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Production of Xylooligosaccharides from Xylans by Extracellular Xylanases from *Thermobifida fusca*

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Xylooligosaccharides are produced for use as a valuable food sweetener or additive. They have many beneficial biomedical and health effects. In this study, a process for producing xylooligosaccharides from lignocellulolytic agricultural waste was developed. Bagasse, corncob, wheat bran, and peanut shell were used as carbon sources for production of xylanolytic enzymes from *Thermobifida fusca* NTU22. When using bagasse as the carbon source, the xylanolytic enzymes that simultaneously accumulated in the broth in a 500 mL Hinton flask after 72 h of cultivation at 50 °C were measured as xylanase (14.0 U/mL), β-xylosidase (74.1 mU/mL), and acetyl esterase (29.1 mU/mL). The optimum pH and temperature for xylanases were 6.0−8.0 and 70 °C, respectively. Six proteins with xylanase activity were identified by zymogram analysis of isoelectric focusing gel. This was followed by heat treatment at 70 °C for 30 min that eliminated 90% of the β-xylosidase activity. The xylanase and acetyl esterase activities were still 100%. Two percent of xylan extracted from the bagasse was then hydrolyzed by heat-treated crude xylanase preparation at 60 °C, pH 7.0, for 10 h. The xylooligosaccharides that accumulated in the broth were about 23.7%. After the purification process by activated charcoal chromatography, the purity of xylooligosaccharides was 71.4%.

KEYWORDS: ***Thermobifida fusca*; xylooligosaccharide; xylanase; β-xylosidase; acetyl esterase; xylan**

INTRODUCTION

Plant cell walls are a major reservoir of fixed carbon sources in nature. They have three major polymers: cellulose (30−45%), hemicellulose (25−45%), and lignin (15−30%) (*1*). Hemicellulose is a collective name for a group of branched heteropolysaccharides. Xylans, the most abundant of the hemicellulose of land plants, are β-1,4-linked polymers of xylopyranosyl units with a degree of polymerization ranging from 70 to 200 (*2*, *3*). In most xylans, the xylan backbone carries acetyl and arabinofuranosyl side chains in varying proportions.

For most hydrolysis processing, xylan must be converted to xylose or xylooligosaccharides. They may be achieved by acid hydrolysis or through the use of xylanolytic enzymes. The xylanolytic enzymes include endo-β-1,4-xylanase (xylanase), β-xylosidase, and debranching enzymes (acetyl esterase). Xylanase catalyzes the random hydrolysis of xylan to xylooligosaccharides while β-xylosidase releases xylose residues from nonreducing ends of xylooligosaccharides. However, a complete degradation requires the synergistic action of acetyl esterase to remove the acetyl substituents from the β-1,4-linked D-XYLOSE BACKBONE OF XYLAN (*4*, *5*).

Xylooligosaccharides with degrees of polymerization from 2 to 5 xylose residues have been produced for use as a valuable food sweetener or additive (*6*). A xylooligosaccharide-containing diet can improve the gastrointestinal health of rats and can increase resistance of mice toward infection by *Clostridium difficile* (*7*, *8*). Imaizumi et al. found that a xylooligosaccharide-based diet reduced the blood concentrations of sugars and lipids of diabetic rats (*9*). In relation to human health, xylooligosaccharides selectively enhance the growth of bifidobacterium, thus promoting a favorable intestinal environment (*10*, *11*).

Bagasse, corncob, wheat bran, and peanut shell are important lignocellulolytic agricultural waste in the world. They are important biomass resources because of some benefits including abundance, ready availability, and low value. These lignocelluloses can also be used as carbon sources for the growth of microorganisms and can induce the production of lignocellulolytic enzymes. To produce enzymes for the development of enzymatic degradation of renewable lignocellulose, we isolated a potent extracellular lignocellulolytic enzyme-producing thermophilic actinomycete, *Thermobifida fusca* NTU22, from compost soils collected in Taiwan (*12*). The xylanase gene from *Thermobifida fusca* was cloned and expressed in *Escherichia coli*, *Streptomyces lividans*, and *Pichia pastoris* (*13*, *14*).

