Corncob-Induced *endo*-1,4-β-D-Xylanase of *Aspergillus oryzae* MTCC 5154: Production and Characterization of Xylobiose from Glucuronoxylan

Ayyappan Appukuttan Aachary and Siddalingaiya Gurudutt Prapulla*

Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore 570 020, Karnataka, India

Eight different fungi were cultivated in a peptone−yeast extract medium containing 1% oat spelt xylan (OSX) to evaluate *endo*-1,4-β-xylanase secretion for xylooligosaccharide (XOS) production. *Aspergillus oryzae* MTCC 5154, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus ochraceus* showed significant titers of endoxylanases, which were further used for the production of XOS from birch wood xylan (BWX). *A. oryzae* produced 89.5 ± 1.13% XOS in the hydrolysate at 24 h of reaction. The effect of OSX, BWX, and raw corncob on the induction of endoxylanase in *A. oryzae* was studied, and the xylanase activity was maximum at 96 h of cultivation in 3% corncob containing medium. XOS produced at 36 h of reaction was 5.87 ± 0.53 mg/mL (12 ± 2% xylose, 48 ± 2.43% xylobiose, and 40 ± 3.6% higher oligomers) from 1% BWX . HPLC/refractive index detection and ESI/MS analysis of fractions obtained by GPC corresponded to neutral and 4-*O*-methyl-α-D-glucuronic acid substituted acidic oligosaccharides. The major fraction, β-D-xylopyranosyl-(1→4)-D-xylanopyranose was characterized using ^{13}C NMR.

KEYWORDS: *Aspergillus oryzae* MTCC 5154; *endo*-1,4-β-xylanase; corncob; xylooligosaccharides; xylobiose; ^{13}C NMR

INTRODUCTION

Xylan, a β-1,4-linked polymer of xylose, is the most abundant hemicellulose in the majority of plants. The β-1,4-linked D-xylosyl backbone of xylan is decorated through an α-1,2 linkage with glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid, whereas α-L-arabinofuranosyl residues are linked through α-1,3 to the polymer (*1, 2*). 1,4-β-D-Xylan xylanohydrolase (EC 3.2.1.8), generally known as endoxylanase, hydrolyzes β-1,4-xylosisdic bonds within these polysaccharide backbones, producing β-anomeric xylooligosaccharides consisting of 4-*O*-methyl-α-D-glucuronic acid residues. These oligosaccharides are a class of functional food ingredients generally regarded as prebiotic (*3*). In addition, these oligosaccharides often possess a more unique structural feature or unusual property that is of special value to the biological entity (*4*).

Xylooligosaccharides (XOS) find potential applications in a variety of fields, including pharmaceuticals, feed formulations, and food industries. In the food industry, XOS can be used as soluble dietary fiber since it is not metabolized by the human digestive system. XOS has diverse beneficial health effects such as revitalizing the growth of intestinal bifidobacteria, immunity activation, and noncariogenicity. XOS exhibits a water retention capacity and antifreezing property (*5*) and a variety of biological properties (*6*). XOS has acceptable organoleptic properties and

does not exhibit toxicity or negative effects on human health (*7*). The preferred degree of the polymerization range of XOS is 2−4 for food related applications (*8*). The sweetness of xylobiose is equivalent to 30% that of sucrose, and the sweetness of higher XOS is moderate and possesses no off taste. XOS, as a low calorie sweetner, finds use in an anti-obesity diet (*9*).

The production of XOS on an industrial scale is carried out from lignocellulosic materials (LCMs) rich in xylan. The strategies for the production of XOS from LCMs are grouped into three categories: enzyme treatment of native xylan containing LCMs, chemical fractionization of suitable LCMs, followed by enzymatic hydrolysis for the production of XOS and hydrolytic degradation of xylan to XOS by steam, water, or dilute mineral acids (*10*). However, alkaline extraction and acidic pretreatment are not suitable for the production of XOS because the former causes corrosion of the equipment used as well as alkali pollution and the latter produces an excess of xylose in the hydrolysate (*11*). Enzymatic production of XOS using endoxylanase is preferred in the food industry because of the lack of undesirable side reactions and products.

endo-1,4- β-Xylanases generally known as endoxylanases are the key enzyme components of microbial xylanolytic systems that hydrolyze β-1,4-xylosidic linkages of the xylan backbone to XOS of various chain lengths. A number of microorganisms, including bacteria, yeasts, and filamentous fungi, have been reported to produce xylanases (*12*). They are involved in the production of xylose, a primary carbon source for cell metabo-

---

* Corresponding author. Phone: +91-821-2515792; fax: +91-821-2517233; e-mail: prapullasg@yahoo.co.in.

lism and in plant cell infection by plant pathogens. The microbial xylanases have been used commercially in the pulp and paper industry for the past several years. Apart from this, they also are used as food additives to poultry; in wheat flour for improving dough handling and quality of baked products; in the extraction of coffee, plant oils, and starch; and in combination with cellulases and pectinases for clarification of fruit juices and degumming of plant fibers such as flax, hemp, and jute. Xylanases from fungi have been well-documented and studied (*13*), and there are also reports on its use for the production of XOS (*14, 15*).

For commercial applications, the production of xylanase from simple and inexpensive substrates is economical. Abundantly available agro-residues such as corncobs, wheat bran, wheat straw, and rice straw as substrates are an obvious choice. Corncobs are an important byproduct of the corn industry that are used either as animal feed or returned to the harvested fields. In recent years, interest in the microbial utilization of food processing waste into value-added products has increased. The xylan content of corncob is about 35% (*11*) and can be used as a carbon source for various microorganisms for their growth and the production of food grade enzymes.

In the present study, eight different fungal strains were evaluated for the production of endoxylanases, which were used further for the production of XOS by the hydrolysis of birch wood xylan (BWX), 4-*O*-methyl-α-D-glucurono xylan. The study also focused on the production of endoxylanase by a selected fungus *Aspergillus oryzae* MTCC 5154 using three different xylan sources including corncobs under submerged cultivation conditions and the evaluation of these endoxylanases for XOS production. Product characterization using HPLC, ESI/MS, and NMR also is discussed.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Standards of xylose, xylobiose, arabinose, glucose, oat spelt xylan (OSX), and BWX were from Sigma-Aldrich. Corncobs were obtained from a local maize field in Mysore, Karnataka, India and were dried, powdered (60−80 mesh), and stored in polycarbonate containers.

Microorganisms and Culture Conditions. *A. oryzae* MTCC 5154, *Aspergillus ochraceus* MTCC 1877, and *Penicillium citrinum* MTCC 2553 were from MTCC, IMTECH, Chandigarh, India; *Trichoderma harzianum* ATCC 42459 was from ATCC; and *Aspergillus flavus*, *Aspergillus niger*, *Aureobasidium pullulans* CFR 77, and *Trichoderma viridae* were from the type Culture Collection of CFTRI, Mysore, India. All the strains were maintained on potato dextrose agar (Himedia Laboratories Ltd.) slants except for *A. ochraceus* and *P. citrinum*, which were maintained on malt extract agar and Czepadox yeast extract agar (Himedia Laboratories Ltd.) slants, respectively, at 4 °C.

