Heterologous Expression of *Aspergillus niger* β-D-Xylosidase (XlnD): Characterization on Lignocellulosic Substrates

**Michael J. Selig · Eric P. Knoshaug ·
Stephen R. Decker · John O. Baker ·
Michael E. Himmel · William S. Adney**

Abstract The gene encoding a glycosyl hydrolase family 3 xylan 1,4-beta-xylosidase, *xlnD,* was successfully cloned from *Aspergillus niger* strain ATCC 10864. The recombinant product was expressed in *Aspergillus awamori*, purified by column chromatography, and verified by matrix-assisted laser desorption ionization, tandem time of flight (MALDI-TOF/TOF) mass spectroscopy of tryptic digests. The T_{max} was determined using differential scanning microcalorimetry (DSC) to be 78.2 °C; the K_m and k_{cat} were found to be 255 μM and 13.7 s^{-1}, respectively, using pNP-β-D-xylopyranoside as substrate. End-product inhibition by D-xylose was also verified and shown to be competitive; the K_i for this inhibition was estimated to be 3.3 mM. XlnD was shown to efficiently hydrolyze small xylo-oligomers to monomeric xylose, making it a critical hydrolytic activity in cases where xylose is to be recovered from biomass conversion processes. In addition, the presence of the XlnD was shown to synergistically enhance the ability of an endoxylanase, XynA from *Thermomyces lanuginosus*, to convert xylan present in selected pretreated lignocellulosic substrates. Furthermore, the addition of the XynA/XlnD complex was effective in enhancing the ability of a simplified cellulase complex to convert glucan present in the substrates.

Keywords Xylosidase · Xylanase · Xylo-oligomer · Pretreatment · Lignocellulose · Cellobiohydrolase

Introduction

Xylan is a major structural heterogeneous polysaccharide found in plant biomass and represents the second most abundant polysaccharide in the biosphere, after cellulose. The abundance of xylan and its integral association within the plant cell wall makes it a key

M. J. Selig (✉) · E. P. Knoshaug · S. R. Decker · J. O. Baker · M. E. Himmel · W. S. Adney
National Renewable Energy Laboratory, Chemical and Biosciences Center, 1617 Cole Blvd., Golden, CO 80401, USA
e-mail: michael_selig@nrel.gov

target for hydrolysis and conversion to fermentable sugars. However, because of its complex structure and heterogeneity, the natural bioconversion of xylan requires a diverse suite of synergistic enzymes [1, 2]. After pretreatment, it is often necessary to enzymatically hydrolyze the remaining xylan and xylo-oligomers present in hydrolyzates. In the context of a large-scale biorefinery, xylan breakdown and conversion represents a significant cost barrier to the development of the lignocellulose-to-ethanol industry [3].

To convert xylan to chemical fuels, it must first be reduced to monomeric components, primarily D-xylose; however, other side-chain sugars such as arabinose are also candidates for conversion. Enzyme activities associated with the complete hydrolysis of the xylan backbone include xylan 1,4-β xylosidase (β-xylosidase, EC 3.2.1.37) which removes successive D-xylose residues from the nonreducing ends of xylo-oligosaccharides and endo-1,4-β-xylanase (EC 3.2.1.8), which randomly cleaves 1,4-β-D-xylosidic backbone linkages releasing xylo-oligomers of variable lengths [1, 4]. Exo-xylanases are also involved in this process; however, few examples are known [5]. The random action of endoxylanases typically results in only a portion of xylan hydrolyzed being broken down to monomeric xylose, which makes the presence of β-xylosidase activity key for recovering monomeric xylose from xylan [4]. The production of such xylanolytic activities has been observed in a number of organisms, although the historical production of xylanases for industry, primarily for biobleaching of pulp and paper, has typically been done in fungi [6, 7]. Furthermore, the discovery and improvement of these enzymes is the focus of considerable research for the development of biorefineries and in the pulp and paper industry.

In this study, we report the successful heterologous expression of a functional and well-studied *Aspergillus niger* β-xylosidase (*xlnD*) in *Aspergillus awamori*. The intent was to build a sizable stock of this enzyme activity for use throughout a number of enzymatic studies. Previous experience in our laboratory with the *A. awamori* expression system [8], particularly regarding expression levels and the ease of product purification, led to its' selection as the host organism. After purification, the kinetic and thermal properties of the enzyme were assessed to confirm whether or not the expression produced a functioning form of the protein. To show the utility of the enzyme, we have probed the ability of XlnD to hydrolyze small xylo-oligomers and investigated the ability of this enzyme to work in concert with an endoxylanase, XynA from *Thermomyces lanuginosus,* to hydrolyze xylan in pretreated lignocellulosic substrates. We have also investigated the ability of the XynA/XlnD complex to work in synergy with a simplified cellulase mix to more effectively convert glucan present in pretreated substrates.

