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Enzyme Production by Industrially Relevant Fungi Cultured on Coproduct From Corn Dry Grind Ethanol Plants

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Abstract

Distillers dried grain with solubles (DDGS) is the major coproduct produced at a dry grind ethanol facility. Currently, it is sold primarily as a ruminant animal feed. DDGS is low cost and relatively high in protein and fiber contents. In this study, DDGS was investigated as carbon source for extracellular hydrolytic enzyme production. Two filamentous fungi, noted for their high cellulolytic and hemicellulolytic enzyme titers, were grown on DDGS: *Trichoderma reesei* Rut C-30 and *Aspergillus niger* NRRL 2001. DDGS was either used as delivered from the plant (untreated) or after being pretreated with hot water. Both microorganisms secreted a broad range of enzymes when grown on DDGS. Higher xylanase titers were obtained when cultured on hot water DDGS compared with growth on untreated DDGS. Maximum xylanase titers were produced in 4 d for *A. niger* and 8 d for *T. reesei* in shake flask cultures. Larger amounts of enzymes were produced in bioreactors (5 L) either equipped with Rushton (for *T. reesei*) or updraft marine impellers (*A. niger*). Initial production titers were lower for bioreactor than for flask cultures, especially for *T. reesei* cultures. Improvement of enzyme titers were obtained using fed-batch feeding schemes.

Index Entries: *Aspergillus niger*; biomass; DDGS; cellulases; hemicellulases; *Trichoderma reesei*.

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†Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Introduction

The United States corn ethanol market is rapidly growing; production is 4.26 billion gal/yr (National Renewable Fuel Association, 2005) and is mandated to reach 7.5 billion gal/yr by 2012. The soluble and solid residuals from the ethanol fermentation are combined and marketed as an animal feed ingredient under the name of distillers dried grain with solubles (DDGS). Approximately 17 pounds of DDGS is produced per bushel of corn. Expanding ethanol production has raised concerns about finding a sufficiently sized market for DDGS. DDGS is currently priced at only $65/t and the selling price has been declining for the last several years. Therefore, there is a need to develop further markets for DDGS to preserve its future value.

One possible use for DDGS is for the production of industrial enzymes. DDGS is rich in protein (31%), fat (11%), and fiber/carbohydrates (44%) *(1)*. DDGS's low-cost and relatively high protein and carbohydrate contents make it a promising feedstock for industrial fermentations. In particular, the complex carbohydrate profile of DDGS—it includes starch, cellulose, and a complex xylan—should be particularly well suited for production of carbohydrolytic enzymes, such as hemicellulases and cellulases. Cellulases and hemicellulases have multiple industrial uses including biodegradation of lignocellulosic material in biomass conversion, animal feed, foods, textiles, and biopulping in the paper and pulp industry *(2,3)*. The major obstacles for further industrial application of these enzymes are their cost of production and low fermentation yields. In an attempt to overcome these problems, the production of such enzymes by microbial strains in the presence of inexpensive substrates has been investigated *(4)*.

The most commonly used microorganisms for production of acidic hydrolytic enzymes are filamentous fungi. *Trichoderma reesei* is the most widely studied cellulolytic microorganism and is commonly used for production of both cellulases and xylanases. The most important species of the *Aspergillus* genus for the production and utilization of enzymes are *A. oryzae* and members of the group of black aspergilli. Black aspergilli are favored for production of industrial enzymes because they secrete high levels of proteins, are easily cultured in submerged fermentations, and several species are generally regarded as safe for food and feed applications *(5)*, including *A. niger (6)*. In this article, DDGS was used as a substrate for producing hemicellulases by both *T. reesei* and *A. niger*. DDGS has complex carbohydrate structures. To make the DDGS more digestible to the fungi, the DDGS was pretreated with liquid hot water (LHW) (160°C, 20 min) before being added to the culture. LHW is a promising pretreatment that has been shown to be very effective in preparing corn stover *(7)* and corn fiber *(8)* for subsequent cellulose digestion and ethanol fermentation. The method is currently the subject of an industrial scale-up effort at a 100 million gal ethanol/yr corn wet mill facility (Aventine Renewable Energy, Pekin, IL). However, treating with LHW does not saccharify the

xylan sugars, thereby, readying them for fermentation. Instead, this method relies on using hemicellulases for saccharification. So, as a first application for the hemicellulases described in this article, they were applied to LHW–xylan for production of sugars.