Evaluation of Different Fungi for Production of Endoxylanase. A few fungal strains were evaluated for their endoxylanase activity under submerged cultivation conditions. A loopful of spores from 5 day old slants of the fungi were inoculated into 50 mL of cultivation medium (*16*) containing 1% OSX, 0.5% yeast extract, 0.1% NaNO₃, 0.1% KH₂PO₄, 0.1% peptone, and 0.03% MgSO₄·7H₂O with an initial pH of 5.5. The flasks were incubated at 30 ± 1 °C on a rotary shaker (Emenvee Rotary Shaker, 48N3, Pune, India) up to 144 h. Sampling was performed at regular intervals of 24 h. Experiments were carried out in duplicates. At the end of respective periods of cultivation, the cultures were centrifuged (4 °C, 5000*g*) using a refrigerated centrifuge (Remi cooling centrifuge, C-30, Mumbai, India), and the supernatant was used as the source of endoxylanase and carboxy methyl cellulase (CMCase) without further purification. The pH of the culture fluids (CF) was recorded.

Production of Endoxylanase by *A. oryzae* MTCC 5154. The effect of different sources of xylan such as BWX (1%), OSX (1%), and raw corncob powder (3%) on the production of endoxylanase, using the selected organism *A. oryzae* MTCC 5154 under submerged conditions, was studied. The other media components were similar to those mentioned previously. A total of 0.5 mL of spore suspension containing 6 × 10⁶ spores/mL was inoculated into the media. Growth parameters were maintained as mentioned previously, cultivation was carried out up to 144 h, and the activity was monitored at 24 h intervals. Experiments were carried out in duplicate. The CFs were centrifuged as described previously. The endoxylanase activity, CMCase activity, total cellulase activity, and protein (*17*) content of the CFs were estimated.

Production of XOS from BWX Using Selected Strains of Fungi. XOS production was carried out from standard BWX using endoxylanase obtained by submerged cultivation of *A. oryzae* MTCC 5154, *A. flavus*, *A. niger*, and *A. ochraceus*, which showed significant xylanase activity. The reaction was carried out in a 250 mL conical flask containing 50 mL of reaction mixture, consisting of 0.5 g of BWX and CF corresponding to 100 units (U) of endoxylanase, to a total volume of 50 mL using citrate phosphate buffer (0.05 M, pH 5.4). The enzymatic reaction was carried out in a shaking water bath maintained at 50 °C for 24−36 h. At the end of the specified incubation time, the reaction was stopped by keeping the reaction mixture in a boiling water bath for 10−15 min.

Production of XOS by *A. oryzae* MTCC 5154. XOS production was carried out using CFs obtained after the submerged cultivation of *A. oryzae* MTCC 5154 using BWX, OSX, and corncobs. The CF exhibiting the maximum endoxylanase activity was selected for the reaction. The reaction parameters were maintained as mentioned previously. The reaction was carried out for 36 h with 6 h interval sampling and profiling of products.

Congo Red Staining Method for Detection of Endoxylanase Activity. The endoxylanase activity was detected by the Congo Red staining method (*18*). A loopful spore of each of the eight strains of fungi was inoculated separately onto Potato Dextrose plates containing 1.5% agar (w/v) and 0.5% OSX (w/v) at 30 °C for 5 days. The agar plates were flooded with 0.1% Congo Red (w/v) in water for 15 min. The Congo Red solution was then poured off, and the plate was further washed with 1 M HCl, to arrest the enzyme activity.

Enzyme Assays. Endoxylanase activity of CFs was assayed using standard BWX as the substrate (*19*). A total of 0.9 mL of 1% (w/v) BWX in 0.05 M, pH 5.4 citrate phosphate buffer and 0.1 mL of suitably diluted enzyme solution were incubated at 50 °C in a water bath (Haake SWB 20, Haake, Germany) for 10 min. The amount of reducing sugars liberated was measured by the DNS method (*20*). One unit of endoxylanase activity is defined as the amount of enzyme required to produce 1 μmol of xylose per minute at 50 °C and pH 5.4.

The CMCase activity of CF was assayed using standard carboxy methyl cellulose as the substrate (*21*). A total of 1 mL of 1% (w/v) carboxymethyl cellulose in 0.05 M, pH 4.8 citrate buffer and 0.5 mL of suitably diluted enzyme solution was incubated at 50 °C in a water bath (Haake SWB 20, Haake, Germany) at 100 rpm for 30 min. The amount of reducing sugar that was liberated was measured by the DNS method (*20*). One unit of CMCase activity is defined as the amount of enzyme required to produce 1 μmol of glucose per minute at 50 °C at pH 4.8. The total cellulase as filter paper activity (FPA) was measured according to Wood and Bhat (*21*). One international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose equivalents.

Analysis of XOS Using HPLC. XOS was estimated according to the method of Jeong et al. (*22*). The reaction mixture was filtered through a cellulose nitrate membrane (0.45 μm) to remove the residual xylan. The products of hydrolysis were analyzed by HPLC LC-6A (Shimadzu, Japan) equipped with a refractive index detector RID 6A (Shimadzu, Japan) using an aminopropyl column (250 mm × 4.6 mm SS Excil amino 5 μm (SGE)). Twenty microliters of the hydrolysate was injected using a HPLC injector syringe (Hamilton), and the analysis was carried out at room temperature (26 ± 2 °C) using acetonitrile/water (75:25) as the mobile phase at a flow rate of 1.0 mL/min. Data acquisition was carried out by the AIMIL Chromatography Data Station (AIMIL, New Delhi, India) and processed on a computer using WINACDS software (AIMIL, New Delhi, India). In the preliminary experiments on the evaluation of eight different fungal strains, the final

Corncob-Induced Xylanase for Xylobiose Production

J. Agric. Food Chem., Vol. 56, No. 11, 2008 **3983**

XOS yield was expressed as the percentage of the total sugars in the hydrolysate. In further experiments, the XOS formed was quantified by comparing the peak area of XOS with the standard peak area of xylose and is expressed as milligrams per milliliter of hydrolysate.

Purification of XOS Using GPC. A total of 100 mL of XOS mixture was prepared as described previously and was concentrated to 5 mL by lyophilization and then filtered through a cellulose nitrate membrane (0.45 μm) and centrifuged. A total of 0.5 mL of this sample was loaded onto a gel filtration column (100 cm × 1 cm) packed with BioGel P-2 (BioRad Laboratories). Elution was carried out at room temperature (26 ± 2 °C) using triply distilled water at a flow rate of 6 mL/h, and 1 mL fractions were collected. The fractions were analyzed by HPLC as mentioned previously. The major fractions were pooled, lyophilized, and used for ESI/MS and NMR studies.