Materials and Methods

Cloning of xlnD Sequence

Genomic DNA from *A. niger* (ATCC 10864) grown in CM-glucose media [8] was isolated using the DNeasy Plant Maxi Kit as per the manufacturer's directions (Qiagen, cat. no. 68161). The coding sequence for the gene XlnD (Z84377) was amplified with the polymerase chain reaction (PCR) using PfuTurbo polymerase (Stratagene, cat. no. 600250) and the MasterAmp PCR Optimization Kit with ammonium sulfate (Epicentre, cat. no. MOS02N). Primers used for the PCR are listed in Table 1. The following touchdown PCR cycle parameters were used: denaturing parameters, 95 °C for 1 min, annealing parameters, 60 °C for 30 s, extension parameters, 72 °C for 3 min (repeat previous cycles lowering the annealing temperature by 2 °C with every cycle until an annealing temperature of 52 °C is

Table 1 PCR Primers used for cloning and mutagenesis; start and stop codons are underlined, restriction sites used for cloning are in bold (*BbvCI* and *XbaI*) and the base pair change to knockout the BbvCI restriction site is underlined in bold.

Primer name	Sequence
XlnDfor	**CCTCAGC**A<u>ATG</u>GCGCACTCAATGTCTCGT
XlnDrev	**TCTAGA**C<u>TA</u>CTCCTTCCCCGGCCACTTCAGCA
XlnDmutflor	GCTGCCGCTGCCGCC**C**GAGGCCATCCTCGCC
XlnDmutrev	GGCGAGGATGGCCTC**G**GCGGCAGCGGCAGC

reached, then 28 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 3 min, followed by a final extension at 72 °C for 10 min). The PCR product was cloned into pCR2.1-TOPO (Invitrogen, cat. no. K4510–20) for recovery and mutagenesis to remove the internal *BbvCI* site. Site-directed mutagenesis was preformed on the pCR2.1-TOPO-XlnD construct using the QuickChange Site-Directed Mutagenesis Kit as described (Stratagene, cat. no. 200518). The primers used for site-directed mutagenesis are listed in Table 1. The mutated construct was recovered and confirmed to have lost the additional *BbvCI* site by restriction analysis. The mutated gene was then subcloned into pFE2 for fungal expression. Plasmid DNA of the expression vector-gene construction from a large scale isolation (Qiafilter Plasmid Maxi Kit, Qiagen, cat. no. 12262) was sequenced using the Applied Biosystems Automated 3730 DNA Analyzer and Big Dye Terminator chemistry with AmpliTaq-FS DNA Polymerase at the Cornell Biotechnology Resource Center. *A. awamori* (ATCC 22342) was transformed as described [8]. Spores from potential transformants were frozen to −80 °C for later use.

Production of XlnD Protein

Protein production was carried out in 2.8 l Fernbach flasks using CM medium as described previously [8]. Seed cultures of the transformed spores were prepared in CM media containing 300 μg/ml Zeocin and were incubated for 4 to 5 days before transferring to the 2.8-l flasks containing 1 l of CM media with 100 μg/ml Zeocin. XlnD production was monitored during the fermentations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and activity analysis on *p*NP-β-D-xylopyranoside. Upon harvesting, the fermentation broths were clarified by filtration through Miracloth (Novagen, Madison, WI) and 70 μm polypropylene filter cloth; the broth was then frozen and stored at −20 °C. Before protein purification, the broth was thawed and refiltered through a 141-grade Ahlstrom glass microfiber filter.

Protein Purification

The filtered XlnD fermentation broth was initially concentrated into 20 mM Bis–Tris buffer at pH 6.8 in a 300 ml stirred ultrafiltration cell from Amicon (Beverly, MA) with a 30,000 Da molecular weight cut-off membrane. The protein was then bound to a Source Q anion exchange column (96 ml) and eluted over a 0 to 1 M NaCl gradient into 20 mM Bis–Tris at pH 6.8. The eluted XlnD peak was then reconcentrated and rerun on the Source Q column. From this run, the XlnD fractions were then pooled and buffer-exchanged into 20 mM acetate buffer at pH 5.0 with 100 mM NaCl on a Superdex 200 size exclusion column. The purity of the purified fractions was assessed by SDS-PAGE, and the protein content was assessed by measuring the absorbance at 280 nm and by bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) run as a standard.