Materials and Methods

Materials, Microbial Strains, and Medium

Microbial strains were obtained from the Agricultural Research Service (ARS) Culture Collection (NCAUR, Peoria, IL). DDGS was generously gifted by Big River Resources (West Burlington, IA). *T. reesei* Rut C-30 and *A. niger* NRRL 2001 were routinely propagated in potato dextrose broth (PD) (0.4% potato and 2.0% dextrose) supplemented with 2% agar for solid medium. All chemicals and medium ingredients were of research quality and were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemicals (St. Louis, MO).

LHW Pretreatment of DDGS

For flask experiments, 2.0 g of DDGS (10% moisture) was combined with 8 mL water in a stainless steel tube reactor and treated at 160°C for 20 min using a fluidized heating bath. The tube reactors and heating bath arrangement have been previously described *(7)*. The pretreated DDGS, along with 20 mL of distilled wash water used to remove residual solids, was transferred to a presterilized 250-mL baffled Erlenmeyer flask. For destarched DDGS, following the heat treatment, the solids were recovered on glass fiber filter (GF/A, Whatman, England) and washed with distilled water to further remove solids. The solids were transferred to a tared 250-mL Erlenmeyer flask and distilled water was added until the solids and liquid had a weight of 30.0 g. The material was next autoclaved for 15 min. Pretreated DDGS for use in fed-batch experiments was prepared in multiple batches as follows: 45.0 g of DDGS was added to 105 mL of water and treated in the same conditions as described previously. The resulting material was transferred to flasks after three washes with 50 mL water (final volume = 300 mL).

T. reesei *and* A. niger *Flask Cultures*

Two inoculation loops of *T. reesei* or *A. niger* were transferred to shake flasks (250 mL) containing 50 mL PD medium. Flasks were shaken at 250 rpm at 28°C for 48 h. For both microorganisms, a 5% (v/v) inoculum was transferred to 50 mL of production medium in a 250-mL Erlenmeyer flask. The production media for *T. reesei* and *A. niger* contained per liter 15.0 g KH_2PO_4, 20.0 g corn steep liquor (Sigma Chemicals), 0.5 g NH_4SO_4, 0.5 g $Mg(SO_4)_2{\cdot}7H_2O$, 1.0 mL Tween 80, and 30 mL solution of untreated or HW-treated DDGS containing a total of 2.0 g DDGS. The basal medium was

adjusted to pH 4.8 and autoclaved separately from the HW treated DDGS. Both microorganisms were grown at 28°C with agitation (250 rpm) for 8 d. Flasks were sampled daily (2 mL). Alternatively, HW-treated DDGS was washed to remove starch before being used for fermentation.

T. reesei *and* A. niger *Bioreactor Cultures*

Two inoculation loops of *T. reesei* or *A. niger* were transferred to Erlenmeyer flasks (250 mL) containing 50 mL PD medium. Flasks were shaken at 250 rpm at 28°C for 48 h. For both microorganisms, a 5% (v/v) inoculum was transferred to 450 mL of PD medium in a 2800-mL flask and grown under identical conditions as previously described. These cultures were then used to inoculate the bioreactor at 5% (v/v).

Bioreactor runs were conducted in electronically controlled 5-L laboratory bioreactors (BIOSTAT B, B. Braun Biotech International, Melsungen, Germany). The production medium was supplemented with HW-DDGS (at the same concentration used for flask fermentations) and 1 mL of antifoam (PPG2000 from Bayer, Pittsburgh, PA). The oxygen level was set at 20% of saturation and controlled by varying stirring (300–900 rpm). The pH was set at 4.8 and controlled by automatic additions of either 3 *M* H_3PO_4 or 4 *M* NH_4OH. Samples (approx 10 mL) were taken daily, centrifuged, and the recovered liquid stored at –20°C. At the end of the fermentation, the broth was harvested, centrifuged to remove cell mass, and the recovered liquid (containing the excreted enzymes) was stored at –20°C. Fed-batch fermentations were run in a similar manner to batch fermentations, except they were manually fed at days 3, 6, and 9 by adding 300 mL of a 15% (w/v) DDGS solution.