ESI/MS of XOS. ESI/MS analysis was carried out on a Waters platform ZMD 4000 system composed of a Micromass ZMD spectrophotometer, a Waters 2690 HPLC instrument, and a Waters 996 photodiode array detector (Waters Corporation). Data were collected and processed via a personal computer running Mass Lynx software version 3.1 (Micromass, a division of Waters Corporation). The ESI/MS analysis of XOS was performed using underivatized samples (*23*). Positive ion mode ESI/MS was acquired, setting the needle voltage at 3000 V with an ion source at 80 °C and desolvation temperature at 150 °C maintaining the cone voltage at 30 °C. ESI/MS was performed using argon as the cone gas (50 L/h) and hydrogen as the desolvation gas (50 L/h).The tandem mass spectra of molecular ions were obtained using collision-induced dissociation (CID). The electron spray probe flow was adjusted to 70 mL/min. Each spectrum was produced by accumulating data during 1−2 min. The hydrolysate was filtered through 0.45 μm cellulose nitrate membranes to remove the unreacted xylan and was further used for the analysis. The major fraction obtained in GPC also was analyzed. Mass spectra of pure xylose and xylobiose also were recorded.

^{13}C NMR Spectrometry of Xylobiose. The NMR spectrum was recorded at 27 °C using a Bruker AQS 500 MHz instrument. Approximately 10 mg of dried sample of xylobiose (major fraction) was dissolved in D_2O (99.996%), and ^{13}C chemical shifts were measured relative to the internal deuterium nucleus. The spectra were obtained at 125 MHz. The typical acquisition parameters for 1-D ^{13}C NMR were a 90° pulse of 12 μs, a typical spectral width of 26 455 Hz, and a repetition time of 4 s. The repetition time was long enough to obtain quantitative data.

RESULTS

Evaluation of Xylanase Production by Different Fungi. All eight organisms studied tested positive for endoxylanase by the Congo Red staining method. However, the zone of hydrolysis was very small in the case of *Aureobasidium pullulans*, *T. viridae*, and *Trichoderma harzianum*. The endoxylanase activity and CMCase activity of the CF obtained by these strains under submerged cultivation were measured. The xylanase activities were estimated, and the pH was recorded during the course of 144 h of cultivation. Among the organisms evaluated for xylanase production, *A. oryzae* MTCC 5154, *A. flavus*, *A. niger*, and *A. ochraceus* were found to produce a high titer of extracellular endoxylanase.

***A. oryzae* MTCC 5154.** Among the eight fungal strains studied, the endoxylanase activity was higher in *A. oryzae* MTCC 5154 as shown in **Table 1**. The pH of CF increased from an initial value of 5.5 to 7.67 by 24 h and reached a maximum of 8.08 at 96 h of cultivation, and thereafter, it decreased (data not shown). The maximum endoxylanase activity was observed at 96 h of cultivation, beyond which it decreased with an increase in cultivation time. The extracellular CMCase activity at 96 h of cultivation was negligible (0.103 ± 0.03 U/mL/min)

The extracellular xylanase activity was 55.8 ± 0.53 U/mL/min at 96 h of cultivation, and the resultant XOS produced using

Table 1. Evaluation of Fungi for the Production of Endoxylanase Activity: Maximum Xylanase Activity and Corresponding CMCase Activity

fungus	cultivation time (h)	xylanase activity[a] (U/mL/min)	CMCase activity[a] (U/mL/min)
A. oryzae	96	55.8 ± 0.53	0.103 ± 0.03
A. flavus	72	34.05 ± 0.56	0.094 ± 0.03
A. ochraceus	96	39.93 ± 2.27	0.090 ± 0.01
A. niger	72	40.97 ± 0.55	0.087 ± 0.04
Au. pullulans	72	1.09 ± 0.01	0.066 ± 0.02
P. citrinum	72	12.30 ± 0.13	0.069 ± 0.02
T. viridae	96	0.20 ± 0.02	0.059 ± 0.03
T. harzianum	72	0.16 ± 0.01	0.068 ± 0.01

[a] Mean and SD for $n = 2$.

Table 2. Evaluation of Selected Strains of Fungi for XOS Production

	24 h reaction		36 h reaction	
	xylooligosaccharides[a]			
fungus	% xylobiose	total XOS (%)	% xylobiose	total XOS (%)
A. oryzae	62.57 ± 1.83	89.5 ± 1.13	67.10 ± 0.84	71.7 ± 1.15
A. flavus	53.01 ± 1.56	79.0 ± 1.12	49.4 ± 1.13	79.3 ± 1.13
A. ochraceus	19.95 ± 1.19	83.92 ± 2.24	24.82 ± 2.00	81.04 ± 0.59
A. niger	32.45 ± 1.41	71.81 ± 1.56	36.82 ± 1.30	68.40 ± 0.70

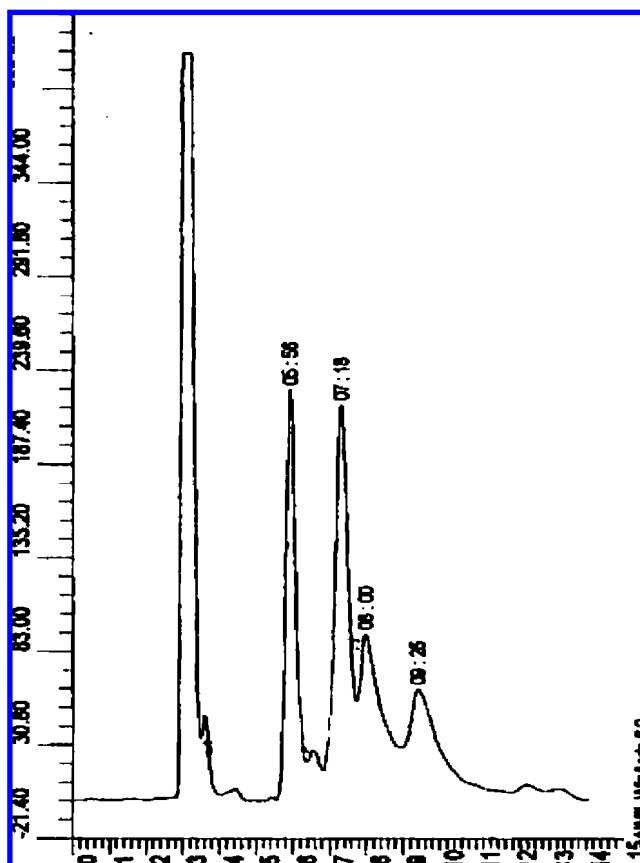[a] Values are mean and SD for $n = 2$.

