for 10 min and then centrifuging at 12 000×g and 4°C for 10 min.

1.9 Enzyme assay

The activity of xylanase was assayed at 30°C using oak spelt xylan as the substrate[14]. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugar(as D-xylose) per min. The specific activity was defined as units per mg of protein. Protein concentrations were measured by the method of Lowry *et al*[15].

The activity of β-xylosidase was assayed at 30°C using

p-nitrophenyl-β-D-xylopyranoside as the substrate[16]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min.

1.10 Purification of xylanase

Xylanase was purified from the culture fluid (1.3 liters) of *B. pumilus* X-6-9 strain U-3 by fractionation with $(NH_4)_2SO_4$ and chromatographies on CM52 cellulose and CM-Toyopearl 650S. All operations for the purification were carried out at 0~4°C. The purity of the enzyme was determined by polyacrylamide gel electrophoresis (PAGE).

1.11 Determination of molecular masses

The molecular mass of the native enzyme was measured by gel filtration, and that of the enzyme subunits was measured by SDS-PAGE[17]. Size markers used for the gel filtration were those from the calibration proteins gel chromatography kit made by Boehringer Mannheim, Mannheim, Germany. The electrophoresis calibration kit LMW (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as size markers for SDS-PAGE.

1.12 Determination of NH_2-terminal amino acid sequence

Purified xylanase was electroblotted using the method of Matsudaira[18] and then the NH_2-terminal amino acid sequence were determined by automated Edman degradation with a Shimadzu PPSQ-10 protein sequencer (Shimadzu).

1.13 Effects of temperature and pH on the activity and stability of the purified xylanase

The effect of temperature on the enzyme activity was determined at temperatures 20~80°C in 5°C increments. The thermostability was determined by measuring the remaining activity after the treatment for 10 min at temperatures from 20~80°C and at pH 8.0. The optimum pH was determined in the following 50 mmol/L buffers: sodium acetate (pH 4.0~5.5), sodium-potassium phosphate (pH 5.0~8.0), Tris-HCl (pH 7.0~9.5), and sodium carbonate (pH 9.0~11.0). The pH stability was determined by measuring the remaining activity after treatment for 24 h at 4°C between pH 4.0~11.0.

1.14 Nucleotide sequence accession number

The nucleotide sequence of a16S rRNA gene from *B. pumilus* X-6-19 reported in this paper has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AB212862.

2 Results

2.1 Taxonomy of strain X-6-19

Strain X-6-19 was a spore-forming rod of (0.7~0.9) × (3.5~4.2) μm and motile with peritrichous flagella. It was facultatively anaerobic, gram-positive, and catalase-and oxidase-negative. Acids were produced from eight sugars. The nucleotide sequence of a 16 S rRNA gene of the strain was 99.7% identical with *B. pumilus* TUT1009 (GenBank Accession no. AB098578). On the basis of these results, together with the description in Bergey's manual[19], we identified strain X-6-19 as *B. pumilus*.

2.2 Production of xylooligosaccharides by *B. pumilus* X-6-19

The xylooligosaccharides produced in the culture of *B. pumilus* X-6-19 were analyzed. As shown in Fig. 1, the xylooligosaccharides (xylose, xylobiose, xylotriose, and xylotetraose) were separated by chromatography on Bio gel P-2. Fraction V in the Fig. 1 probably contained xylopentaose and the other xylooligosaccharides with longer chains than xylopentaose. The overall recovery of the final xylooligosaccharide preparations from the culture fluid was 48%.

Since the yield of the xylooligosaccharides generated from xylan was 10% as D-xylose, we tried to increase the production of the xylooligosaccharides by *B. pumilus* X-6-19 in the basal medium containing various carbon and nitrogen sources. Among them, the addition of 1%(*W*/*V*) sodium acetate trihydrate to the basal medium produced a 1.5-fold increase in the production of reducing sugars from xylan, compared to that in the absence of the acetate. Chromatographic patterns of the xylooligosaccharides on TLC were essentially the same as those in Fig. 1. D-Glucose and lactose repressed the production of the xylooligosaccharides, indicating that the two sugars produced catabolite repression to the xylan metabolism of *B. pumilus* X-6-19. The other examined carbon and nitrogen sources had no effects on the production of the xylooligosaccharides in the bacterium.

