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Production, Characterization, and Application of the Cellulase-Free Xylanase from *Aspergillus niger*

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ABSTRACT

The effects of carbon source on xylanase and cellulase production were studied. The extract of steam-exploded corn stover was found to be the best raw material for producing cellulase-free xylanase. The xylanolytic enzymes of *Aspergillus niger* An-76 were purified by chromatography, and the properties of the four purified components were analyzed. When the enzyme was used to treat the birch Kraft pulp, and followed by a subsequent CEH bleaching, a brightness of 6.8% SBD more than that of the untreated one using the same chlorine dosage, or a saving of nearly 50% of chlorine consumption with the same brightness, was realized.

Index Entries: Xylanase; cellulase-free; *Aspergillus niger*; pulp bleaching.

INTRODUCTION

Xylanolytic enzymes play an important role in the bioconversion of lignocellulosic materials. For example, the energy used for lignin degradation during white rot decay is obtained from hydrolysis of xylan *(1)*. In 1986, Viikari et al. found that when Kraft pulp was pretreated with xylanase, a higher brightness was obtained after bleaching *(2)*. Since then, many reports dealing with the pretreatment of pulp with xylanases have been published. These results show that the xylanase treatment enables significant reduction of chlorine usage, resulting in less production of toxic chlorinated compounds from pulp mills. The xylanase preparations should be free from cellulase activities, since the cellulase would destroy the strength properties of the pulp *(3,4)*.

It has been reported that a large number of mesophilic and thermophilic fungi, e.g., *Aspergillus* strains *(5–7)*, *Trichoderma* strains *(3,6)*, *Chaetomium cellulolyticum (1)*, and *Thermomyces lanuginosus (8)*, can produce xylanases. Most of them produce xylanase and cellulase simultaneously when they were grown on plant residues, but the synthesis of the two enzymes is regulated under a separate control system *(5)*.

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In this article, we report the production of a high level of cellulase-free xylanase by using the extract of steam-exploded corn stover, and report on the characterization and application of the cellulase-free xylanase from *Aspergillus niger* An-76.

MATERIALS AND METHODS

Microorganisms

Six filamentous fungi, two bacteria, and one yeast were obtained from the stock cultures in our laboratory *(5)*. The white rot basidiomycetes, *Phanerochate chrysosporium* ME 448, was kindly offered by the Wood Research Institute, Kyoto University, Japan.

Substrates

The steam explosion of corn stover was carried out in pilot-scale equipment with a working volume of 1 m^3. The corn stover was heated to 180°C by steam under a pressure of 1 MPa for 20 min, and exploded by releasing the pressure instantaneously. The exploded corn stover was extracted by hot water (about 75°C) with a water ratio of 1:10. About 15 g/L of total sugar were obtained in the extract. More than 85% of it was pentoses, and the ratio of monose to olignose was 1:9.

Medium and Cultivation

The basic mineral medium of Mandels and Reese *(9)* was used. Twenty grams per liter of carbon source and 10 g/L of wheat bran were added to the medium. After inoculation, the 300-mL flasks containing 80 mL medium were shaken on an orbital shaker at 200 rpm and 28°C for 3–4 d (*P. chrysosporium* was at 39°C for 6 d). The cultured medium was centrifuged at 5000*g* for 10 min, and the clear supernatant was used for enzyme assay and purification.

Enzyme Assay

Endo-1,4-β-xylanase activity was determined by estimating the xylose liberated from 1% of beechwood xylan (Sigma, St. Louis, MO) suspended in acetate buffer (0.05*M*, pH 5.0) after 20 min of reaction at 50°C. CMCase activity and filter paper activity (FPA) were assayed as recommended by IUPAC *(10)*, and the reducing sugar was determined by the DNS method using glucose as standard sugar. One unit of enzyme activity was defined as the amount (μmol) of reducing sugar released per minute.

