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Purification and Characterization of *Clostridium thermocellum* Xylanase from Recombinant *Escherichia coli*

KOO, BON-JOON*, HWA-GYUN OH, KI-HAENG CHO, CHANG-KUN YANG, KYUNG-HWA JUNG[1], AND DAI-YOUNG RYU

Department of Fermentation, R&D Center of TS Corp. 6-14, 1 Ka, Buksung-Dong, Chung-Gu, Inchon, Korea,
[1]Korea Reseach Institute of Bioscience and Biotechnology, KIST, P.O.Box 115, Yusong, Taejon, Korea

The *xyln*X gene encoding a xylanase from *Clostridium thermocellum* ATCC27405 was cloned in the plasmid pJH27, an *E. coli-Bacillus* shuttle vector and the resultant recombinant plasmid, pJX18 was transformed into *E. coli* HB101. The overexpressed xylanase was found to be secreted into the periplasmic space of the recombinant *E. coli* cells. The crude enzyme was obtained by treating the *E. coli* cells with lysozyme, and purified by DEAE-Sepharose column chromatography. Molecular wieght of the xylanase was estimated to be 53 kDa by gel filtration. The pI value was determined to be pH 8.8. The N-terminal sequence of the enzyme protein was Asp-Asp-Asn-Asn-Ala-Asn-Leu-Val-Ser-Asn which was considered to be the sequence of that of the mature form protein. The Km value of the enzyme for oat spelt xylan was calculated to be 2.63 mg/ml and the Vmax value was 0.47 μmole/min. The xylanase had a pH optimum for its activity at pH 5.4 and a temperature optimum at 60°C. The enzyme hydrolyzed xylan into xylooligosaccharides which were composed mainly of xylobiose (40%) and xyloltriose (12%) after 5 hour reaction. This result indicates that the xylanase from *C. thermocellum* ATCC27405 is an endo-acting enzyme.

Xylan is a major component of hemicelluolse, and the second most abundant polysaccharide next to cellulose in nature (18). It is found in large amounts in the cell walls of plants. The great biodegradative capabilities of microorganisms are now being exploited to expand the use of this biomass resource. The enzymatic process for the industrial hydrolysis of xylan has advantages over chemical reactions in many respects such as the high specificity of the enzyme, the mildness of the reaction conditions, and the absence of substrate loss caused by chemical modification etc (13, 14).

Many biodegradative microorganisms including bacteria and fungi utilize xylan as a carbon and energy source, provided they produce necessary xylanolytic enzymes such as endo-xylanases, β-xylosidases, α-glucuronidase, α-arabinofuranosidase and esterases. Thus, these organisms are contributing to the maintenance of the carbon cycle of this biomass in nature.

Amomg the enzyme described above, the two xylanolytic enzymes, xylanase and β-xylosidase are directly involved in the hydrolysis of the characteristic backbone of xylans which consist of β-1,4 linked D-xylosyl residues (2, 20).

Recently, we cloned a xylanase gene (*xylnX*) from *Clostridium thermocellum* ATCC27405 which was distinct from other xylanases so far cloned, and its nucleotide sequence was determined (7). It was found that the *xylnX* gene was on the 3.3 kb fragment of the cloned chromosomal DNA and deletion experiments showed that deletion of 1.7 kb from the 3' end of the insert DNA increased the xylanolytic activity. Therefore, the 1.6 kb fragment of the 5' terminal side of the original insert DNA was subcloned into pJH27, *E. coli-Bacillus* shuttle vector and the resultant plasmid was named as pJX18 (Fig. 1). The present work describes overproduction, purification and characterization of the xylanase from *E. coli* transformants.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli HB101 (F^- *hsd*S20 (r_B^-, m_B^-) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 (st^r) *xyl*1-5 *mtl*-1 *sup*E44λ^-) was used as the host for the overexpression of the xylanase.