In the present paper, we evaluate the xylanolytic enzymes obtained from the *T. fusca* NTU22 on the lignocellulolytic agricultural waste and the xylooligosaccharides accumulated from the xylans hydrolyzed by xylanolytic enzymes. The methods of recovering the xylooligosaccharides are also discussed.

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MATERIALS AND METHODS

Chemicals. Czapek-dox powder, yeast extract, casamino acids, and agar were purchased from Difco (Detroit, MI). Xylobiose, xylotriose, and xylotetrose were purchased from Megazyme (Bray, Wicklow, Ireland). Inorganic salts and all other chemicals were purchased from Sigma (St. Louis, MO). Bagasse sample was collected from Taiwan Sugar Company. Corncob, wheat bran, and peanut shell samples were all collected from a local market. Lignocellulolytic samples were washed thoroughly with tap water to remove the dust and then were air-dried. These were chopped and milled to 100-mesh size using a Cyclone Mill (Tecator AB, Hoganas, Sweden).

Alkaline Extraction of Xylan. The milled lignocellulolytic samples were soaked in 4% (w/v) NaOH and then were steamed at 100 °C for 3 h. After alkaline treatment, the supernatant was recovered by centrifugation (5000*g*, 20 min) and was acidified with 12 N HCl to pH 5.0. Then, 1.5-fold volume 95% ethanol was added to precipitate the xylan. After centrifugation, the xylan was dried. Subsequently, it was milled to 100-mesh, using a Cyclone Mill (*15*).

Microorganism and Growth Conditions. A thermophilic actinomycete, *Thermobifida fusca* NTU22, which was isolated from compost soils collected in Taiwan, was used in this study (*12*). The stock culture was maintained on a CYC plate consisting of 33.3 g Czapek-dox broth, 2.0 g yeast extract, 6.0 g casamino acid, and 15.0 g agar per liter of distilled water (pH 7.3). They were kept at 4 °C and were transferred monthly. The microorganism was initially cultured in 50 mL of CYC medium in a 500 mL Hinton flask. Cultivation was carried out on a reciprocal shaker at 125 rpm and 50 °C for 24 h. The culture broth was used as the seed culture.

Preparation of Crude Xylanase. For investigation of various substrate effects on extracellular xylanase production, aliquots of 2.5 mL of the seed culture were transferred to a 500 mL Hinton flask loaded with 50 mL of the xylanase-producing medium consisting of 20 g lignocellulose, 4 g yeast extract, 4 g ammonium sulfate, 13.6 g KH_2PO_4, 0.2 g $MgCl_2.6H_2O$, and 11.0 mg $CaCl_2$ per liter of distilled water (pH 9.0). After the fermentation was complete, the culture broth was centrifuged at 10 000*g* for 30 min at 4 °C, and finally the supernatant was used as crude xylanases preparation (*13*).

Enzymatic Hydrolysis of Xylan. The reaction mixture contained 2% (w/v) xylan, appropriately diluted crude enzyme preparation, and 100 mM sodium-phosphate buffer (pH 7.0). After incubation at 60 °C for 24 h, the reaction was stopped by chilling the mixture on ice followed by centrifugation at 3000*g* for 1 min. The amount of xylose or xylooligosaccharides produced in the mixture was determined by high-performance liquid chromatography (HPLC). The xylooligosaccharide yield (w/w) was (xylobiose + xylotriose + xylotetrose)/pentosan weight.

Purification of Xylooligosaccharides. After enzymatic hydrolysis, the supernatant was recovered by centrifugation at 8000*g* for 30 min. The xylooligosaccharides were adsorbed by the activated charcoal powder that was packed in the column (26 × 400 mm). The activated charcoal powder was then washed twice with pure water and then the xylooligosaccharide syrup was eluted with 30% ethanol (*16*). The purity of xylooligosaccharides syrup was (xylobiose + xylotriose + xylotetrose)/total sugar weight.