Figure 1. Typical HPLC chromatogram of XOS from BWX. *X*-axis: retention time (RT) in minutes and *Y*-axis: millivolt area of peaks. The sugars corresponding to RTs 05:56, 07:18, 08:00, and 09:25 are xylose, xylobiose, xylotriose, and xylotetraose, respectively.

this xylanase was 89.5 and 71.7% at 24 and 36 h of reaction, respectively, at 50 °C (**Table 2**). Among the eight fungi studied, the maximum percentage of XOS in the hydrolysate was obtained from *A. oryzae* endoxylanase. The xylobiose in the hydrolysate was 62.57 ± 1.83% after 24 h of reaction, and it

Table 3. Xylanase and CMCase Activity of CF Obtained from Three Different Media by *A. oryzae* MTCC 5154 under Submerged Cultivation Conditions[a]

| | activity (U/mL/min) | | | | | |
| | media containing 3% raw corncob | | media containing 1% BWX | | media containing 1% OSX | |
FT (h)	xylanase	CMCase	xylanase	CMCase	xylanase	CMCase
24	32.3 ± 4.24	0.207 ± 0.07	3.12 ± 0.28	ND[c]	3.56 ± 0.28	ND[c]
48	60.2 ± 3.46	0.201 ± 0.12	35.72 ± 1.48	0.111 ± 0.08	16.56 ± 1.10	0.036 ± 0.01
72	71.3 ± 3.39	0.179 ± 0.05	54.98 ± 0.91	0.173 ± 0.06	24.01 ± 1.41	0.063 ± 0.03
96	81.1 ± 3.74	0.120 ± 0.10	62.78 ± 2.46	0.151 ± 0.06	76.73 ± 0.28	0.178 ± 0.05
120	70.7 ± 3.74	0.189 ± 0.08	51.23 ± 2.82	0.094 ± 0.02	65.17 ± 0.98	0.158 ± 0.06
144	69.9 ± 1.83	0.203 ± 0.05	40.95 ± 5.13	0.106 ± 0.05	47.33 ± 0.94	0.175 ± 0.04

[a] FT: cultivation time; BWX: birch wood xylan; and OSX: oat spelt xylan. [b] Values are mean and SD for *n* = 2. [c] ND: not detectable.
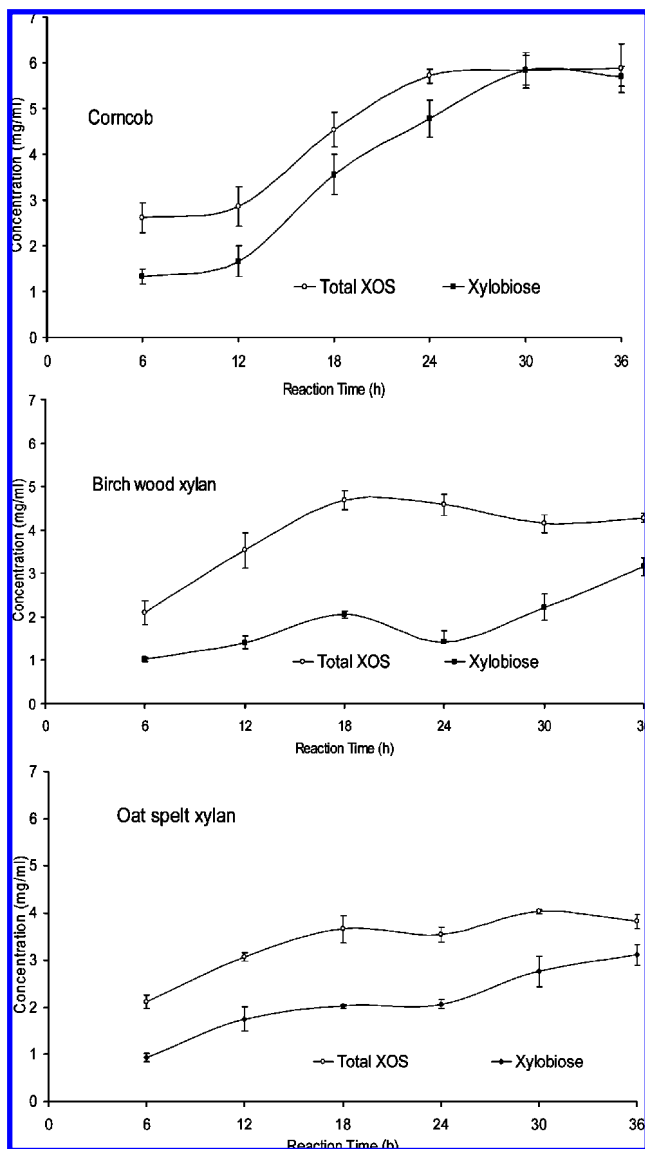

Figure 2. Production of xylooligosaccharides from BWX (10 mg/mL) using endoxylanases produced by *A. oryzae* in medium containing different xylan inducers at 96 h of cultivation.

increased to 67.10 ± 0.84% by the end of 36 h of reaction, indicating XOS hydrolysis of a higher degree of polymerization (DP) to xylose and xylobiose with an increase in the reaction time. **Figure 1** shows a typical HPLC chromatogram of the XOS mixture obtained.

A. flavus, A. ochraceus,* and *A. niger. In the case of *A. flavus*, the endoxylanase activity was maximum (34.05 ± 0.56 U/mL/

min) at 72 h of cultivation (**Table 1**). The percentage of XOS produced in the hydrolysate was 79.0 ± 1.12 and 79.3 ± 1.13% at 24 and 36 h of incubation, respectively, at 50 °C. This indicates that there is no significant increase in the percentage of XOS beyond 24 h of reaction. *A. ochraceus* showed a maximum endoxylanase activity at 96 h of cultivation as in the case of *A. oryzae*; however, it was less than that of *A. oryzae*. The endoxylanase activity at 96 h of cultivation was 39.93 ± 2.27 U/mL/min. The percentage of XOS in the hydrolysate was 83.92 ± 2.24 and 81.04 ± 0.59% at 24 and 36 h of incubation, respectively, at 50 °C. The xylobiose in the hydrolysate was 19.95 ± 1.19% by 24 h of reaction and increased to 24.82 ± 2.00% by 36 h of incubation, indicating the hydrolysis of XOS of higher DP to xylose and xylobiose with an increase in reaction time.

Unlike other *Aspergillus* species evaluated, *A. niger* showed a considerable titer of endoxylanase activity of 23.64 ± 0.67 U/mL/min at 24 h of cultivation and a maximum activity of 40.97 ± 0.55 U/mL/min at 72 h of cultivation. The percentage of XOS was 71.81 ± 1.56 and 68.40% at 24 and 36 h of incubation, respectively, at 50 °C. The percentage of xylobiose was 32.45 ± 1.41% by 24 h of reaction and increased to 36.82 ± 1.30% by 36 h of incubation, indicating the hydrolysis of XOS of higher DP to xylose and xylobiose with an increase in reaction time.

The endoxylanase activity of the other four species of fungal strains (*Au. pullulans, P. citrinum, T. viridae,* and *T. harzianum*) was very insignificant, and hence, these were not evaluated for XOS production. *A. oryzae* MTCC 5154, *A. flavus, A. niger,* and *Aspergillus* were found to be efficient for the production of XOS. Among these four *Aspergillus* species, *A. oryzae* was selected for further studies as it exhibited a maximum endoxylanase activity and high percentage of XOS in the hydrolysate by 24 h of incubation.