Plasmid pJH27 is an *E. coli-Bacillus* shuttle vector which has the strong promoter (BJ27) screened from the chromosomal DNA of *B. subtilis* 168 (8) and pJX18 is a

*Corresponding author
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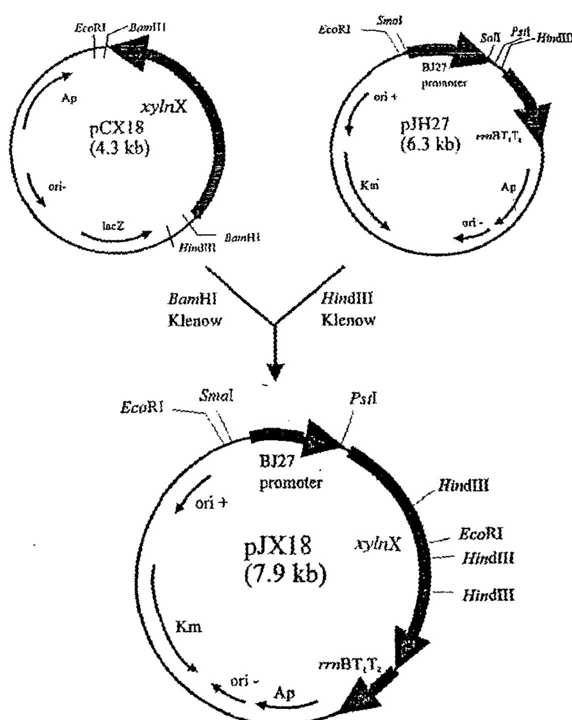

Fig. 1. Construction of pJX18.
A *Bam*HI 1.6 kb fragment of *xylnX* from pCX18 was inserted to the *E. coli-Bacillus* shuttle vector, pJH27. ori+, replication origin for *Bacillus subtilis*; ori-, replication origin for *E. coli*.

pJH27 derivative containing 1.6 kb *xylnX* gene fragment from *C. thermocellum* ATCC27405.

Materials and Reagents

Xylose, oat spelts xylan, TEMED, SDS and lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-sepharose CL-6B and Superdex 75 HR 10/30 were from Pharmacia Co., and molecular weight standard protein from Bio-Rad Co.

Enzyme Assay

The xylanase activities were determined by measuring the increase of reducing sugars released from hydrolysis of xylan substrate. One ml of reaction mixtures containing 1% (W/V) xylan and appropriately diluted enzyme solution in 50 mM sodium citrate buffer (pH 5.4) were incubated for 15 minutes at 60°C. After removal of solids by centrifugation, 3 ml of DNS reagent was added, and then the tubes were placed in boiling water for 5 minutes. After cooling with tap water, absorbance was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of xylose equivalent per minute.

Determination of Protein Concentration

Concentratin of protein was determined by Bradford's method (3). One ml of Bradford working buffer (100 mg Coomassie brilliant blue G-250, 50 ml 95% ethanol and 100 ml 85% phosphoric acid per liter) was added to 5-10 μl of protein solution along with 100 μl of the experimental buffer. After 3 min, A_{570} was measured. Bovine serum albumin (BSA) was used as a standard protein.

Purification Procedure

E. coli HB101 carrying the pJX18 plasmid was grown in 3 liters of LB medium containing 25 μg/ml ampicillin. Cells were harvested by centrifugation for 15 min at 6,000×g and washed with 100 ml of STE buffer (20% sucrose, 30 mM Tris-HCl, 1 mM EDTA, pH 8.0). The periplasmic fraction was obtained by the treatment of 100 ml of lysozyme (1 mg/ml) in STE buffer at 0°C for 10 min and centrifugation for 10 min at 4°C and 12,000×g. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and then the dialyzed sample was applied on a DEAE-sepharose column (150 ml) equilibrated with sodium phosphate buffer (pH 7.0). The column was eluted with stepwise gradient from 50 mM to 500 mM KCl in the same buffer at a flow rate of 5 ml/min and the fraction volume was 10 ml.

Determination of Molecular Weight

The molecular weight of the native xylanase was determined by FPLC using Superdex 75 HR 10/30 which was a kind of gel filtration column. The flow rate was 0.1 ml/min. Protein peaks were monitored as absorbance at 280 nm.

SDS-polyacrylamide Gel Electrophoresis of Proteins

Protein samples were analyzed by electrophoresis in 10% (W/V) polyacrylamide gel containing 0.1% (W/V) SDS as described by Laemmli (9). Proteins were stained with Coomassie brilliant blue R-250.

