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Saccharification of Corn Fiber Using Enzymes from *Aureobasidium* sp. Strain NRRL Y-2311-1

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ABSTRACT

Crude enzyme preparations from *Aureobasidium* sp. strain NRRL Y-2311-1 were characterized and tested for the capacity to saccharify corn fiber. Cultures grown on xylan, corn fiber, and alkaline hydrogen peroxide (AHP)-pretreated corn fiber produced specific levels of endoxylanase, amylase, protease, cellulase, and other activities. Using equal units of endoxylanase activity, crude enzymes from AHP-pretreated corn fiber cultures were most effective in saccharification. Multiple enzyme activities were implicated in this process. Pretreatment of corn fiber with AHP nearly doubled the susceptibility of hemicellulose to enzymatic digestion. Up to 138 mg xylose, 125 mg arabinose, and 490 mg glucose were obtained per g pretreated corn fiber under conditions tested.

Index Entries: Corn fiber; enzymatic hydrolysis; hemicellulose; saccharification; xylanase.

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INTRODUCTION

Corn fiber is an abundant co-product of wet-milling, principally consisting of the outer seed coat (pericarp) fraction of corn kernels as separated early in the wet-milling process (*1*). Purified corn bran from pericarp has been reported to contain about 70% hemicellulose (primarily arabinoxylan), 23% cellulose and about 0.1% lignin (*2*). Corn fiber contains an average of 20% adherent starch, although this amount can vary widely based on processing conditions (*3*). Currently, corn fiber is combined with other co-products, such as steep liquor and/or stillage residues, to produce corn gluten feed, which is marketed as a cattle feed.

Because of the relatively low commercial value and increasing supply of corn fiber, interest has grown in finding new, value-added uses for this material (*4*). Sugar components of corn fiber are potential substrates for a variety of bioconversions, including fermentation to ethanol. The abundant pentose sugar xylose could be converted to the sugar alcohol xylitol, valued as a natural, anticariogenic sweetener (*5*).

Although hemicellulose is more readily hydrolyzed than cellulose by chemical means, even mild conditions such as dilute acid may lead to the production of biologically inhibitory compounds, such as furfural and lignin degradation products (*6*). Enzymatic saccharification may prove to be an attractive alternative, particularly in the development of integrated or simultaneous processes for fermentative conversions of corn fiber.

A recent survey of commercially available enzymes found that none, alone or in combination, were able to efficiently hydrolyze the hemicellulose fraction of corn fiber (*7*). The yeastlike fungus *Aureobasidium pullulans* has been reported to produce a variety of hydrolytic enzymes (*8–11*). Furthermore, naturally occurring "color variant" strains of *Aureobasidium* produce elevated levels of endoxylanase (EC 3.2.1.8) with high specific activity (*12–14*). In preliminary studies, we found that partially purified xylanase from *Aureobasidium* sp. strain NRRL Y-2311-1 effected only limited saccharification of corn fiber. We consequently tested crude enzyme preparations from cultures grown on xylan or corn fiber. Our objective was to determine whether such enzyme mixtures from *Aureobasidium* could be effective in the saccharification of corn fiber.

MATERIALS AND METHODS

Organism and Enzyme Preparation

Aureobasidium sp. strain NRRL Y-2311-1 was obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL. So-called color variant strains of *Aureobasidium*, such as NRRL Y-2311-1, constitute a taxonomically distinct group that is likely to be named a new species (*15*). Working stocks were maintained on solid medium containing yeast extract and malt extract (YM). Liquid culture

conditions were essentially as previously described (*13,14*), with growth substrates at 1.0% w/v in a defined basal medium. Pre-inocula were grown to saturation on xylose and used at 1.0% v/v to inoculate experimental cultures containing xylan or corn fiber. Xylan was practical grade from oat spelts (Sigma, St. Louis, MO). Corn fiber was the gift of Pekin Energy, Pekin, IL. Crude culture supernatants were clarified by certrifugation at 7000*g* for 20 min, and then concentrated approximately 10-fold by ultrafiltration, using low protein-binding membranes with a 10,000 MW cut-off (YM-10, Amicon, Beverly, MA).