Evaluation of Different Xylan Sources for the Production of Endoxylanase by *A. oryzae* MTCC 5154. In the evaluation experiments, OSX was the carbon source as well as the inducer in the cultivation medium and was replaced with 1% BWX or 3% corncob powder (to equalize the amount of xylan in the medium, as corncob contains only 30−35% xylan). The efficiency of these xylan sources for the xylanase production was evaluated. The cultivation media (50 mL) was inoculated with 0.5 mL of spore suspension containing 6 × 10^{6} spores/ mL from a 5 day old slant.

Among the three xylan sources evaluated, the production of endoxylanase was more in media containing 3% corncob (**Table 3**). The maximum endoxylanase activity was observed at 96 h of growth, beyond which it decreased with an increase in cultivation time. The CMCase activity (**Table 3**) and total cellulase activity based on FPA were negligible (data not shown). As can be seen from the results, *A. oryzae* produced
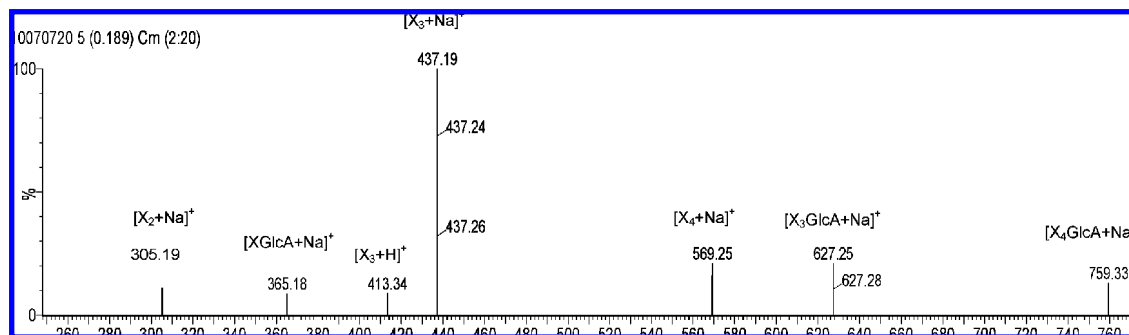
Corncob-Induced Xylanase for Xylobiose Production

J. Agric. Food Chem., Vol. 56, No. 11, 2008 **3985**

Figure 3. ESI/MS spectrum of xylooligosaccharides obtained by hydrolyzing BWX using endoxylanase produced by *A. oryzae* in a medium containing corncob as the inducer.
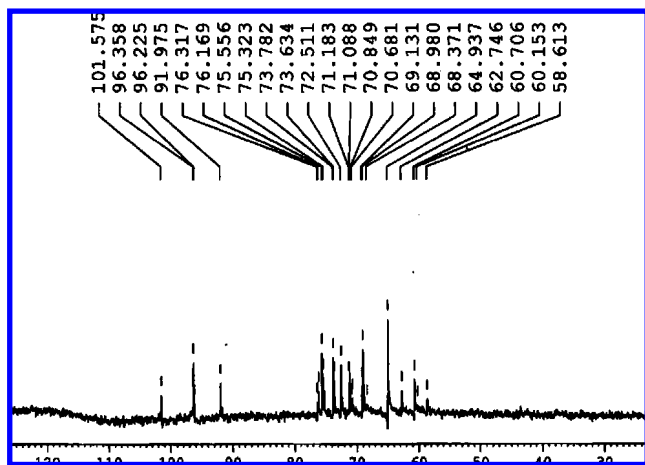

Figure 4. ^{13}C NMR spectrum of xylobiose.

higher titers of xylanases when powdered corncob was used as the substrate in comparison to that of BWX or OSX.

The endoxylanase activity at 96 h was 81.1 ± 3.74 U/mL/ min, and the total maximum protein content was 1.79 mg/mL. Similar to corncob powder, when BWX was used as the xylan source, the maximum endoxylanase activity (62.78 ± 2.46 U/mL/min) was at 96 h of cultivation (**Table 3**). The CMCase and FPA activity of CF also was recorded; however, this was not significant. A maximum xylanase activity of 76.73 ± 0.28 U/mL/min was observed at 96 h of cultivation when OSX was the inducer in the media. The CMCase and FPA activities of the culture fluid were found to be insignificant (**Table 3**).

Production of XOS by *A. oryzae* 5154. XOS production is maximum with xylanase obtained at 96 h of cultivation in medium containing corncob as the inducer (**Figure 2**). Maximum XOS produced was 5.87 ± 0.53 mg/mL from BWX (an initial substrate concentration of 10 mg/mL). The resultant hydrolysate consisted of 12 ± 2.0% xylose, 48 ± 2.43% xylobiose, and 40 ± 3.6% other XOS. The xylobiose content by 6 h of reaction was 1.33 ± 0.16 mg/mL, which increased to a value of 5.8 ± 0.33 mg/mL by 30 h. There was a slight increase in the concentration of XOS from 5.70 ± 0.15 mg/mL at 24 h of reaction to 5.87 ± 0.53 mg/mL at the end of 36 h. The amount of XOS produced using the enzyme obtained at 96 h of cultivation in the medium using BWX as the inducer was comparatively less and was 4.68 ± 0.22 mg/mL at 18 h of reaction; thereafter, it reduced slightly. Xylobiose was at a maximum at 36 h of reaction (3.12 ± 0.21 mg/mL). The amount of XOS produced using OSX as the inducer was much less when compared to the other two and was 4.03 ± 0.15 mg/mL at 36 h of reaction. In this case as well, the maximum xylobiose was 3.12 ± 0.22 mg/mL at 36 h of reaction.

ESI/MS of XOS. All measurements were performed in water. In these conditions, all the oligosaccharides appeared as [M + Na]$^+$ ions except for xylotriose (X$_3$), which formed both [M + Na]$^+$ and [M + H]$^+$ ions. The sodium adducts were found to be more stable in the case of oligosaccharides with a higher degree of polymerization. On the basis of measured mass spectra, six different oligosaccharides were identified. In addition, no in-source fragmentation of oligosaccharide ions appeared under the experimental conditions used. The mass spectra (**Figure 3**) showed the presence of various oligosaccharides at *m/z* values of 305.19, 365.18, 437.19, 413.34, 569.25, 627.25, and 759.33. The mass spectra of the major fraction obtained by GPC showed the presence of two compounds at *m/z* 301.19 and 365.18.