In order to detect xylanase activity, the customary SDS-PAGE of the samples was simultaneously carried out on the same polyacrylamide gels containing 0.5% oat spelts xylan. After electrophoresis, sodium dodecyl sulfate (SDS) was removed by washing the gel two times in 50 mM sodium citrate buffer (pH 5.4) for 4 h at room temperature. Subsequently, the gel was stained by 1% (W/V) Congo red solution for 30 min and then washed with 1 M NaCl solution.

Analysis of Xylan Hydrolysis Products

Hydrolysis products of xylan by the purified xylanase were analyzed by high pressure liquid chromatography (HPLC) using a carbohydrate analysis column (Waters Co.) with 78% acetonitrile as the eluent at a flow rate of 1.2 ml/min. For the identification of xylooligomers, 10% solution of commercial xylooligosaccharide-70 (Suntory Ltd.) was used as standard solution.

RESULTS AND DISCUSSION

Overexpression of the Xylanase Gene

The *E. coli* HB101 harboring pJX18 showed growth-

associated xylanase production during the course of culturing in LB medium at 37°C (Fig. 2). The maximum xylanase activity in the sonicated culture broth was 19.2 units/ml. The growth curve of the *E. coli* transformant was similar to that of the reference strain, HB101. This result indicates that the overexpression of xylanase from the cloned *xylnX* gene did not affect growth of the host *E. coli* cells. Thus, this recombinant *E. coli* strain was proved to be suitable for the industrial process. Most xylanase activity was detected in the periplasmic space (data not shown), demonstrating that the signal peptide of the *C. thermocellum* xylanase had effectively functioned in the Gram-nagative *E. coli* host cells.

Secreting into the periplasmic space has also made the enzyme purification steps be simpler.

Purification of Xylanase from the Recombinant *E. coli*

The xylanase was purified to homogeneity as judged by SDS-PAGE (Fig. 3). There were multiple bands showing the xylanase activity on the gel loaded with crude lysate sample from the recombinant *E. coli* cells (Fig. 3, lane D). The multiple banding may be due to protein aggregation or protein degradation, and it is also possible that some of the xylanases detected in the culture broth were precursors carrying the peptide signal sequence (19).

Elution patterns of protein and xylanase activity during the DEAE-Sepharose chromatography were shown in Fig. 4.

The overall steps of the purification procedures and the results obtained were summarized in Table 1. After

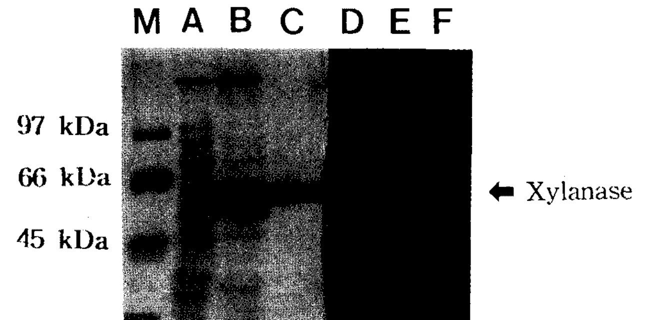

Fig. 3. SDS-PAGE (A, B, C) and activity staining (D, E, F) of the fractions from each purification step.
SDS-PAGE was performed on a 10% polyacrylamide gel. Lane M, protein molecular weight markers; lane A, crude lysate of the cell; lane B, crude extract from periplasmic fraction; lane C, pooled fractions from DEAE-sepharose column. Lane D, E, F showed the xylanase-activity bands that matched lane A, B, C, respectively.

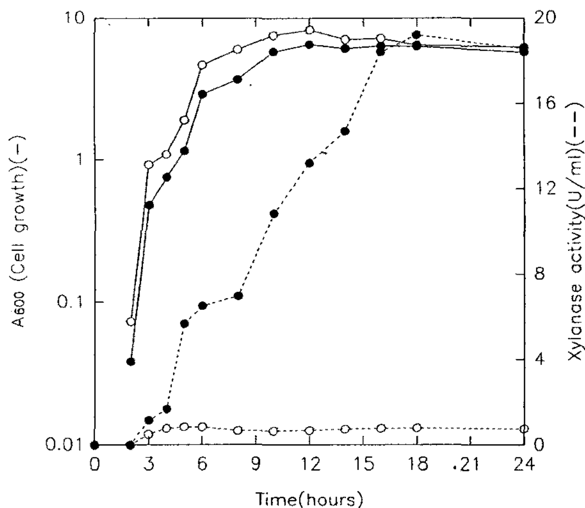

Fig. 2. Xylanase activity (··) and cell growth (—) of *E. coli* transformants.
E. coli HB101 harboring pJX18 (●) grown in LB media containing 50 μg/ml ampicillin was compared with *E. coli* HB101, wild type (○) growin in LB media.