Enzyme and Protein Assays

All assays were performed at 37°C, in 50 m*M* sodium acetate buffer at pH 5.0. Endoxylanase, carboxymethylcellulase (CM-cellulase), and total amylase activities were determined by reducing sugar microassays as described elsewhere (*12,13,16*). Xylosidase and arabinosidase were estimated using the synthetic substrates *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl α-L-arabinofuranoside, respectively (Sigma), as previously described (*12*). Acetyl esterase activity was estimated according to MacKenzie et al. (*17*), using *p*-nitrophenyl acetate. Total protease activity was estimated using azocoll substrate as described by Chavira et al. (*18*). All enzyme activities are expressed in International Units (IU, μmol end product produced per min), with the exception of protease activity, expressed as change in OD_{520} per h. Protein was estimated by the Bio-Rad protein assay (Richmond, CA), based on the Bradford method (*19*). All assays are reported as the mean and standard deviation of replicate cultures.

Corn Fiber Pretreatment and Saccharification

Corn fiber was baked to dryness and finely ground in a mortar and pestle, and used directly or after pretreatment with alkaline hydrogen peroxide (AHP) according to Gould (*20,21*). Specifically, corn fiber was suspended at 2.0% w/v in distilled water containing 1.0% hydrogen peroxide. The suspension was adjusted to pH 11.5 with sodium hydroxide, and was stirred slowly overnight at room temperature. The suspension was adjusted to pH 7.0 with hydrochloric acid, and then precipitated with 2 vol of ethanol. Precipitates were recovered by centrifugation at 7000*g* for 30 min, and then dried and ground as before. For enzymatic saccharification, substrates were suspended at 1.0% w/v in 50 m*M* sodium acetate, pH 5.0, and boiled for 5 min to gelatinize starch and to enhance substrate solubilization and emulsification. Substrates contained no detectable free sugars prior to enzymatic digestion. Partially purified xylanase from *Aureobasidium* used at 3 IU/mg corn fiber was found to effect a limited saccharification of corn fiber within approximately 8 h (unpublished results). This xylanase to corn fiber ratio was consequently maintained in studies of crude enzymes. Crude enzyme preparations from cultures grown on various carbon sources were used in amounts adjusted to contain equal

Table 1
Neutral Sugars in TFA Hydrolysates of Corn Fiber

Substrate	Glucose,[a] mg/g	Xylose, mg/g	Arabinose, mg/g
Corn fiber	200 ± 7	199 ± 3	98 ± 7
Pretreated corn fiber	200 ± 17	204 ± 16	102 ± 8

[a]Includes glucose from starch (estimated as 182 ± 6 mg/g corn fiber by enzymatic analysis) and from hemicellulose, but not from cellulose.

units of xylanase. Although xylanase from *Aureobasidium* is optimally active at 45° to 50°C (*14*), saccharifications were carried out at 37°C to reduce the possibility of inactivating other enzymes in crude preparations. Saccharifications typically involved 3 mL substrate samples, shaken at 200 rpm in 25 mL stoppered flasks. Reactions were terminated by boiling for 5 min to inactivate enzymes, and clarified by centrifugation prior to analysis. Saccharification controls (substrates incubated without enzymes) showed no detectable free sugars after 48 h. Saccharifications were monitored for microbial contamination, which was found to be extremely rare under conditions tested.

Sugar and Carbohydrate Analyses

Sugars were quantitated by high-performance liquid chromatography (HPLC), using an ion moderated partition chromatography column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad, Hercules, CA), eluted under isocratic conditions with 0.85 m*M* sulfuric acid at 0.65 mL/min at 35°C, detected with a Waters 410 differential refractometer (Milford, MA). The hemicellulose plus starch content of corn fiber substrates was estimated by trifluoroacetic acid digestion (*22,23*). The starch content of initial substrates was estimated by digestion with glucoamylase, as described by Osborn and Chen (*3*). Commercial cellulase was from *Penecillium funiculosum* (Sigma). All values are reported as the mean and standard deviation of replicate digestions.