LHW-Treated DDGS Digestion Assays

A stock supply of LHW pretreated DDGS was prepared as follows for the digestion assay. Pipe reactors were each filled with 1.5 g DDGS (90% wt dry) and 8.5 mL H_2O. Using a fluidized heating bath, the reactors were heated to 160°C and kept at that temperature for 20 min. The pipes were quickly cooled by quenching in water. The contents of eight reactors were transferred to a single flask along with 10 mL of 1 *M* sodium citrate buffer (pH 4.5, final concentration of 50 m*M*) and 1 mL of thymol stock solution (50.0 g/L in 70% [v/v] ethanol). Enough water was added for a final volume of 200 mL and a stir bar added to the flask. For individual enzyme assays, DDGS solution was mixed on a stirring plate and 4-mL aliquots transferred to scintillation vials. Following that, appropriate enzyme mixtures were added to the vials. The vials were mixed using a mini tube roller (Belco Glass, Inc., Vineland, NJ) placed in a constant temperature incubator (Innova 4230, News Brunswick, NJ) set to 50°C. The reactions were allowed to proceed for 72 h. Digestion reactions were clarified by centrifugation and the supernatant analyzed for total soluble and monomeric carbohydrates as described in the following sections.

Enzyme Assays

Enzyme activities in the presence of 1% oat-spelt xylan and carboxymethylcellulose (CMC, low viscosity, Sigma Chemicals), and 2.5 m*M* *p*-nitrophenyl (*p*-NP) conjugated substrates were determined at 50°C in the presence of 50 m*M* sodium acetate buffer, pH 4.8 using published methods. Filter paper activity was assayed as described by Mandels et al. *(9)*. The release of reducing sugars was determined in according to Miller *(10)*. One unit of cellulase (for CMC as substrate) and xylanase activities was defined as the release of one µmol of either glucose or xylose per min. For *p*-NP conjugated substrates, one unit of activity was defined as one µmol of *p*-NP released per min.

Feruloyl esterase activity was assayed measuring the conversion of methyl-ferulate (prepared as 100 m*M* in 50% DMSO [v/v] and added to a final concentration of 2 m*M*) to ferulate. Ferulic acid and methyl ferulate were measured by reverse phase SpectraSYSTEM liquid chromatography system (Thermo Finnigan, San Jose, CA) using an Inertsil C18 xolumn (5 µm, ODS3, PN 0396-250X046, Varian, Torrance, CA) combined with a UV2000 ultraviolet detector (310 nm; Thermo Finnigan). Samples were run at room temperature and eluted at 0.8 mL/min with a linear gradient from 5 to 50% acidified methanol (containing 0.25% acetic acid) run over 15 min. One unit of feruloyl esterase activity was defined as the release of one µmol of ferulic acid per min.

Analysis of Soluble Carbohydrates

Total soluble carbohydrates were analyzed by HPLC after being hydrolyzed by treating with 2 *N* trifluoroacetic acid (TFA) for an hour at 100°C, as previously described *(2)*. Samples were analyzed for sugars and acids using a SpectraSYSTEM liquid chromatography system with an organic acids column (Aminex HPX-87H Column, 300 × 7.8 mm^2, Bio-Rad Laboratories, Inc, Hercules, CA) and a refractive index detector (RI-150, Thermo Finnigan).