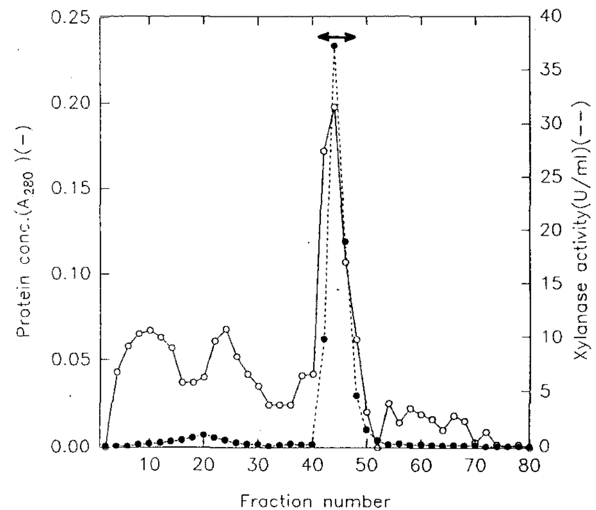

Fig. 4. DEAE-sepharose chromatography of xylanase during its purification.
Protein concentration was measured at 280 nm (○) and xylanase activity (●) at 550 nm. Pooled fractions were indicated as arrow.

Table 1. Summary of Purification steps of Xylanase from *E. coli* HB101 harboring pJX18.

Purification step	Total Protein (mg)	Total Activity (Unit)	Specific Activity (Unit/mg)	Yield (%)	Purification Folds
Cell (soluble protein)	92.7	2684	28.9	100	1.0
Lysozyme treatment	8.9	1726	193.7	64.3	6.7
DEAE-sepharose	3.4	850.4	247.7	31.7	8.6

purification, specific activity was increased by 8.6 fold, and the yield was 31.7% of original activity.

Physicochemical Properties of Xylanase

Apparent molecular weight of the xylanase determined by SDS-PAGE was estimated to be about 55 kDa. Gel filtration analysis showed that the native xylanase was a monomer of 53 kDa (Fig. 5).

The isoelectric focusing using the Phast sytem (Bio-Rad Co.) showed that the pI value of the xylanase was pH 8.8, indicating that the enzyme was a basic protein (Fig. 6).

N-terminal analysis of the purified xylanase was performed by the Edman degradation method (10) using a Milligen 6600B protein sequencer at Korea Basic Science Institute. *N*-terminal sequence of xylanase was Asp-Asp-Asn-Asn-Ala-Asn-Leu-Val-Ser-Asn which was supposed to be the sequence of the mature form of this enzyme (7). Whereas, some xylanases are reported to be produced as the precursor forms (4, 5).

Enzyme Kinetics

The kinetic constants of purified xylanase were determined under the assay conditions of pH 5.4 and 60°C. The apparent Km value for oat spelts xylan was 2.63 mg/ml calculated by using the Lineweaver-Burk method (Fig. 7). The value of maximal velocity (V_{max}) was 0.47 µmole/min.

Effect of pH and Temperature on the Enzymatic Activity

The xylanolytic activity of the purified xylanase toward oat spelt xylan was measured from pH 3.0 to 10.0 using various buffers and at various temperatures at pH 5.4 (Fig. 8). The maximum activity was observed at