2.3 Production of xylooligosaccharides by mutant strain U-3

Mutant strain U-3 obtained from *B. pumilus* X-6-19 by the UV light irradiation, produced a 3.2-fold increase in the production of reducing sugars from xylan on the basal medium containing 1%(*W*/*V*) each of xylan, D-glucose, and sodium acetate trihydrate after incubation for 36 h, when compared with that of the parent strain. At that time, D-glucose in the culture was completely consumed and the chromatographic patterns of the xylooligosaccharides on TLC were essentially the same as those in Fig. 1, although the amount of the xylose decreased. The overall recovery of the final xylooligosaccharide preparations from the culture fluid was 56%. The yield of the xylooligosaccharides generated from xylan increased to 32% as D-xylose.

The enzymes xylanase and β-xylosidase responsible for the xylan degradation were analyzed in the culture and cells of *B. pumilus* strain U-3. The activities of xylanase and β-xylosidase were exclusively observed in

the culture and cells, respectively. In Fig. 2, typical data show that the activity of β-xylosidase in the cells of the strain markedly decreased in the presence of D-glucose. On the other hand, that of xylanase was mostly unchanged.

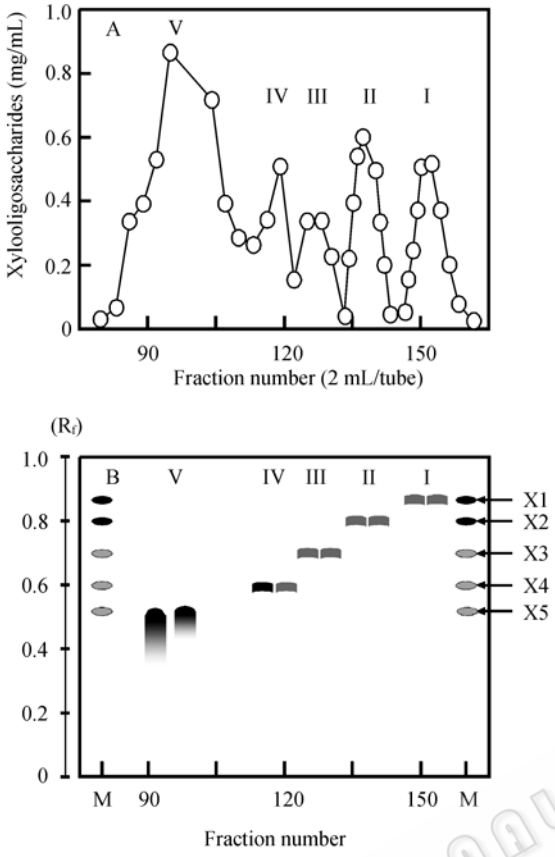

Fig. 1 Separation of xylooligosaccharides on Bio Gel P-2 (A) and TLC of the separated xylooligosaccharides (B)

A: the cultural fluid(100 mL) was dried on an evaporator and then the residues were extracted with ethanol. The extracts were then passed through activated charcoal. The fractions containing xylooligosaccharides were chromatographed on Bio Gel P-2. B: the separated xylooligosaccharides were detected on a TLC plates. See the text for details.

M: marker; X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose

2.4 Purification of xylanase

Table 1 is a summary of a typical enzyme purification for xylanase. The specific activity of the final preparation of the enzyme was 45 u/mg with an overall recovery of 3%. The final enzyme preparation had a 6.6-fold increase in the specific activity and showed a single protein band on a native polyacrylamide gel.

2.5 Molecular properties of the enzyme

The final preparations of xylanase showed a single protein band on a SDS-polyacrylamide gel and the apparent molecular mass was determined to be 26 kD. The apparent molecular mass of the native enzyme was 24 kD by gel filtration. These findings indicated that the enzyme was a monomer.

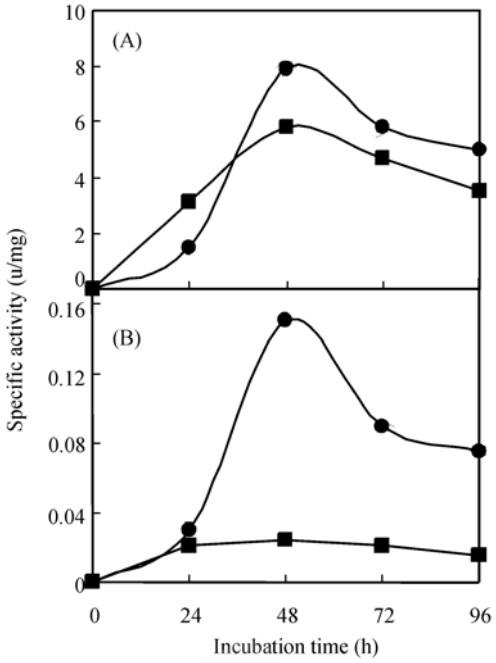

Fig. 2 Synthesis of xylanase (A) and β-xylosidase (B) in the presence (closed square) and absence (closed circle) of D-glucose

Table 1 Purification of xylanase from strain U-3

Fraction	Total activity (u)	Total protein (mg)	Specific activity (u/mg)	Recovery (%)
Supernatant	3200	470	6.8	100
Ammonium sulfate	1800	130	14	56
CM52	170	3.6	46	5.3
CM-Toyopearl 650S	100	2.2	45	3.0

2.6 Analysis of amino acid sequence

The NH_2-terminal amino acid sequence of the purified xylanase was RTITNNEMG.