Results and Discussion

Fermentation Results Using Untreated and LHW-Treated DDGS

T. reesei and *A. niger* were grown on either untreated or hot water treated (20 min, 160°C) DDGS and the cultures sampled for production of enzymes. The goal of this experiment was to determine if LHW-treated or -untreated DDGS would be a more suitable carbon source for producing enzymes. The plotted data demonstrate that hot water treated DDGS gave higher final xylanase titers for both *A. niger* and *T. reesei* cultures (Fig. 1A,B). The xylanase enzyme profile for *T. reesei* also showed a long lag phase (5 d) for xylanase production. One possible explanation for the delay in xylanase production is that residual starch present in the DDGS is repressing its production. To test this hypothesis, hot water treated DDGS was

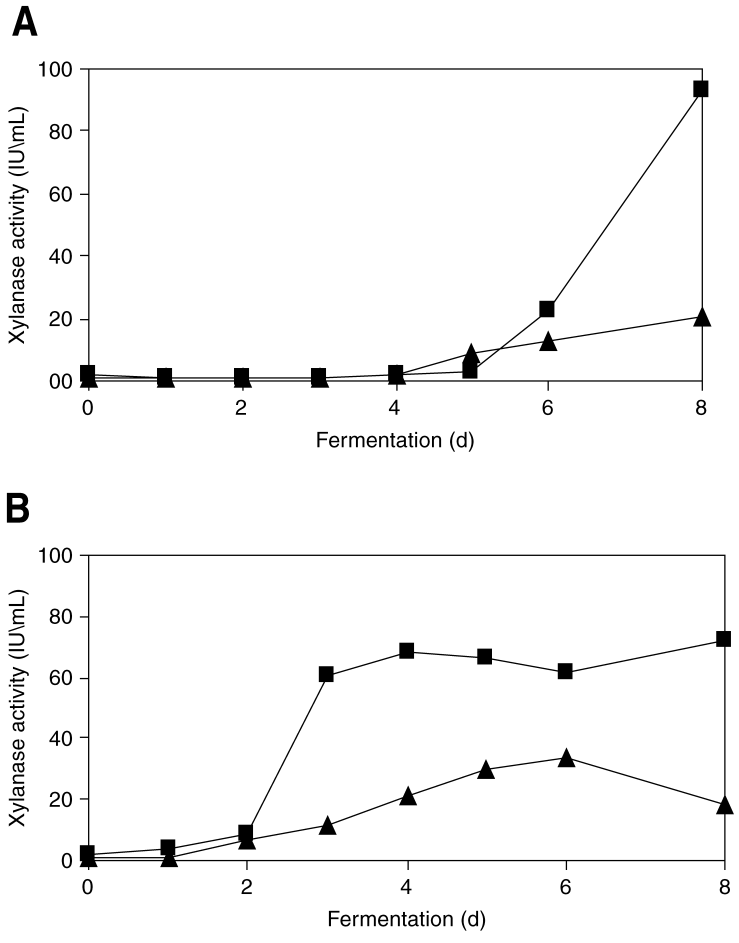

Fig. 1. Xylanase production under flask culturing conditions of *T. reesei* Rut C-30 **(A)** and *A. niger* NRRL 2001 **(B)** grown on either DDGS (▲) or hot water pretreated DDGS (■).

washed to remove starch before being used for fermentation. Earlier results have shown that LHW pretreatment solubilizes starch. Therefore, by only adding the washed solids following LHW pretreatment, starch was effectively removed from the medium. Disappointedly, removing the starch failed to shorten the lag phase and only served to lower the final xylanase titer (Fig. 2).

An alternative explanation for the long lag phase of xylanase production might be the adsorption of the xylanases to insoluble DDGS during the early stage of *T. reesei* cultures. A major xylanase produced by *T. reesei* has been found to bind strongly to insoluble xylan (unpublished). This statement is in agreement with the observation that the HW–DDGS resulted in earlier production of xylanase activities than untreated DDGS did (Figs. 1 and 2). Other enzyme activities were also measured. The results are summarized in Table 1. As expected, based on the complex composition of the