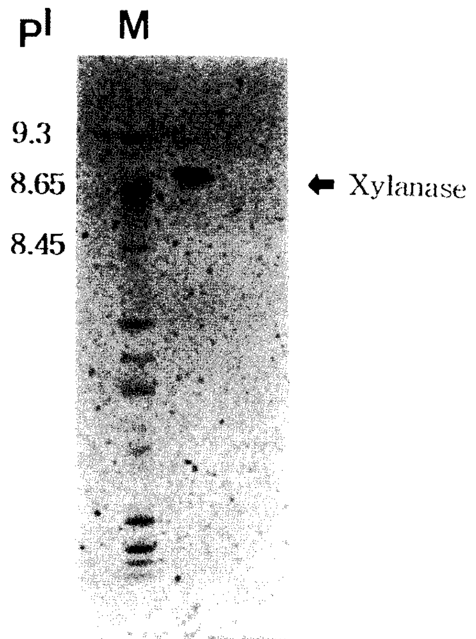

Fig. 6. Isoelectric focusing of xylanase purified from *E. coli* transformant.

This work was carried out on PhastGel IEF3-9 at 75 Vh and then at 15 Vh.; lane M, isoelectric focusing standards. The isoelectric point of the standard protein was represented by pH number; Xylanase was indicated by arrow.

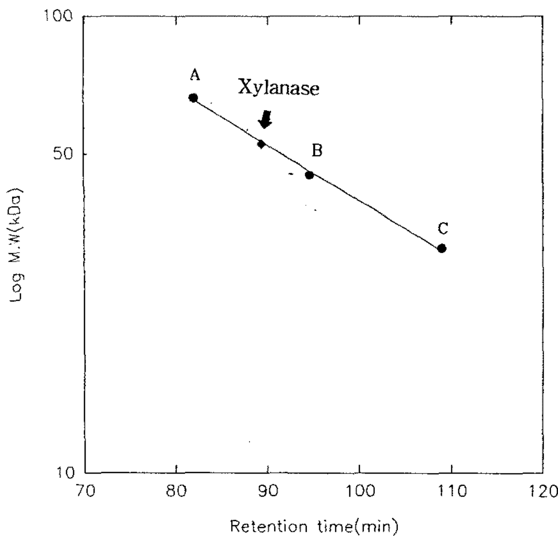

Fig. 5. Molecular weight estimation of xylanase by gel filtration.

Superdex 75 HR 10/30 in FPLC system was used. The standard proteins used were as follows; A, bovine serum albumin (66 kDa); B, ovalbumin (45 kDa); C, carbonic anhydrase (31 kDa).

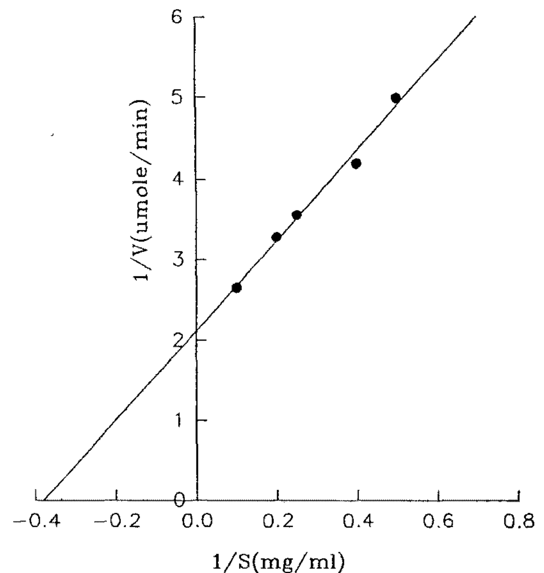

Fig. 7. Lineweaver-burk plot for the xylanase with oat spelts xylan as substrate.

Enzyme reaction was measured at 60°C in 2-10 min interval with various concentrations of substrate until the maximum velocity was reached.