Enzyme Activities. Xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan (*17*). One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmole D-XYLOSE PER MIN AT 50 °C. ACETYL ESTERASE AND B-XYLOSIDASE ACTIVITIES WERE ASSAYED AS DESCRIBED BY KODEMI ET AL. (*18*) AND TUNCER ET AL. (*19*). THE SUBSTRATES USED WERE *P*-NITROPHENYL ACETATE AND *P*-NITROPHENYL-B-D-xylopyranoside, respectively. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmole *p*-nitrophenol per min at 50 °C. The isoelectric focusing (IEF) gel electrophoresis of xylanases was described by Bachmann and McCarthy (*20*).

Xylooligosaccharides Assays. The enzymatic hydrolytic products of xylanase from xylan were determined by high-performance liquid chromatography (Shimadzu LC-3A, Kyoto, Japan) using a Lichrospher NH_2 (250 × 4.5 mm) column (MetaChem Technologies Inc., Torrance, CA) and refractive-index detector. A mobile phase composed of acetonitrile and water (70:30, v/v) was used as the eluant at a flow rate of 1.0 mL/min, and xylose, xylobiose, xylotriose, and xylotetrose were used as standards.

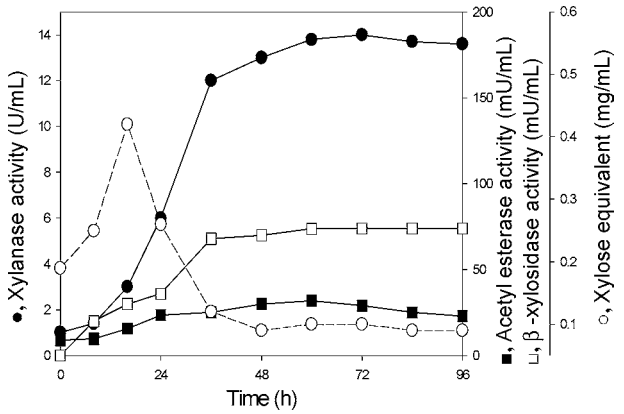

Figure 1. Time course for production of extracellular xylanolytic enzymes in a 500 mL Hinton flask by *T. fusca* NTU22. Cells were grown aerobically in a 500 mL Hinton flask loaded with 50 mL of medium consisting of 2% bagasse, 0.4% ammonium nitrate, 0.05% yeast extract, and 0.68% potassium phosphate, pH 9.0, and were incubated at 50 °C, 125 rpm for 96 h.

Reducing Sugar and Total Sugar Assays. The reducing sugar in the samples was directly determined by the dinitrosalicyclic acid method (*21*). The total sugar was determined by the same method after refluxing the sample in 7% H_2SO_4 for 2 h to hydrolyze xylan into monosaccharides. The pentosan content of the xylan was determined by phloroglucinol method (*22*).

Statistical Analysis. All analytic measurements were performed at least in triplicate.