^{13}C NMR Spectrometry of Xylobiose. Analysis of purified disaccharide by ^{13}C NMR spectrometry was carried out to specify the structure of xylobiose (**Figure 4**). Assignments of signals were carried out using ^{13}C NMR and were based on published values for some related compounds (*24*). **Table 4** gives the complete assignments of the signals that were made by ^{13}C NMR. The anomeric carbons of the probable disaccharide appeared at δ_{ppm} 91.97, 96.3, and 101.57, which were assigned as reducing α-D-xylopyranoside, reducing β-D-xylopyranoside, and nonreducing β-D-xylopyranoside, respectively (**Figure 5**).

DISCUSSION

Several species of *Aspergillus* have been reported to produce xylanase including *A. niger*, *A. ochraceus*, *A. oryzae*, *A. awamori*, *A. tamarii*, and *A. fumigatus* (*12, 25, 26*). In the present study, all the strains evaluated tested positive for extracellular endoxylanase production as was evident from the clear zone of xylan hydrolysis observed in the Congo Red plate assay. *A. oryzae* is well-known for the production of a variety of industrially important enzymes. The xylanase activity was at a maximum (55.8 ± 0.53 U/mL/min) at 96 h of cultivation, and the pH was 8.08. Alkaline pH is found to be suitable for the production of high titers of endoxylanase. The results of preliminary screening experiments with the other organisms indicated that a pH range of 6.5−8.5 is best for xylanase production, with an exception of *A. niger*, wherein the pH drastically reduced during the initial hours of cultivation.

The extracellular CMCase activity of *A. oryzae* MTCC 5154 at 96 h of cultivation was negligible (0.103 ± 0.03 U/mL/min) (**Table 1**), which has an important bearing when lignocellulosic materials are used as substrates for XOS production. In general, LCMs rich in cellulose and xylan are the preferred substrates for the production of XOS. Since most of the fungal cellulases and xylanases exhibit a maximum activity at 50 °C, a cellulase-free xylanase or xylanase with less cellulase activity is advantageous because the glucose released by the action of cellulases

Table 4. Assignments of Signals in the ^{13}C NMR[a] Spectra of Xylobiose

xylobiose	chemical shifts (δ_{ppm})[b]									
	C1	C2	C3	C4	C5	C1'	C2'	C3'	C4'	C5'
α-form[c]	91.97	71.18	70.08	76.3	58.6	101.57	72.5	75.32	75.55	64.9
β-form[d]	96.3	73.6	73.7	76.1	62.7	101.57	72.5	75.32	75.55	64.9

[a] In D_2O. [b] Values are chemical shifts relative to acetone nucleus. [c] β-D-Xylopyranosyl-(1→4)-α-D-xylanopyranose. [d] β-D-Xylopyranosyl-(1→4)-β-D-xylanopyranose.
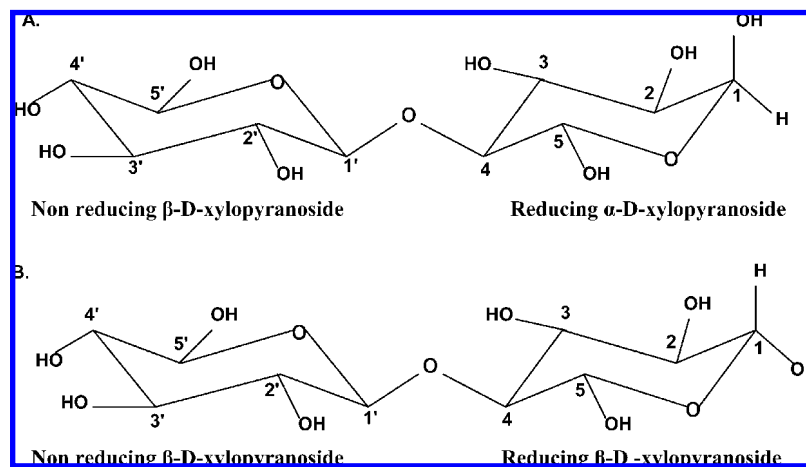
Figure 5. Structure of xylobiose: (A) β-D-xylopyranosyl-(1→4)-α-D-xylanopyranose and (B) β-D-xylopyranosyl-(1→4)-β-D-xylanopyranose.

on the cellulose matrix is an undesirable calorific sugar in the final product (XOS). *A. oryzae* and *A. fetidus* have been reported to be noncellulolytic, but *A. fumigatus* and *A. terreus* are cellulolytic (*25*). Similarly, *A. ochraceus* has been reported to produce cellulase-free xylanolytic enzymes when grown on pulverized grass (*27*). In the present study, all the four species of *Aspergillus* showed only trace amount of cellulase activity.

The endoxylanase activity of the other four species of fungal strains (*Au. pullulans*, *P. citrinum*, *T. viridae*, and *T. harzianum*) was very insignificant, even though some of them already were reported as good xylanase producers. The results clearly indicate that there are significant differences in the endoxylanase activity produced by the different strains of organisms. *A. oryzae* MTCC 5154, *A. flavus*, *A. niger*, and *A. ochraceus* were found to be efficient for the production of XOS. *A. oryzae* and *A. flavus* produced more than 50% of xylobiose in the hydrolysate by 24 h of reaction, but *A. niger* and *A. ochraceus* produced comparatively less xylobiose, even though their endoxylanase resulted in 71.81 ± 1.56 and 83.9 ± 2.24% of XOS by 24 h. This indicates that the endoxylanase of *A. oryzae* and *A. flavus* are more active.

The vast majority of xylanases is excreted into the extracellular environment as the large size of the substrate generally prevents its penetration into the cell. Xylanases are usually inducible enzymes secreted into the media containing pure xylan or xylan-rich residues (*28*). In fact, the current belief is that xylanase production is induced by means of the products by their own action (*29–31*). It is believed that small amounts of constitutively produced enzymes liberate xylooligomers that may be transported into the cell where they are further degraded by β-xylosidases or indeed by intracellular xylanases (*32*) and where they induce further xylanase synthesis. Induction is mostly by xylan in *Trametes trogii* (*33*) and *Aspergillus awamori*. Induction of xylanases by several other compounds such as L-sorbose, various xylooligosaccharides, and xylose has been reported (*34*). In the evaluation experiment using eight different fungi, the xylan source in the cultivation medium was OSX. The use of purified xylan as an inducer increases the cost of enzyme production. For this reason, different lignocellulosic

residues, including wheat bran, wheat straw, corncobs, and sugar cane bagasse have been used as substrates for the growth and production of xylanases (*12*). There are many reports on the different degrees of xylanase induction by xylan from various sources. Induction is affected by the type of xylan, particularly the extent of branching, the type and frequency of the sidegroup on the xylan backbone, and the degree of polymerization of the xylan molecule (*35*). In the present study, the efficiency of BWX and raw corncob to induce xylanase production also was studied.