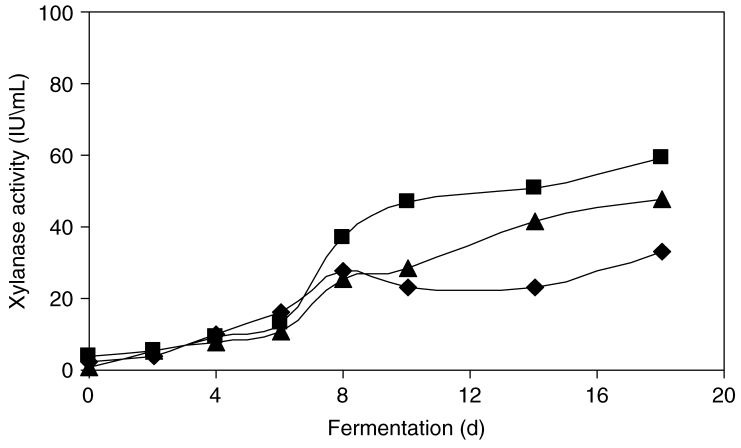

Fig. 2. Xylanase production under flask culturing conditions of *T. reesei* Rut C-30 grown on DDGS (◆), hot water pretreated DDGS (■), or washed DDGS for starch removal (▲).

DDGS substrate, a broad range of different enzyme activities were detected. The final enzyme titers using hot water treated DDGS as the substrate were similar or higher than those using DDGS for both microorganisms. A broad range of different enzyme activities was also produced by *T. reesei* QM 9414 and *T. reesei* Rut C-30 grown on corn fiber fractions *(11)*.

As this is the first time DDGS has been reported as a feedstock for production of xylanase, no direct comparison can be made of our results with those from literature. However, there are previous studies that report xylanase yields for fungi grown on other agricultural residues (summarized in Table 2). The xylanase enzyme titers obtained for both microorganisms in shake flask conditions were higher than *Penicillium janthinellum (12)* cultivated in corn cob, and oat husk hydrolysates and *Thermomyces lanuginosus* CAU 44 *(13)* cultivated in wheat straw and rice straw (Table 2). Also, the results for *T. reesei* and *A. niger* were comparable with *T. reesei* QM 9414 and Rut C-30 cultivated in crude corn fiber. On the other hand, much higher enzyme titers were obtained for these two *Trichoderma* strains when grown in corn fiber arabinoxylan (Table 2) *(11)*. Also, Jiang et al. *(13)* reported a xylanase activity of 3260 U/mL when cultivating *T. lanuginosus* CAU 44 in corncob xylan (Table 2).

Batch and Fed-Batch Bioreactor Cultures for Production of Enzymes

The fungi were next grown in bioreactors to produce larger volumes of enzymes. The first attempt to grow *A. niger* in a bioreactor was unsuccessful (data not shown) because the culture was too viscous to obtain good mixing. It was found that replacing two Rushton impellers with the marine type impellers achieved more homogenous mixing in the reactor. However, the xylanase titer was still lower for *A. niger* (48.8 IU/mL) in the bioreactor compared with flask cultures. In an attempt to increase enzyme titers, the fungi were next grown under fed-batch conditions using the marine impellers.

Table 1
Other Measured Enzyme Activities From Untreated and LHW Treated DDGS Flask Fermentations

Organism	Substrate	Filter paper (50.0 mg) (FPU)	CMC (1%) (CMCase IU/mL)	*p*-NPG (2.5 m*M*) (β-glucosidase IU/mL)	*p*-NP-gal (2.5 m*M*) (α-galactosidase IU/mL)	*p*-NPX (2.5 m*M*) (β-xylosidase IU/mL)	*p*-NPA (2.5 m*M*) (α-arabinofuranosidase IU/mL)	Ferulic acid (100 mM) (feruloyl esterase IU/mL)
A. niger	Untreated	0.96[a]/0.00[b]	3.68[a]/8.21[b]	1.73[a]/5.71[b]	2.99[a]/5.6[b]	0.37[a]/1.20[b]	1.46[a]/1.86[b]	0.12[a]/0.01[b]
A. niger	LHW DDGS	1.11[a]/0.39[b]	4.20[a]/22.0[b]	2.13[a]/3.60[b]	2.13[a]/4.13[b]	0.78[a]/1.33[b]	1.60[a]/1.60[b]	0.22[a]/0.37[b]
T. reesei	Untreated	0.50[a]/0.70[b]	2.10[a]/21.8[b]	0.25[a]/1.53[b]	0.28[a]/1.86[b]	0.22[a]/1.06[b]	0.40[a]/0.99[b]	nd[c]
T. reesei	LHW DDGS	0.39[a]/1.16[b]	1.29[a]/44.6[b]	0.61[a]/4.50[b]	0.00[a]/0.68[b]	0.80[a]/2.93[b]	0.98[a]/1.86[b]	nd[c]

[a]Activity measured after 4 d.
[b]Activity measured after 8 d.
[c]Not determined (*T. reesei* does not produce feruloyl esterase).