Chromatography

The crude enzyme in the clear supernatant was precipitated with ammonium sulfate at 80% saturation, and the precipitate was collected by centrifugation at 5000*g* for 20 min. Crude enzyme precipitate (about 2.5 g of dry wt) was dissolved in 20 mL of 0.05*M* phosphate buffer (pH 6.8). The enzyme solution was chromatographed on Sephadex G-100 column (26 × 1000 mm), DEAE-Biogel A, and CM-Biogel A column (26 × 200 mm). Eluted fractions were analyzed for xylanase, CMCase, and FPase activities.

Table 1
Xylanase and Cellulase Production in the Corn Stover and Wheat-Bran-Based Medium by Various Microorganisms[a]

Strains	Xylanase, IU/mL	CMCase, IU/mL	FPase, IU/mL
Bacillus subtilus 921	8.4	12.0	n.d.
Xanthomonas sp.	7.2	8.4	n.d.
Endomyces 9304	8.6	4.3	n.d.
A. niger An-76	38.6	11.3	0.6
A. niger L22	32.4	24.2	1.2
Chaetomium globosum SM27	4.0	8.2	0.6
Neurospora crassa 1606	4.1	11.2	0.5
Trichoderma harzianum 912	8.2	10.3	0.4
Lentinus lepidens	10.2	4.7	n.d.
P. chrysosporium ME446	14.3	10.2	0.7

n.d., not detectable.
[a]All data in the tables (except some of data in Table 5) are means of three tests.

Gel electrophoresis on a 10% polyacrylamide gel using the discontinuous buffer system and thin-layer gel isoelectric focusing (LKB-2117 Multiphor) were used to analyze enzyme purity, molecular weight, and pI (stained by Coomassie blue). Bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 45,000), and chymotrypsinogen (mol wt 25,000) were used as the standard proteins (Bio-Rad, Hercules, CA).

Treatment of Pulp by Xylanase

The treatment was carried out in plastic bags. Thirty grams of Kraft pulp were added to each bag with various amounts of enzyme. The pulp consistency was 5%, temperature was 45°C, and the pH was adjusted to 4.8 by HAc-NaAc buffer. Incubation time was 3 or 4 h.

After enzyme treatment, the pulp was washed to neutrality and bleached by the CEH three-stage bleaching. The pulp and paper were tested according to the standard methods.

RESULTS AND DISCUSSION

High Level Cellulase-Free Xylanase Production Using the Extract of Steam-Exploded Corn Stover

The effects of carbon source on xylanase and cellulase productions were tested by adding three different carbon sources to the basic mineral medium. As shown in Table 1, the yields of both xylanase and cellulase were high in the corn stover–wheat bran-based media. When xylose was selected as the carbon source, the production of both enzymes was repressed (Table 2). The best results of cellulase-free xylanase production were obtained by using the extract of steam-exploded corn stover as the carbon source (Table 3). In this case, the xylooligosaccharide acted not only as a carbon source, but also as an inducer for xylanase production. Small

Table 2
Xylanase and Cellulase Production
in the Xylose-Based Medium by Various Microorganisms

Strains	Xylanase, IU/mL	CMCase, IU/mL	FPase, IU/mL
B. subtilus 921	n.d.	n.d.	n.d.
Xanthomonas sp.	n.d.	n.d.	n.d.
Endomyces 9304	n.d.	n.d.	n.d.
A. niger An-76	3.2	n.d.	n.d.
A. niger L22	0.3	1.2	n.d.
C. globosum SM27	0.2	n.d.	n.d.
N. crassa 1606	0.4	n.d.	n.d.
T. harzianum 912	0.8	n.d.	n.d.
L. lepidens	1.2	n.d.	n.d.
P. chrysosporium ME446	0.6	n.d.	n.d.

n.d, not detectable.