RESULTS AND DISCUSSION

Sugar Composition of Corn Fiber

Corn fiber and AHP-pretreated corn fiber were hydrolyzed by TFA, and total neutral sugars were quantitated by HPLC. TFA hydrolyzes starch and hemicellulose, but not cellulose (*24*). By this analysis, corn fiber used in this study contained approximately 20% glucose, 20% xylose, and 10% arabinose (Table 1). By digestion with commercial glucoamylase,

we estimated that corn fiber included 18.2% ± 0.6% starch. AHP-pretreatment had no apparent effect on corn fiber digestion by TFA or glucoamylase. However, pretreatment had a dramatic effect on the susceptibility of corn fiber to cellulase digestion. Using a crude cellulase preparation from *P. funiculosum*, which included significant amounts of amylase activity, untreated corn fiber yielded 24.3 ± 3.5% glucose. Most of this sugar was probably derived from adherent starch, based on the susceptibility of untreated corn fiber to glucoamylase. Cellulase digestion of AHP-pretreated corn fiber produced nearly twice as much glucose (43.7 ± 5.0%). Most of this additional glucose was presumably derived from cellulose, although a portion may have also come from hemicellulose. AHP-pretreatment is known to increase the susceptibility of various celluloses to enzymatic digestion (*16*). AHP partially dissociates lignin and hemicellulose from cellulose, and induces physical changes in cellulose, such as swelling, increased pore volume, and decreased crystallinity (*16,25*). Using the phenol sulfuric acid method, we previously estimated that corn fiber contained approx 67% total saccharides (*26*).

Enzyme Activities from Cultures of *Aureobasidium* sp. Strain NRRL Y-2311-1 Grown on Xylan or Corn Fiber

Aureobasidium strain NRRL Y-2311-1 was cultured on oat spelt xylan, corn fiber, or AHP-pretreated corn fiber as growth substrates, and culture supernatants were assayed for levels of xylanase and other enzymes (Table 2). As shown, relatively low levels of xylanase were produced by cultures grown on untreated corn fiber, which may reflect limited accessibility of xylan in this material. Similarly, protease levels were low in untreated corn fiber-grown cultures (Table 2). All cultures produced pNP-acetyl esterase, which may enhance xylan degradation (*27*). Also found in all cultures were relatively low levels of CM-cellulase, pNP-arabinosidase, and pNP-xylosidase. Amylase levels were highest from cultures grown on corn fiber and especially pretreated corn fiber.

Saccharification of Corn Fiber by Enzymes from *Aureobasidium* sp. Strain NRRL Y-2311-1

As shown in Table 3, enzyme preparations released from 119 to 280 mg of glucose per g of untreated corn fiber. This sugar may predominantly represent adherent starch hydrolyzed over the course of the reaction, although glucose from hemicellulose or even cellulose might be included. Enzymes from cultures grown on xylan and AHP-pretreated corn fiber liberated significant amounts of xylose and arabinose from untreated corn fiber (Table 3). However, enzymes from untreated corn fiber cultures produced approximately equal amounts of xylose and xylobiose from untreated corn fiber, and failed to produce arabinose. This result was unexpected, since untreated corn fiber-grown cultures included both

Table 2
Enzyme Activities from Cultures of *Aureobasidium* sp. Strain NRRL Y-2311-1 Grown on Various Carbon Sources

Carbon source	Enzyme activities							
	Xylanase, IU/mL	pNP-Xylosidase, IU/mL	pNP-Arabinosidase, IU/mL	pNP-Acetyl esterase, IU/mL	Amylase, IU/mL	CM-Cellulase, IU/mL	Protease, U/mL	Protein mg/mL
Xylan	294 ± 15	0.001 ± 0.000	0.011 ± < 0.001	1.00 ± 0.03	0.012 ± 0.001	0.031 ± 0.002	0.045 ± 0.003	0.081 ± 0.002
Corn fiber	8 ± 1	0.022 ± 0.003	0.003 ± < 0.001	0.13 ± < 0.01	0.116 ± 0.006	0.030 ± 0.001	< 0.001	0.050 ± 0.006
Pretreated corn fiber	90 ± 23	0.046 ± 0.011	0.007 ± 0.001	0.69 ± 0.15	0.63 ± 0.12	0.055 ± 0.004	0.057 ± 0.019	0.121 ± 0.012

Table 3
Saccharification of Untreated Corn Fiber
by Enzymes from *Aureobasidium* sp. Strain NRRL Y-2311-1[a]

Enzyme source (cultures grown on)	Sugars release, mg/g substrate			
	Glucose	Xylose	Xylobiose	Arabinose
Xylan	119 ± 13	72 ± 12	<5	83 ± 3
Corn fiber	235 ± 35	46 ± 2	47 ± 10	<5
AHP-pretreated corn fiber	280 ± 43	85 ± 7	<5	56 ± 2

[a]All enzymes adjusted to final concentrations of 3.0 IU xylanase per mg substrate, 48 h digestion.