Table 2
The Effect of Various Carbon Sources on the Production of Xylan-degrading Enzymes by Different Microorganisms in Submerged Cultivations

Organism	Substrate	Cultivation conditions	Substrate for determination of enzyme activity	Xylanase (IU/mL)	Reference
T. reesei Rut C-30	LHW–DDGS	Shake flask 28°C, 132 h	1% oat spelt xylan	93.4	This work
A. niger NRRL 2001	LHW–DDGS	Shake flask 28°C, 132 h	1% oat spelt xylan	72.2	This work
P. janthinellum CRC 87M-115	Corncob	Shake flask 30°C, 132 h	1% birchwood xylan suspension	55.3	*12*
P. janthinellum	Oat husk	Shake flask 30°C, 132 h	1% birchwood xylan suspension	58.8	*12*
T. lanuginosus	Wheat straw	Shake flask 50°C, 96 h	1% birchwood xylan suspension	53	*13*
T. lanuginosus	Rice straw	Shake flask 50°C, 96 h	1% birchwood xylan suspension	25	*13*
T. lanuginosus	Corncob xylan	Shake flask 50°C, 96 h	1% birchwood xylan suspension	3260	*13*
T. reesei QM 9414	Corn fiber	Shake flask 28°C, 192 h	1% oat spelt xylan	98.5	*11*
T. reesei Rut C-30	Corn fiber	Shake flask 28°C, 192 h	1% oat spelt xylan	86.1	*11*
T. reesei QM 9414	Corn fiber xylan	Shake flask 28°C, 192 h	1% oat spelt xylan	221	*11*
T. reesei Rut C-30	Corn fiber xylan	Shake flask 28°C, 192 h	1% oat spelt xylan	621	*11*
T. reesei Rut C-30	LHW–DDGS	4 L fermentation (fed batch) 28°C, 264 h	1% oat spelt xylan	148	This work
A. niger NRRL 2001	LHW–DDGS	4 L fermentation (fed batch), 28°C, 264 h	1% oat spelt xylan	64.3	This work
P. pinophilum NTG III/6	Avicel + barley straw	12 L fermentation, 35°C, 240 h	1% birchwood xylan suspension	114	*14*
A. awamori mutant AANTG 43	Ball-milled oat straw	4 L fermentation 30°C, 48 h	2% oat spelt xylan	820	*15*

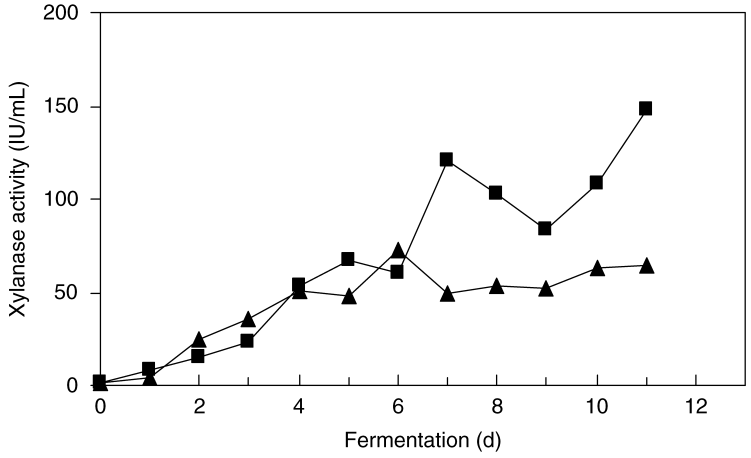

Fig. 3. Xylanase production of *T. reesei* Rut C-30 (■) and *A. niger* NRRL 2001 (▲) in repeated fed-batch culture conditions. Three cycles of medium feeding were used (after 3, 6, and 9 d of growth).