RESULTS AND DISCUSSION

T. fusca NTU22 was capable of growth in a basal salts-yeast extract medium containing bagasse, corncob, wheat bran, and peanut shell. However, production of extracellular xylanolytic enzymes including xylanases, β-xylosidase, and acetyl esterase by *T. fusca* NTU22 was found to vary between the different carbon sources. A typical growth curve and enzyme production by *T. fusca* NTU22 in basal salts-yeast extract media containing 2.0% (w/v) bagasse is shown in **Figure 1**. The accumulation of reducing sugar increased slowly from 0.19 to 0.42 mg/mL by 16 h. The rapid consumption of the reducing sugars was parallel with an increase in production of extracellular xylanolytic enzymes during the growth phase of the culture. This indicates that the production of extracellular xylanolytic enzymes involved in lignocellulose degradation is growth associated in *T. fusca* NTU22 when it is grown in a bagasse medium. It is similar to the results from Tuncer et al. (*19*). After 72 h cultivation, the xylanolytic enzyme activities that simultaneously accumulated in the 500 mL Hinton flask were xylanases (14.0 U/mL), β-xylosidase (74.1 mU/mL), and acetyl esterase (29.1 mU/mL). Similar growth curves and enzyme production patterns were observed for *T. fusca* NTU22 grown in all other carbon sources. The highest extracellular xylanase activity was found when *T. fusca* NTU22 was grown in a medium containing bagasse (14.0 U/mL) (**Table 1**). Corncob was a poor inducer with a maximum production of xylanases of only 1.31 U/mL. Ghosh and Deb described the production of xylanases from black rot fungus *Thielaviopsis basicola* on different carbon sources. They found that the peanut shell had the lowest xylanase production compared to corncob, rice, straw, and xylan (*23*). This is different from our results using *T. fusca* NTU22.

Table 1. The Xylanolytic Enzymes Produced by *T. Fusca* NTU22 Incubated in Various Lignocellulolytic Agricultural Wastes[b]

	substrates			
enzyme activity[a]	corncob	bagasse	wheat bran	peanut shell
xylanase (U/mL)	1.31	14.00	6.68	5.19
β-xylosidase (mU/mL)	2.40	74.10	18.40	30.00
acetyl esterase (mU/mL)	15.00	29.10	32.00	42.40

[a] Basal medium: 2% carbon source, 0.4% NH_4NO_3, 0.05% yeast extract, 0.68% KH_2PO_4, pH 9.0. [b] Cells were grown aerobically in a 500 mL Hinton flask loaded with 50 mL of a basal medium containing various concentration of substrates and were incubated at 50 °C 125 rpm for 72 h.

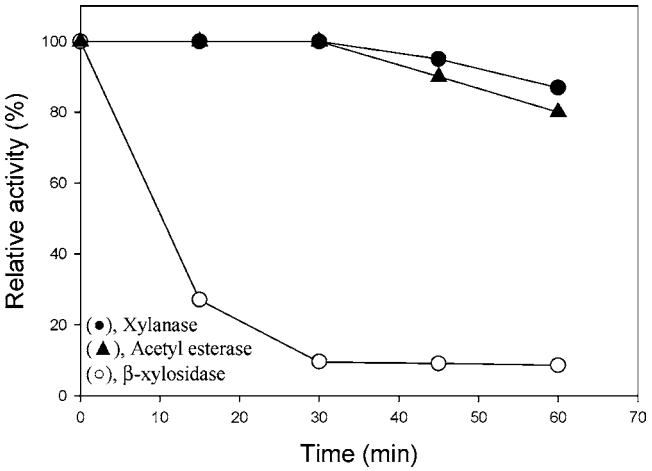

Figure 2. Thermal stability of xylanase, acetyl esterase, and β-xylosidase from *T. fusca* NTU22 at 70 °C.

The enzyme productions by different microorganisms from the same carbon sources were variable.

The optimum pH and temperature for xylanases were 6.0−8.0 and 70 °C, respectively. About 70% of the original activity still remained at 70 °C for 3 h. A total of six bands of xylanase activities (pI 5.6, 6.5, 7.4, 8.45, 8.65, 9.30) were identified by release of remazol brilliant blue from RBB-xylan on isoelectric focusing gel. They are similar with the results from *T. fusca* MT100 (*24*).

It is necessary to remove the acetic acid moieties from acetylxylan before hydrolysis of the substrate by xylanase. Acetyl esterases remove acetyl groups from a wide variety of substrates carrying acetyl substitutions. The acetyl esterase might not be identical to acetylxylan esterase, which is capable of cleaving the acetyl ester bonds in xylan. Determining whether the acetyl esterase is an acetylxylan esterase or not needs more evidence (*20*).