2.7 Properties of the purified enzyme

The purified xylanase showed a maximum activity at pH 6.0 and 55°C. In addition, the enzyme exhibited more than 90% of the maximum activity between pH 5.0 and 7.5. It was stable between pH 5.0 and 9.5. The enzyme maintained more than a 60% activity up to 50°C. The K_m value of the enzyme was 14 mg xylan per mL.

2.8 Production of xylooligosaccharides by purified enzyme

The process of the hydrolysis of xylan with the purified xylanase was analyzed by TLC (Fig. 3). The amount of xylotriose generated from xylan markedly increased, as the incubation time was prolonged. Although the spots showing xylotetraose and xylopentaose were obscure on the TLC chromatogram, the two oligosaccharides were distinctly detected by HPLC (Fig.

4). Xylose was not observed on the both chromatograms.

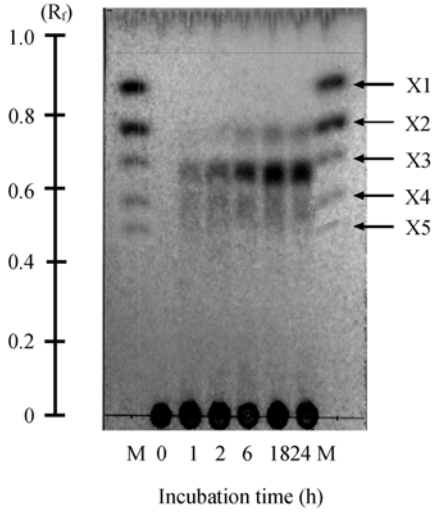

Fig. 3 TLC analysis of reaction products with the purified xylanase

The reaction mixture (7.5 mL) containing 13 μg of enzyme and 1% (*W*/*V*) xylan were incubated under the standard conditions. After incubation for 1, 2, 6, 18, and 24 h, 0.5 mL of sample was withdrawn and then applied to the TLC sheet. See the text for details.
M: marker; X1: xylose; X2: xylobiose; X3: xylotriose;
X4: xylotetraose; X5: xylopentaose

3 Discussion

In this study, we isolated *B. pumilus* X-6-19 that produced xylooligosaccharides with long chains from xylan and accumulated them in the culture. By improving the culture conditions and mutating the bacterium, a 3.2-fold increase in the production of the xylooligosaccharides was obtained, when compared to the original culture conditions of *B. pumilus* X-6-19. This is the first report describing the production and accumulation of xylooligosaccharides by a microbial culture.

Although the activity of the xylanase of the mutant *B. pumilus* X-6-19 strain U-3 was not very different in the presence and absence of D-glucose, that of β-xylosidase in the cells of the strain markedly decreased in the presence of D-glucose (Fig. 2). These results suggest that strain U-3 was resistant to the catabolite repression for the xylanase biosynthesis by D-glucose if the xylanase protein was not degraded during cultivation, but not for β-xylosidase. Since β-xylosidase has an exo-type activity for xylan and xylooligosaccharides, xylose may be formed during the process of xylan degradation, which will then be used as an energy and carbon source[20,21].

When xylooligosaccharides is produced by a microbial culture, the strain responsible for this manufacturing process requires carbon and energy sources for growth. If it exclusively utilizes xylan or produced xylo-oligosaccharides as carbon and energy sources, the conversion efficiency of xylooligosaccharides from xylan will be decreased. Because *B. pumilus* X-6-19 strain U-3 can grow on D-glucose as a carbon and energy source without the decrease of xylanase activity, it was favorable for the production of the xylooligo-saccharides. However, strain U-3 was not deficient of the β-xylosidase gene, but repressed the synthesis of the enzyme by D-glucose. Therefore, if a mutant deficient β-xylosidase gene is totally obtained, it will be possible to efficiently produce xylooligo- saccharides with long chains.