Table 3
Other Measured Enzyme Activities From Hot Water Treated DDGS Fermentations Under Fed-Batch Conditions After 11 d of Culturing

Organism	Filter paper (50.0 mg) (FPAase IU/mL)	CMC (1% [w/v]) (CMCase IU/mL)	*p*-NPG (2.5 m*M*) (β-glucosidase IU/mL)	*p*-NP-gal (2.5 m*M*) (α-galactosidase IU/mL)	*p*-NPX (2.5 m*M*) (β-xylosidase IU/mL)	*p*-NPA (2.5 m*M*) (α-arabinofuranosidase IU/mL)	Ferulic acid (100 m*M*) (feruloyl esterase IU/mL)
T. reesei	1.90	59.8	4.52	5.59	5.98	8.51	ND[a]
A. niger	0.31	5.50	4.12	8.65	1.99	6.38	0.38

[a]Not determined (*T. reesei* does not produce feruloyl esterase).

Growing the fungi under fed-batch conditions achieved higher xylanase titers: 3X improved for *T. reesei* (148 IU/mL) and about 1.5X for *A. niger* (64 IU/mL) (Fig. 3).

In comparison with other microorganisms grown in bioreactors (Table 3), the xylanase titers obtained for *T. reesei* and *A. niger* were higher than those reported for *P. pinophilum* NTG III/6 *(14)*, but considerable lower than those for a *A. awamori* mutant (AANTG 43) *(15)*. Other enzyme activity titers are given in Table 3. The data obtained demonstrate that under fed-batch culturing conditions a broad range of different enzyme activities were produced by both fungi. The final enzyme titers were either similar or higher in comparison with the results obtained for flask culturing cultures, except for cellulase production by *A. niger*.

Enzymatic Digestion of Pretreated DDGS

As a first application, the enzymes were evaluated for production of sugars from LHW–DDGS. It was hypothesized that the enzymes would be particularly well suited for this application because the fungi were cultured on LHW–DDGS. LHW is a favorable pretreatment method that has been investigated for preparing biomass for conversion to ethanol. However, although this pretreatment method is effective at increasing the digestibility of cellulose, it does not completely saccharify the xylan fraction. As a result, the method relies on hemicellulases for completing the conversion of xylan to monosaccharides for ethanol fermentation. Converting xylan from DDGS is particularly challenging because it is largely made up of corn pericarp xylan, which is noted in the literature as being particularly recalcitrant to enzymes.

We examined the effects of adding enzymes produced by *T. reesei* and/or *A. niger*, produced in separate fed-batch cultures that used pretreated DDGS, for digesting pretreated DDGS. When added separately, *A. niger* enzymes released more xylose (64%) than *T. reesei* (48%) (Fig. 4A,B), which was expected as a result of superior auxiliary activities (xylanase, feruloyl esterase) found in the *A. niger* preparation. On the other hand, *T. reesei* enzymes released more glucose (84%) compared with *A. niger* (77%) (Fig. 4A,B). This result was expected because *T. reesei* is known as a good cellulase producer with superior activities compared with *A. niger*. The maximum yields for glucose and xylose (99 and 71%, respectively) were obtained using the combined enzymes, albeit there was not improvement in the yield of arabinose (Fig. 4C). The lack of complete conversion of xylan demonstrates the recalcitrant nature of the substrate and the need to identify limiting factors during future research.

Conclusion

Untreated and LHW–DDGS were evaluated as substrate for producing hydrolytic enzymes using *A. niger* and *T. reesei*. The cultures had xylanase titers higher or comparable with those reported for fungi cultured on other agricultural residues, except for when purified xylan was used as the carbon source. Not surprisingly, these latter gave much higher xylanase titers, but the isolated xylan fractions can also be expected to be a much more expensive fermentation substrate than LHW–DDGS. In addition to having high xylanase titers, the LHW–DDGS grown cultures also contained cellulase and multiple other activities. The mixture of *A. niger* and *T. reesei* enzymes was found to be a highly effective hemicellulase preparation as evidenced by its ability to convert 71% of the xylan from LHW–DDGS to xylose.