Table 3
Xylanase and Cellulase Production in the Extract of Steam-Exploded
Corn Stover-Based Medium by Various Microorganisms

Strains	Xylanase, IU/mL	CMCase, IU/mL	FPase, IU/mL
B. subtilus 921	4.2	0.2	n.d.
Xanthomonas sp.	3.1	0.3	n.d.
Endomyces 9304	6.3	0.2	n.d.
A. niger An-76	27.3	0.1	0.02
A. niger L22	18.6	0.1	0.01
C. globosum SM27	7.4	0.3	n.d.
N. crassa 1606	12.1	0.2	n.d.
T. harzianum 912	14.3	0.1	n.d.
L. lepidens	8.2	0.2	n.d.
P. chrysosporium ME446	7.3	0.4	0.04

n.d., not detectable.

amounts of glucose existed in the extract (<10% of total sugar), and the production of cellulase was repressed. Corn stover is a cheap agricultural residue, and the cost of its pretreatment (steam-explosion process) is also relatively inexpensive. The extract of steam-exploded corn stover was used for the further studies. *A. niger* An-76 was selected as the xylanase producer according to its xylanase productivity.

Various nitrogen compounds were added to the culture media with the same concentration as the nitrogen content of ammonium sulfate in the basic mineral medium. The xylanase activities after 2, 3, or 4 d of cultivation were determined, and the effects of nitrogen source are shown in Table 4. Except in the medium containing urea, the final pH values of all broth were 3.6–3.8 after 4 d of incubation. According to the data in Table 4, $NaNO_3$ was chosen as the optimal nitrogen source for enzyme production.

Table 4
Effects of Various Nitrogen Sources on the Production of Xylanase by *A. niger* An-76 inthe Extract of Steam-Exploded Corn Stover-Based Medium

Nitrogen source	Xylanase, IU/mL		
	48 h	72 h	96 h
$(NH_4)_2SO_4$	20.5	40.3	46.2
$(NH_4)_2HPO_4$	24.3	36.2	38.2
NH_4NO_3	10.2	30.1	36.5
$NaNO_3$	8.4	34.2	48.0
Beef extract	16.8	28.4	30.1
Peptone	14.6	28.6	48.4
Yeast extract	12.0	24.3	38.8
Urea	10.2	18.3	22.0

Purification and Characterization of Xylanolytic Enzymes from *A. niger* An-76

The procedure used for the fractionation of the xylanolytic enzymes from *A. niger* An-76 is shown in Fig. 1. Four peaks appeared in the eluted fractions from Sephadex G100 column by eluting with 10 mmol/L phosphate buffer, pH 6.8. Two of them (fraction I and fraction II) showed β-xylosidase and endoxylanase activities. Fraction I was concentrated and applied to a DEAE-Biogel A column equilibrated with 0.05*M* phosphate buffer, pH 6.8. After a nonadsorbed protein peak was washed out with the same buffer, the column was eluted using a 0–0.4 mol/L NaCl linear gradient. A β-xylanase component was obtained. The fraction II showing endoxylanase activity was also applied to a DEAE-Biogel A column and eluted as was done with the fraction I. Three endoxylanase components were obtained. The first peak (fraction II-1) was further purified by CM-Biogel A column equilibrated with 0.05*M* acetate buffer, pH 3.8, and eluted with a 0–0.3 mol/L NaCl linear gradient. The endoxylanase I was obtained.

All four components of the xylanolytic enzymes appeared as a single band on SDS polyacrylamide gel electrophoresis. Some of their chemical and physical properties are shown in Table 5.

It was reported that the cellulase and xylanase were usually produced at the same time in the strains of *Trichoderma reesei (11)*. It is difficult to produce cellulase-free xylanase using these strains. The tests of substrate specificity in this study demonstrated that the three endoxylanase components from *A. niger* An-76 showed no CMCase and FPase activity, and the β-xylosidase could not hydrolyze cellobiose. This suggests that the xylanases from *A. niger* An-76 are specific for xylan. This property is very useful for the production of cellulase-free xylanase.