Table 4
Saccharification of AHP-Pretreated Corn Fiber
by Enzymes from *Aureobasidium* sp. Strain NRRL Y-2311-1[a]

Enzyme source (cultures grown on)	Sugars release, mg/g substrate			
	Glucose	Xylose	Xylobiose	Arabinose
Xylan	133 ± 4	102 ± 4	<5	114 ± 1
Corn fiber	451 ± 24	73 ± 1	21 ± 1	145 ± 1
AHP-pretreated corn fiber	490 ± 28	138 ± 8	<5	125 ± 8

[a]All enzymes adjusted to final concentrations of 3.0 IU xylanase per mg substrate, 48 h digestion.

pNP-xylosidase and pNP-arabinosidase activities (Table 2). It may be that some assay substrates, such as synthetic nitrophenyl derivatives, are inadequate models for corn fiber substrates, or that additional enzymes play a role in saccharification. Since untreated corn fiber-grown cultures were deficient in protease activity, it is also possible that corn fiber protein restricted saccharification.

AHP-pretreatment made an obvious difference in the gross appearance and behavior of corn fiber, which became lighter in color and more smoothly dispersed in suspensions. As reported above, AHP-pretreatment enhanced the hydrolysis of corn fiber by commercial cellulase. As shown in Table 4, pretreatment also made corn fiber more susceptible to saccharification by enzymes from *Aureobasidium*. Enzymes produced from 1.5- to 2-fold higher levels of pentose sugars from AHP-pretreated corn fiber than from untreated fiber (Tables 3 and 4). Enzymes produced on corn fiber also liberated much higher levels of glucose from pretreated than from untreated corn fiber.

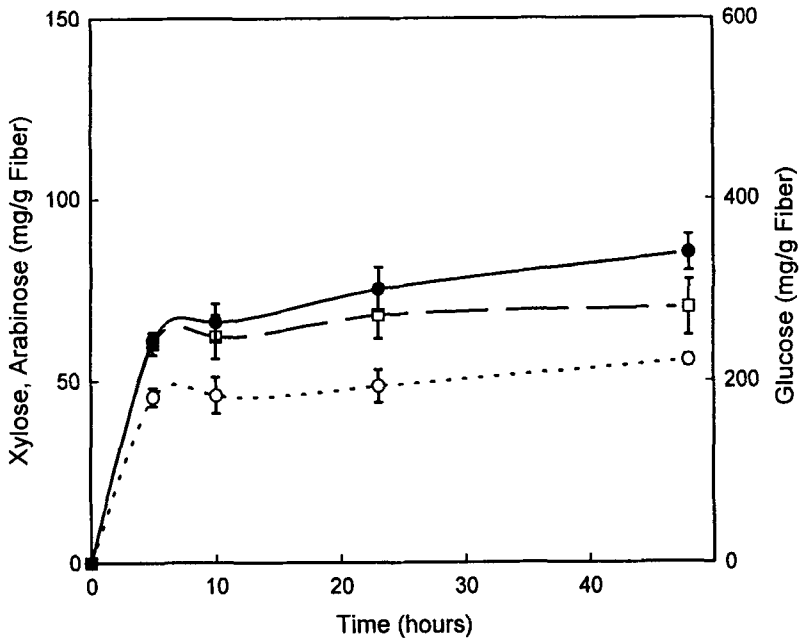

Fig. 1. Time-course of saccharification of untreated corn fiber by enzymes from *Aureobasidium* sp. strain NRRL Y-2311-1. □, Glucose; ●, Xylose; ○, Arabinose.

Time-Course of Enzymatic Saccharification

Figure 1 illustrates the saccharification of untreated corn fiber over a 48 h period, using crude enzymes from cultures grown on AHP-pretreated corn fiber. As shown, approx 200 mg of glucose, 60 mg of xylose, and 50 mg of arabinose/g corn fiber were solubilized within the first 10 h of digestion. Only xylose significantly increased (to about 90 mg/g fiber) with additional time. These results suggest that most of the adherent starch and a significant portion of corn fiber hemicellulose were accessible to enzymes without pretreatment.