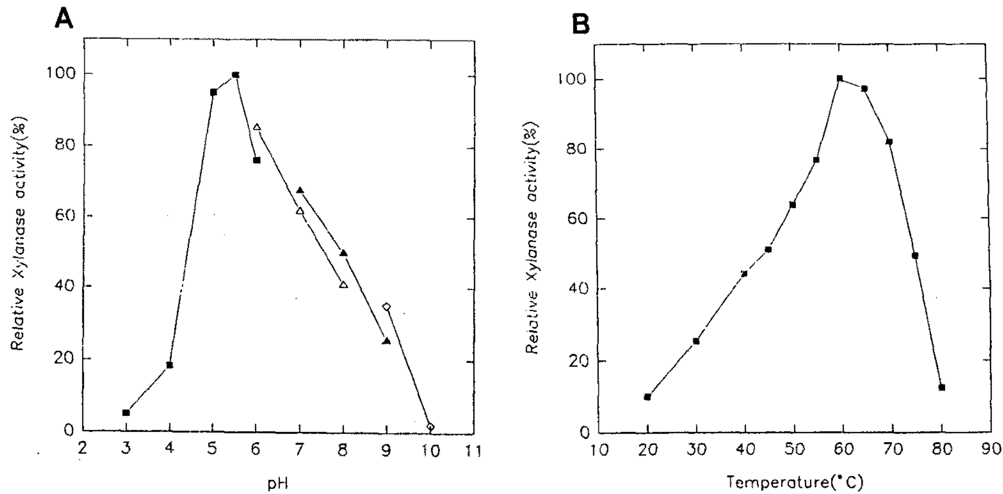

Fig. 8. Effects of pH (A) and temperature (B) on the activity of the purified xylanase.
The enzyme was first assayed at various buffers: 50 mM sodium citrate buffer (■) at pH 3.5-6, phosphate buffer (△) at pH 6-8, Tris buffer (▲) at pH 7-9, and glycine buffer (◇) at pH 9-10. Relative activity was determined relative to the enzyme activity at pH 5.4 and at 60°C.

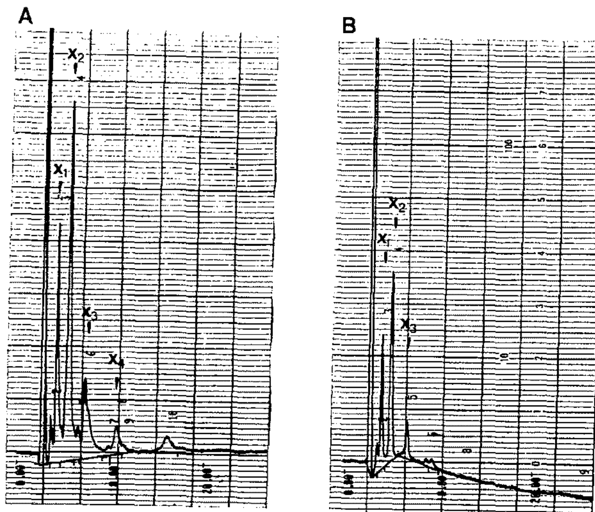

Fig. 9. HPLC anaylsis of hydrolysate after xylanase reaction.
Periodically taken reaction mixture, carried out at 60°C, was boiled and pretreated with Sep-Pak catridge. 10 μl of sample was separated on carbohydrate analysis column (Waters Co.) using 78% acetonitrile as eluent. (A) Mixed standard of 10% xylooligosaccaharide (Suntory Ltd), (B) Hydrolysate of xylan reaction carried out with 5 U/ml xylanase after 5 h. X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetrose.

60°C and pH 5.4. The xylanase was found to be highly heat-stable (data not shown).

Thermal stability is a highly desirable characteristic for the industrial enzymes because it permits enzyme recycling to be possible and also makes enzyme handling and storage be easer (1, 6, 12).

Enzymatic Production from Xylan

The HPLC analysis showed that the levels of xylobiose (X_2) and xylotriose (X_3) in the hydrolyzate increased to 45% and 12%, respectively when xylan was hydrolyzed for 5 h with 5 units of xylanase per ml of the reaction mixture (Fig. 9). The result indicates that the xylanase is an endo-acting enzyme which is very useful for industrial applications.

The high proportions of xylobiose and xylotriose in the hydrolysis products of xylan is an encoraging result, because these two xylooligomers are of high value sugars in food industry (15-17). Especially, xylobiose is known to be a selective growth stimulant to the intestinal *Bifidobacteria* that are regarded to be beneficial microorganisms for the maintenance of healthy intestinal microflora (11). Therefore, the xylanase can be effectively used for production of xylobiose from xylan biomass which can be used as a food enriching material.

The characteristics of the *Clostridium* xylanase are judged to be suitable for industrial utilization of the enzyme in producing xylooligomers from xylan, and further studies on the improvement of the hydrolysis yield, immobilization of the enzyme, and development of an efficient new type of bioreactor are desirable to be carried out.

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