The efficiency of corncobs in the maximum induction of xylanase production over other complex carbon sources such as sawdust, wheat bran, rice bran, and bagasse also was reported. As can be seen from the results, *A. oryzae* produced higher concentrations of xylanases when powdered corncob was used as the substrate in comparison to that of BWX or OSX. In the case of media containing corncob as the inducer, a high xylanase activity of 32.2 ± 4.24 U/mL/min was observed by 24 h of cultivation. This could be attributed to the presence of soluble fragments of xylooligomers in the corncob itself. The possibility of methyl xyloside, xylobiose, and other xylooligomers inducing xylanase is reported in *A. oryzae*, *A. fetidus*, *A. fumigatus*, and *A. terreus* (*25*). In all cases, the maximum xylanase production was observed at 96 h of cultivation, with a considerable reduction in the activity as the cultivation proceeded. The CMCase and total cellulase activities of the culture fluids were found to be very insignificant. These results are in agreement with literature reports (*36, 37*). Yet, the reason and regulation of xylanase secretion by microorganisms is still not completely understood. Since xylan is unable to enter the microbial cell, the induction of xylanase is stimulated by low molecular weight xylan fragments, which are produced in the medium by a small amount of constitutively produced xylanase (*38, 39*).

The results of XOS production using endoxylanase from *A. oryzae* indicated that the maximum production occurred using the enzyme produced in the medium containing corncob as the inducer. In all cases, the amount of XOS produced by 6 h of reaction was around 2 mg mL^{-1}, and it reached a maximum of 5.84 ± 0.39 and 4.03 mg/mL by 30 h of reaction when enzymes

Corncob-Induced Xylanase for Xylobiose Production

J. Agric. Food Chem., Vol. 56, No. 11, 2008 **3987**

obtained in medium containing corncob powder and OSX, respectively, were used for hydrolysis. A maximum of 4.68 ± 0.22 mg/mL XOS was produced by 18 h of reaction when birch wood-induced xylanase was used. In the initial stages of the enzymatic reaction, the amount of XOS produced was almost the same; however, the maximum amount of XOS produced varied significantly. These results suggest diversity in the mode of action of xylanases obtained from different sources.

Xylobiose was the major oligomer of XOS obtained in all cases. This is in agreement with reports on XOS production by xylanase obtained from *Aspergillus kawachii* (*40*). This indicates the true endoxylanase activity of these enzymes since for an exoenzyme the proportion of xylose should have increased soon after the start of hydrolysis and should have been the major product. The action of purified acidic xylanase from *Aspergillus nidulans* on OSX resulted in xylopentose with a lesser amount of xylotriose and xylobiose by 7 h, whereas xylobiose was the main product after prolonged incubation of 24 and 72 h (*41*). The results indicate the potential use of *A. oryzae* MTCC 5154 for the production of xylanases (with a low cellulase activity) by submerged cultivation using raw corncob powder as the inducer, which can be further used for the production of XOS with a higher concentration of xylobiose for food and non-food applications.

ESI/MS indicated the presence of two disaccharides in the XOS mixture, mainly xylobiose with m/z 305.19 for its sodium adduct $[X_2 + Na]^+$. Another disaccharide detected had an m/z value of 365.18 $[XGlcA + Na]^+$, which is probably a disaccharide of xylose and 4-*O*-methyl-α-D-glucuronic acid. Both sodium and hydrogen adducts of xylotriose were detected with m/z values of 437.19 $[X_3 + Na]^+$ and 413.34 $[X_3 + H]^+$, respectively. The mass spectrum shows the absence of a trisaccharide containing a 4-*O*-methyl-α-D-glucuronic acid residue. Two types of tetrasaccharides were observed: xylotetraose and the other having one 4-*O*-methyl-α-D-glucuronic acid residue with m/z values of 569.25 $[X_4 + Na]^+$ and 627.25 $[X_3GlcA + Na]^+$, respectively. An m/z value of 759.33 $[X_4GlcA + Na]^+$ indicates the presence of a pentasaccharide with 4-*O*-methyl-α-D-glucuronic acid residue. The mass spectra of the major fraction obtained by GPC showed the presence of a disaccharide of xylose and 4-*O*-methyl-α-D-glucuronic acid in addition to the xylobiose. ^{13}C NMR spectrometry of purified disaccharide revealed the same structural details. The signal at δ_{ppm} 101.57 confirmed the β-(1→4)-xylosidic linkage between the two xylose residues. In this way, the major disaccharide was determined as β-D-xylopyranosyl-(1→4)-D-xylanopyranose (xylobiose). The structure of xylobiose based on these data is given in **Figure 5**. The formation of xylobiose during the hydrolysis of BWX explains the structural features of the backbone of xylan and also is useful as basic data for the identification of analogous XOS (*2*).

ABBREVIATIONS USED

XOS, xylooligosaccharides; CMCase, carboxymethyl cellulase; FPU, filter paper unit activity for total cellulase; OSX, oat spelt xylan; BWX, birch wood xylan.

LITERATURE CITED

(1) Zui, F.; Kaneko, S.; Kuno, A.; Kobayashi, H.; Kusakabe, I.; Mizuno, H. Crystal structure of decorated xylooligosaccharides bound to a Family 10 xylanase from *Streptomyces olivaceoviridis* E-86. *J. Biol. Chem.* **2004**, *279* (10), 9606–9614.

(2) Ishihara, M.; Nojiri, M.; Hayashi, N.; Nishimura, T.; Shimizu, K. Screening of fungal β-xylanases for production of acidic xylooligosaccharides using in situ reduced 4-*O*-methylglucuronoxylan as substrate. *Enzyme Microb. Technol.* **1997**, *21*, 170–175.

(3) Okazaki, M.; Fujikawa, S.; Mastumoto, N. Effects of xylooligosaccharides on growth of *Bifidobacteria*. *Bifidobacteria Microflora* **1990**, *9*, 77–86.

(4) Pazur, J. H. Oligosaccharides. In *The Carbohydrates—Chemistry and Biochemistry*; Pigman, W., Horta, D., Eds.; Academic Press: New York, 1970; Vol. IIA, pp 69–73..

(5) Koga, K.; Fugikawa. S. *Oligosaccharides*; Nihon Shokuhin Kako Ltd.: Shizuoka, Japan, 1985; Vol. 3, pp 130–143..

(6) Moure, A.; Gullon, P.; Dominiguez, H.; Parajo, J. C. Advances in the manufacture, purification, and applications of xylooligosaccharides as food additives and nutraceuticals. *Proc. Biochem.* **2006**, *41* (9), 1913–1923.

(7) Nabarlatz, D.; Farriol, X.; Montane, D. Autohydrolysis of almond shells for the production of xylooligosaccharides: Product characteristics and reaction kinetics. *Ind. Eng. Chem. Res.* **2005**, *44*, 7746–7755.

(8) Loo, J. V.; Cummings, J.; Delzenne, N.; Englyst, H.; Franck, A.; Hopkins, M.; Kok, N.; Macfarlane, G.; Newton, D.; Quigley, M.; Roberfroid, M.; van Vliet, T.; van den Heuvel, E. Functional food properties of nondigestible oligosaccharides: A consensus report from the ENDO Project (DGXII-AIRII-CT94-1095). *Br. J. Nutr.* **1999**, *81*, 121–132.