β-Xylosidase can hydrolyze xylooligosaccharides to xylose. To produce xylooligosaccharides, it is necessary to eliminate the activity of β-xylosidase. Previously, research decreased the β-xylosidase activity by mutagenesis or purification of xylanase (*25*−*27*). Comparing the thermostability between xylanases, acetyl esterase, and β-xylosidase from *T. fusca* NTU22, we found that the xylanase and acetyl esterase have better thermostability than β-xylosidase. After incubation at 70 °C for 30 min, the β-xylosidase activity retained was less than 10% but the xylanase and acetyl esterase activity was still 100% (**Figure 2**). In this study, we simply treated the crude enzymes at 70 °C for 30 min to eliminate the β-xylosidase activity.

The enzymatic hydrolysis conditions for production of xylooligosaccharides were investigated. Two percent of oat-spelt xylan was hydrolyzed by 10 U/mL of heat-treated crude

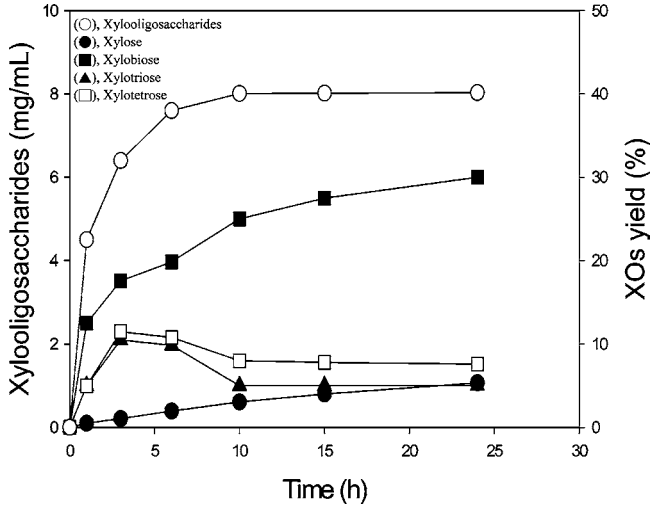

Figure 3. Time course for production of xylooligosaccharides from hydrolysis of oat-spelt xylan. The reaction mixture consisted of 2% oat-spelt xylan, phosphate buffer (50 mM, pH 7.0), and 10 U/mL heat-treated crude xylanase preparation (xylanase 10 U/mL, β-xylosidase 3.5 mU/mL, acetyl esterase 20.8 mU/mL). The reaction conditions were as follows: reaction volume, 50 mL; shaking speed, 100 rpm; temperature, 60 °C; reaction time, 24 h.

xylanase preparation (xylanase 10 U/mL, β-xylosidase 3.5 mU/mL, acetyl esterase 20.8 mU/mL) at 60 °C and pH 7.0 for 24 h. As shown in **Figure 3**, the accumulation of xylobiose, xylotriose, and xylotetrose increased rapidly during the initial 3 h. After 3 h incubation, the xylotriose and xylotetrose decreased. The maximum xylooligosaccharide yield was achieved at 10 h. After 10 h incubation, the xylooligosaccharides that had accumulated in the broth were about 40.1% containing 61% xylobiose, 13% xylotriose, and 26% xylotetrose. The residual β-xylosidase activity in the enzyme preparation may increase shorter fragments of xylooligosaccharide products. There was 0.28 mg/mL xylose equivalent retained in the broth. Comparing with the xylooligosaccharides produced by the crude xylanase preparation that were not heat-treated (xylanase 10 U/mL, β-xylosidase 53 mU/mL, acetyl esterase 20.8 mU/ mL), the xylooligosaccharides that accumulated in the broth were about 14.1%. There was 4.52 mg/mL xylose equivalent retained in the broth.