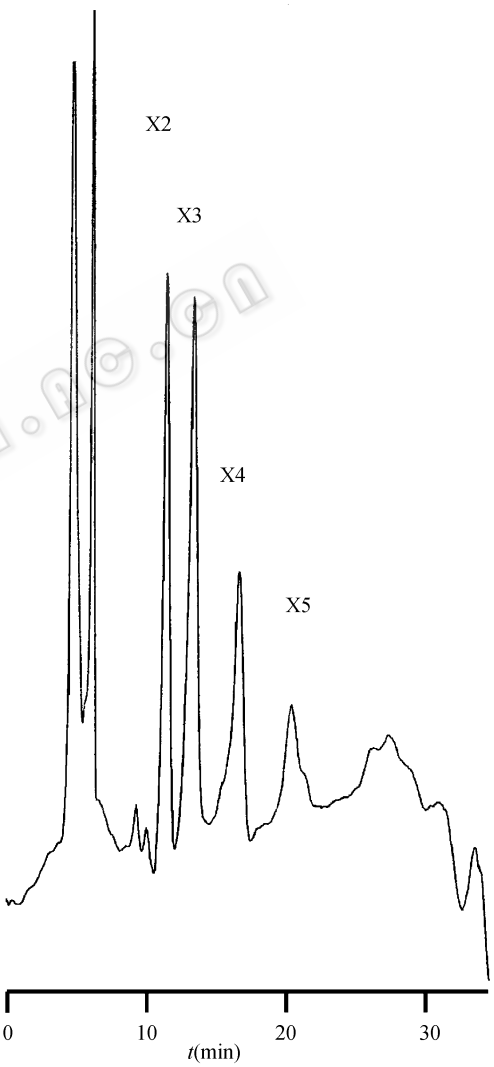

Fig. 4 HPLC analysis of reaction products with the purified xylanase

The enzyme reaction was conducted as described in the legends of Fig. 3. The reaction mixture incubated for 24 h was concentrated on a evaporator. The concentrated sample was analyzed under the following conditions: column, Shodex NH2P-50 4E (Shoko Co., Ltd., Tokyo); mobile phase, CH_3CN-H_2O (65:35, *V*/*V*); flow rate, 0.5 mL/min; and detection, refractive index.
X1: xylose; X2: xylobiose; X3: xylotriose;
X4: xylotetraose; X5: xylopentaose

We purified and characterized xylanase from *B. pumilus* X-6-19 strain U-3 for use as a catalyst for producing the xylooligosaccharides. The xylanase from strain U-3 retained its activity at a higher temperature than those from *B. pumilus* IPO[14] and *Trichoderma lignorum*[16]. In addition, the xylanase from *B. pumilus* X-6-19 strain U-3 showed an activity in a wider range of pH than that of the xylanase from *B. pumilus* IPO[14]. Therefore, although the amino acid sequence up to nine residues of xylanase from strain U-3 was identical to that of the enzyme from the IPO strain, the two xylanases probably have differences in their inner amino acid sequences.

When xylan was incubated with the purified xylanase from strain U-3, xylose was not detected in the reaction mixture (Figs. 3 and 4), while it was accumulated in the cultures of *B. pumilus* X-6-19 and its mutant strain U-3. The production of xylobiose by the xylanase decreased rather than that by the cultivation using *B. pumilus* X-6-19 (Figs. 1 and 3). Therefore, the use of the xylanase may be more advantageous for the production of the xylooligosaccharides with a higher molecular mass than in that for the cultivation of microorganisms. In addition, the xylooligosaccharide mixtures produced by the xylanase from *B. pumilus* X-6-19 strain U-3 contained more xylooligosaccharides with long chains than those produced by the xylanases from *B. pumilus* IPO[14] and *Trichoderma longibrachiatum*[6].

However, the fraction containing xylopentaose and the other xylooligosaccharides with longer chains than xylopentaose, was obtained from the culture fluid of *B. pumilus* X-6-19 strain U-3, as well as from that of the parent strain (Fig. 1), although the xylooligosaccharide mixtures produced by the xylanase from *B. pumilus* X-6-19 strain U-3 contained the low amount of xylopentaose (Figs. 3 and 4). In the purification of xylanase, we found the minor fraction showing a xylanase activity on the chromatography on CM52 cellulose, in addition to the major fraction, as mentioned above. Since the amount of the xylanase in the minor fraction was a little, it was difficult to purify and characterize. This xylanase may be involved in the production of xylopentaose and the other xylooligosaccharides with longer chains than xylopentaose.

For these reasons, *B. pumilus* X-6-19 strain U-3 and the xylanases synthesized by the strain seem to be good tools for the production of xylooligosaccharides with long chains. It will be possible to obtain the saccharides with much longer chains in a high yield by mutating this strain and these enzymes. The efforts for mutation of the strain and cloning of the genes encoding these xylanases are now in progress.

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