(9) Toshio, I.; Noriyoshi, I.; Toshiaki, K.; Toshiyuki, N. Kunimasa, K. Production of Xylobiose. Japanese Patent JP 2119790, 1990.

(10) Vazquez, M. J.; Alonso, J. L.; Dominguez, H.; Parajo, J. C. Xylooligosaccharides: Manufacture and applications. *Trends Food Sci. Technol.* **2000**, *11*, 387–393.

(11) Yang, R.; Xu, S.; Wang, Z.; Yang, W. Aqueous extraction of corncob xylan and production of xylooligosaccharides. *LWT* **2005**, *38*, 677–682.

(12) Haltrich, D.; Nidezky, B.; Kulbe, K. D.; Steiner, W.; Zupancic, S. Production of fungal xylanases. *Biores. Technol.* **1996**, *58*, 137–161.

(13) Pham, P. L.; Taillandier, P.; Delmass, M.; Strehaiano, P. Production of xylanase by *Bacillus polymixa* using lignocellulosic wastes. *Ind. Crop Prod.* **1998**, *7*, 195–203.

(14) Akpinar, O.; Ak, O.; Kavas, A.; Bakir, U.; Yilmaz, L. Enzymatic production of xylooligosaccharides from cotton stalks. *J. Agric. Food Chem.* **2007**, *55*, 5544–5551.

(15) Yang, C. H.; Yang, S. F.; Liu, W. H. Production of xylooligosaccharides from xylans be extracellular xylanase from *Thermobifida fusca*. *J. Agric. Food Chem.* **2007**, *55*, 3955–3959.

(16) Kimura, T.; Suzuki, H.; Furuhashi, H.; Aburatani, T.; Morimoto, K.; Sakka, K.; Ohmiya, K. Molecular cloning, characterization, and expression analysis of the xynF3 gene from *Aspergillus oryzae. Biosci., Biotechnol., Biochem.* **2002**, *66* (2), 285–292.

(17) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 267–275.

(18) Theater, R. M.; Wood, P. J. Use of Congo Red—polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **1993**, *43*, 777–780.

(19) Jiang, Z.; Yang, S.; Yang, Q.; Li, L.; Tan, S. Optimization of xylanase production by a newly isolated strain CAU 44 of the Thermophile *Thermomyces lanuginosus*. *World J. Microbiol. Biotechnol.* **2005**, *21* (6–7), 863–867.

(20) Miller, G. L. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* **1959**, *31* (3), 426–428.

(21) Wood, T. M.; Bhat, K. M. Methods for measuring cellulase activities. *Methods Enzymol.* **1988**, *160*, 94–102.

(22) Jeong, K. J.; Park, I. Y.; Kim, M. S.; Kim, S. C. High-level expression of an endoxylanase from gene *Bacillus* sp. in *Bacillus subtilis* DB104 for the production of xylobiose from xylan. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 113–118.

(23) Reis, A.; Coimbra, M. A.; Domingues, P.; Correia, A. J. F.; Rosario, M.; Domingues, M. Fragmentation pattern of underivatized xylooligosaccharides and their alditol derivatives by electrospray tandem mass spectrometry. *Carbohydr. Polym.* **2004**, *55*, 401–409.

(24) Takeshi, N.; Ishihara, M.; Ishii, T.; Kato, A. Structure of neutral branched xylooligosaccharides produced by xylanase from in situ reduced hard wood xylan. *Carbohydr. Res.* **1998**, *308*, 117–122.

(25) Bailey, M. J.; Poutanen, K. Production of xylanases by strains of *Aspergillus*. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 5–10.

(26) Kadowaki, M. K.; Souza, C. G. M.; Simao, R. C. G.; Peralta, R. M. Xylanase production by *Aspergillus tamari*. *Appl. Biochem. Biotechnol.* **1997**, *66*, 97–106.

(27) Das, A.; Nanda, G. Production of xylanolytic enzymes during growth on pulverized grass by *Aspergillus ochraceus*-42. *Lett. Appl. Microbiol.* **1995**, *20*, 141–144.

(28) Balakrishnan, H.; Srinivasan, M. C.; Rele, M. V. Extracellular protease activities in relation to xylanase secretion in an alkilophilic *Bacillus* sp. *Biotechnol. Lett.* **1997**, *18*, 599–601.

(29) Biely, P. Microbial xylanolytic systems. *Trends Biotechnol.* **1985**, *9*, 83–88.

(30) Defaye, J.; Guillot, J. M.; Biely, P.; Vrsanska, M. Positional isomers of thioxylobiose: Their synthesizing and inducing ability for D-xylan-degrading enzymes in the yeast *Cryptococcus albidus*. *Carbohydr. Res.* **1992**, *228*, 47–64.

(31) Singh, S.; Madlala, A. M.; Prior, B. A. *Thermomyces lanuginosus*: Properties of their strains and their hemicellulases. *FEMS Microbiol. Rev.* **2003**, *27*, 3–16.

(32) Shulami, S.; Gat, O.; Sonenshein, A. L.; Shosham, Y. The glucuronic acid utilization gene cluster from *Bacillus stearothermophilus* T-6. *J. Bacteriol.* **1999**, *181*, 3695–3704.

(33) Levin, L.; Forschiassin, F. Influence of growth conditions on the production of xylanolytic enzymes by *Trametes trogii*. *World J. Microbiol. Biotechnol.* **1998**, *14*, 443–446.

(34) Beg, Q. K.; Kapoor, M.; Mahajan, L.; Hoondal, G. S. Microbial xylanases and their industrial applications: A review. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 326–338.

(35) Anita, J. Production of xylanase by thermophilic *Melanocarpus albomyces* IIS-68. *Proc. Biochem.* **1995**, *30* (8), 705–709.

(36) Samain, E.; Debeire, P. H.; Touzel, J. P. High level production of a cellulase-free xylanase in glucose-limited fed batch cultures of a thermophilic bacillus strain. *J. Biotechnol.* **1997**, *58*, 71–78.

(37) Li, L.; Tian, H.; Cheng, Y.; Jiang, Y.; Yang, S. Purification and characterization of a thermostable cellulase free xylanase from newly isolated *Paecilomyces themophila*. *Enzyme Microb. Technol.* **2006**, *38*, 780–787.

(38) Bastawde, K. B. Xylan structure, microbial xylanases, and their mode of action. *World J. Microbiol. Biotechnol.* **1992**, *8*, 353–368.

(39) Kulkarni, N.; Shendye, A.; Rao, M. Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.* **1999**, *23*, 411–456.

(40) Ito, T.; Ogasawara, H.; Sugimoto, T.; Ishikawa, T. Purification and properties of acid stable xylanase from *Aspergillus kawachii*. *J. Biotechnol. Biochem.* **1992**, *56*, 547–550.

(41) Fernandez-Espinar, M.; Pinaga, F.; de Graaff, L.; Valles, S. Purification, characterization, and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 55–562.