Application of the Cellulase-Free Xylanase as a Pulp Bleaching Booster

The cellulase-free xylanase was used in the treatment of wheat straw pulp before bleaching at 50°C for 3 or 4 h with different enzyme dosages (IU/g pulp). The results show that the more xylanase that was used in the process, the more xylose

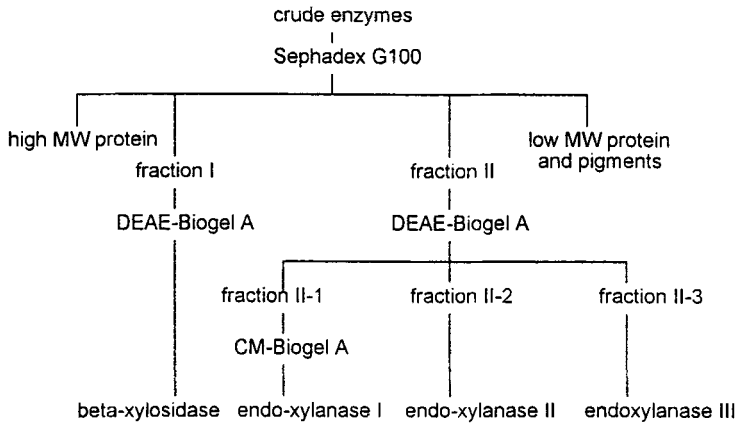

Fig. 1. The procedure used for the purification of xylanolytic enzymes from *A. niger* An-76.

Table 5
Characterization of Four Xylanolytic Enzymes from *A. niger* An-76

	Molecular weight, kDa	pI	Carbohydrate content, %	Temp. optimum, °C	pH optimum
β-xylosidase	147	5.9	4.3	45–55	3.5–4.0
Endoxylanase I	23	4.1	12.5	45–55	5.0
Endoxylanase II	22	3.9	9.4	45–55	5.0
Endoxylanase III	41	4.6	7.8	45–55	3.0

Table 6
The Results of Wheat-Straw Pulp Treatment
Using the Cellulase-Free Xylanase from *A. niger* An-76

	Incubation time, 3 h			Incubation time, 4 h	
Xylanase dosage, IU/g	50	100	200	100	200
Reducing sugar, mg/mL	5.4	5.9	6.7	8.0	8.4
Xylose released, mg/mL	3.0	4.4	5.5	6.0	8.6
Xylose/reducing sugar, %	70	76	82	75	81
κ reduction[a]	2.0	2.8	3.0	5.2	6.0

[a]After bleaching.

that was released from the pulp, which implied that more hemicellulose was hydrolyzed (Table 6). As the incubation time increased, the ratio of xylose to reducing sugar did not increase, but the reduction of κ number increased, which suggests an improvement for the bleaching process and the quality of pulp.

When the birch Kraft pulp was pretreated with the cellulase-free xylanase from *A. niger* An-76 (xylanase dosage 230 IU/g pulp), and followed by a subsequent CEH bleaching, a brightness of 6.8% SBD more than that of the untreated one using the same chlorine dosage, or a saving of nearly 50% of chlorine consumption with the same brightness, could be realized (Table 7). The yield decrease

Table 7
Comparison of the Results of CEH Bleaching on Birch Kraft Pulp with or Without Xylanase Pretreatment

Xylanase dosage, IU/g	Total chlorine dosage, %	Brightness SBD, %	κ Number[a]	Yield[b], %
0	10	69.1	3.7	93.35
0	7	66.3	5.0	94.65
0	5	62.0	5.8	95.01
600	5	70.5	3.3	88.26
320	5	69.4	3.5	88.35
230	5	68.8	3.6	92.95
120	5	67.0	3.6	92.30
60	5	64.6	3.8	93.70

[a]After alkaline extraction.
[b]After bleaching.

was slight, and no adverse effect on the strength properties of pulp was found. The application of this cellulase-free xylanase from *A. niger* An-76 in the pulp-paper industry could be promising.

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