The xylans extracted from bagasse, corncob, wheat bran, and peanut shell were used as the substrates hydrolyzed by 10 U/mL of heat-treated crude xylanase preparation (xylanase 10 U/mL, β-xylosidase 3.5 mU/mL, acetyl esterase 20.8 mU/mL) at 60 °C and pH 7.0 for 10 h. As shown in **Table 2**, the corncob and bagasse xylans had better xylooligosaccharide yields.

Physical access to xylosic linkage in xylan is restricted by the surrounding lignocellulosic components as well as by the substituents on its backbone. The raw materials are conventionally pretreated before enzymatic degradation (*28*). There are several pretreatment methods that can expose the polysaccharide components to enzymatic hydrolysis, including the alkaline extraction method, acidic treatment, and cooking. However, the acidic pretreatment is not suitable for the production of xylooligosaccharides because it may produce a lot of xylose in the hydrolysate (*29*). The modified alkaline extraction method as described in the Materials and Methods was used in this study. According to the results in **Table 3**, the wheat bran had the highest extraction rate (18.5%), but the pentosan content was low. The extraction rates of bagasse and corncob are 15.7% and 12.5%, respectively, and the pentosan content was over 60%

Table 2. Production of Xylooligosaccharides from Various Xylans by the Heat-Treated Crude Xylanases Preparation from *T. Fusca* NTU22

substrate	hydrolysates (mg/mL)				
	X1	X2	X3	X4	XO yield (%)[a]
OSX[c]	0.61	4.70	1.32	1.99	40.1
CX	0.61	3.57	0.34	1.99	29.5
BX	0.57	3.71	0.21	0.82	23.7
WBX	0.39	3.42	1.14	N.D.[b]	7.6
PSX	0.85	3.94	3.66	N.D.	10.1

[a] The reaction mixture consisted of 2% xylan, phosphate buffer (50 mM, pH 7.0), and 10 U/mL heat-treated crude xylanases preparation (xylanase 10 U/mL, β-xylosidase 3.5 mU/mL, acetyl esterase 20.8 mU/mL). The reaction conditions were as follows: reaction volume, 50 mL; shaking speed, 100 rpm; temperature, 60 °C; reaction time, 24 h. [b] N.D.: not detected. [c] OSX, oat-spelt xylan (Sigma); CX, corncob xylan; BX, bagasse xylan; WBX, wheat bran xylan; PSX, peanut shell xylan.

Table 3. Extraction of Xylan from Various Lignocellulolytic Agricultural Wastes by Alkaline Extraction Method

source	xylan (%)	pentosan (%)
corncob	12.5	68.0
bagasse	15.7	62.0
wheat bran	18.5	28.0
peanut shell	3.5	21.0
oat-spelt xylan		60.0

(62% and 68%). These rates are similar to the commercial product sold on the market.

Xylooligosaccharides were then purified by activated charcoal chromatography. One gram of activated charcoal could adsorb 35.1 mg of xylooligosaccharides. The xylose and xylooligosaccharides adsorbed on the activated charcoal could be eluted by water and 30% ethanol, respectively. The recovery of xylooligosaccharides was about 95% and the purity was about 71.4%. It is higher than the commercial xylooligosaccharides syrup (70%) sold on the market.

In this process, we used lignocellulolytic agricultural waste to induce *T. fusca* NTU22 for production of the xylanolytic enzymes. The β-xylosidase activity can be simply eliminated by heat treatment at 70 °C for 30 min. The heat-treated crude xylanase preparation can be used directly to hydrolyze the xylans and to produce the xylooligosaccharides. It is not necessary to purify the xylanases further. As a result, the cost of the enzyme preparation may be reduced.

In summary, the xylanolytic enzymes including xylanase, β-xylosidase, and acetyl esterase were simultaneously accumulated in the broth when using bagasse as a carbon source. We simply treated the crude enzymes at 70 °C for 30 min, which we found can eliminate 90% of the β-xylosidase activity. This means that the heat-treated crude xylanase preparation can successfully be used for the production of xylooligosaccharides.

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