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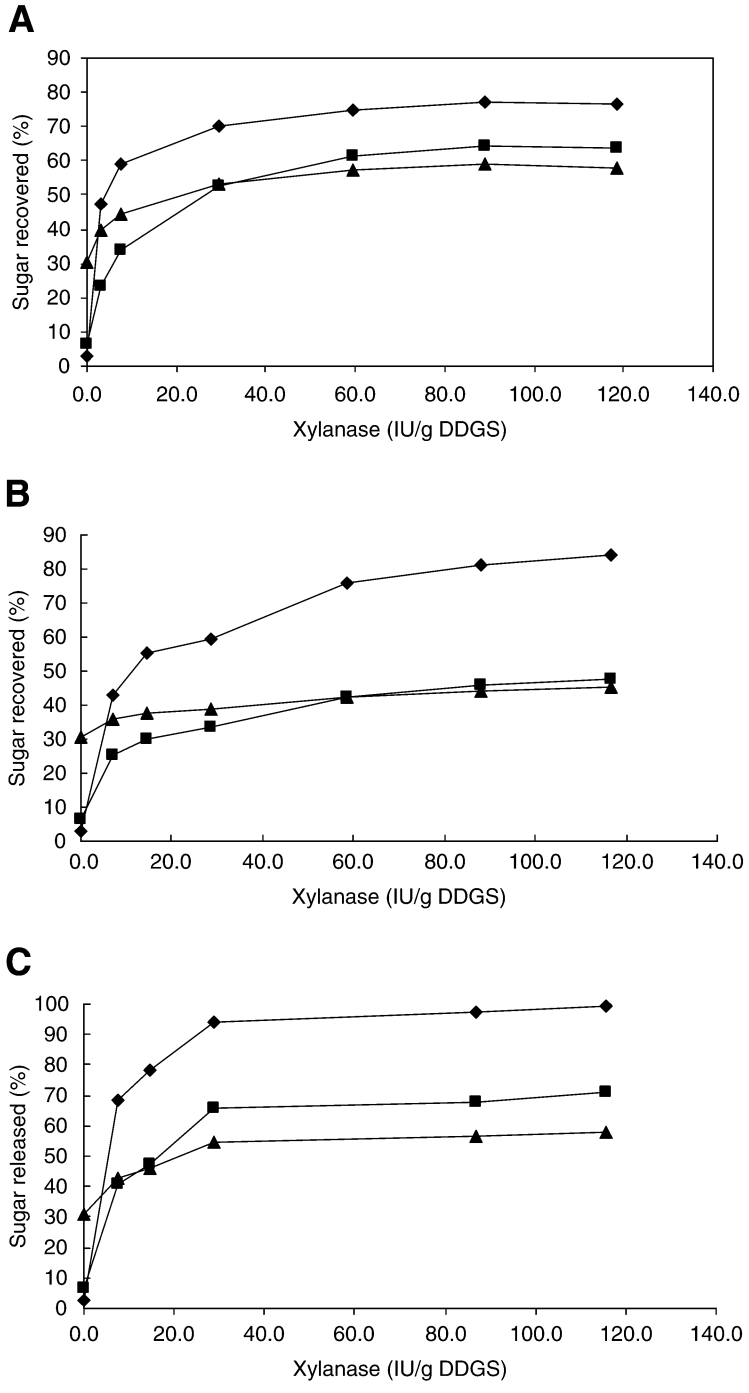

Fig. 4. Sugar yields from saccharifying hot water pretreated DDGS hydrolysates using varying loading of *A. niger* NRRL 2001 **(A)**, *T. reesei* Rut C-30 **(B)**, and an equal mixture of *T. reesei* Rut C-30 and *A. niger* NRRL 2001 enzyme preparation **(C)**. Glucose (◆), xylose (■), and arabionose (▲).

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