Figure 2 illustrates the saccharification of AHP-pretreated corn fiber using the same enzyme preparation. Once again, sugars were most rapidly liberated during an initial 10 h period, although considerably higher levels of xylose and arabinose were produced. Based on TFA analysis of corn fiber composition, essentially all arabinose was solubilized in this phase. Glucose and xylose concentrations continued to increase, although more slowly, during the next 38 h. Xylose levels doubled during this secondary phase, to 138 mg/g corn fiber, representing about 70% of total xylose as estimated by TFA analysis. Thus, AHP pretreatment appeared to increase the portion of corn fiber hemicellulose and cellulose that was readily susceptible to enzymatic attack.

Although corn fiber hemicellulose may be efficiently hydrolyzed by chemical means (*3,28*), enzyme preparations have previously been reported to saccharify corn fiber very poorly (*7*). Gattinger et al. (*29*) found that

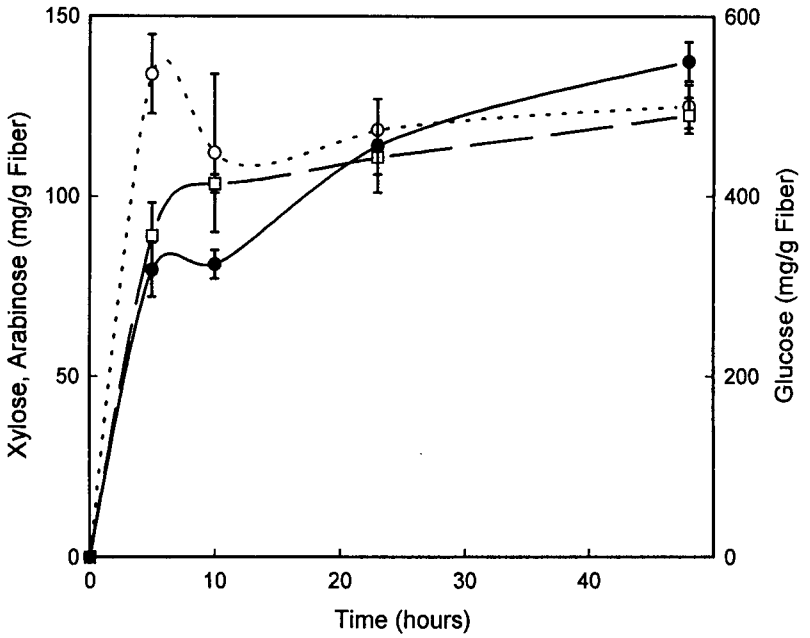

Fig. 2. Time-course of saccharification of AHP-pretreated corn fiber by enzymes from *Aureobasidium* sp. strain NRRL Y-2311-1. □, Glucose; ●, Xylose; ○, Arabinose.

xylanase from *Trichoderma reesei* effected only about 20% saccharification of corn bran. Other studies have reported 10 to 28% of initial xylose released from other hemicelluloses by enzymatic hydrolysis (*30,31*).

CONCLUSIONS

Crude enzymes from *Aureobasidium* sp. strain NRRL Y-2311-1 were effective in saccharification of corn fiber from wet-milling. Under conditions tested, up to an estimated 70% of initial xylose and 100% of arabinose were liberated as free monosaccharides. In addition, most or all associated starch was apparently hydrolyzed to glucose.

Results suggest that a limiting factor in the enzymatic saccharification of corn fiber was the accessibility of cellulose and associated hemicellulose. Pretreatment with alkaline hydrogen peroxide (AHP) increased the susceptibility of corn fiber to enzymes.

The cost of enzymes is often a limiting constraint for industrial applications. However, since best results were obtained using crude enzymes from cultures grown on corn fiber itself, it is possible that an integrated process could be designed that would include the production of enzymes.

Pretreatment of corn fiber with AHP is also a relatively expensive step, and alternatives to this process are being explored. However, results suggest that a practical yield tradeoff might be obtained by pretreating only corn fiber used in enzyme production. Using crude enzymes

from cultures grown on pretreated corn fiber, untreated corn fiber was saccharified to yield approx 42% of initial xylose and 56% of arabinose. Given the abundance and low cost of corn fiber, a more complete saccharification might not be cost effective. Residual fiber from partial saccharifications could be recycled into animal feed products.

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