Additional proteins used in this study, the endoxylanase XynA, the cellobiohydrolase Cel7A, and a cellobiase were all purified from commercial sources. Cel7A and XynA were purified as described previously [9]. In brief, the XynA was isolated from a Sigma *T. lanuginosus* endoxylanase prep by sequential anion exchange and size exclusion chromatography steps. The Cel7A was isolated from a *Trichoderma reesei* cellulase preparation (Spezyme CP, Genencor, Copenhagen, Denmark), by multiple anion exchange steps followed by cellulose affinity chromatography and clean-up by size exclusion chromatography (SEC). The cellobiase was purified as previously described [10] from an *A. niger* cellobiase preparation (Novo 188, Novozymes, Bagsvaerd, Denmark).

Thermal Analysis of Purified XlnD

Thermal analysis of the purified XlnD protein was performed by differential scanning microcalorimetry (DSC) in a Microcal (Northampton, MA) VP-ITC system. All portions of this analysis were conducted in 20 mM sodium acetate buffer at pH 5.0 with 100 mM NaCl (SEC buffer). The XlnD was analyzed at a nominal protein concentration of 50 nM and scanned from 30 to 95 °C at a rate of 60 °C/h.

Determination of Initial Rate Kinetics on *p*-nitrophenol β-ᴅ xylopyranoside

Initial rate kinetic assays were conducted at 37 °C in 50 mM citrate buffer at pH 4.8 in 96-well microtiter plates using *p*-nitrophenol β-ᴅ-xylopyranoside. For all assays, the XlnD was loaded at 1.5 μg/ml of reaction, and initial substrate concentrations were varied from 0.1 to 3.2 mM. The release of *p*NP was monitored every 15 s for the initial 10 min of each reaction by measuring the absorbance at 405 nm on a SpectraMax 190 UV/VIS microplate scanner from the Molecular Devices (Sunnyvale, CA). End product inhibition by ᴅ-xylose was confirmed by running identical assays to those described above with initial ᴅ-xylose concentrations ranging from 3.33 to 40 mM. Triplicate analyses of all assays were run at all conditions. All parameters estimated in this study were calculated using standard Michaelis–Menten kinetics as described previously [11].

Assessment of Efficacy in Converting Simple Xylo-Oligomers

The ability of the XlnD to convert simple xylo-oligomers was initially assessed on low DP (up to DP6) preparations purchased from Megazyme (Wicklow, Ireland). Solutions of xylobiose, xylotriose, xylotetrose, xylopentose and xylohexaose, 1.5 mg/ml, were incubated in 50 mM citrate buffer at 50 °C for 1 and 24 h in the presence of a 5-mg/g xylan loading of XlnD. A mixed solution of xylobiose (~1.5 mg/ml), xylotriose, xylotetraose, and xylohexose (1.0 mg/ml each) was also incubated in the presence of a 0.5-mg/g xylan loading of XlnD and analyzed for oligomeric and monomeric xylose by high-performance liquid chromatography (HPLC) at 10, 30, 60, and 120 min.

Digestions with Oat Spelt Xylan

The ability of XlnD to convert xylo-oligomers resulting from endoxylanase action was determined using oat spelt xylan (Sigma Chemical) and the purified XynA. Digests containing only XynA were run at loadings between 0.25 and 50 mg/g xylan; digestions with XlnD were run at loadings ranging from 0.25 to 10.0 mg/g xylan at a near-optimal

XynA loading (2.5 mg/g xylan). All digestions were run in duplicate at 50 °C in 50 mM, pH 4.8, citrate buffer, and sampled at 24 h for analysis by HPLC.

Digestions on Pretreated Corn Stover

Corn stover used for this study was harvested in 2003 at the Kramer Farm in Wray, Colorado. The stover was pretreated either in-house at the National Renewable Energy Laboratory or received via subcontract from the CAFI [12] pretreatment group members. The samples selected for this study were pretreated by alkaline peroxide (NREL), sulfite steam explosion (UBC), ammonia fiber explosion (MSU), and dilute sulfuric acid (NREL) methods. The composition of the pretreated stover was determined by a two-stage sulfuric acid hydrolysis treatment according to the NREL Laboratory Analytical Procedure titled "Determination of Structural Carbohydrates and Lignin in Biomass" [13]. The pretreatment conditions and compositional information for each substrate are listed in Table 2.

Digestions were run on the pretreated substrates with XlnD and XynA loaded at 1.0 and 2.5 mg/g xylan, respectively. Digestions with these loadings were run with each individual enzyme and the combination of the two. In addition, the same loadings were run in combination with the purified Cel7A and cellobiase at nominal loadings of 50 and 5 mg/g glucan, respectively. All digestions were run for 24 h at 50 °C in 50 mM citrate buffer at pH 4.8; after which, the hydrolysates were sampled for analysis by HPLC.

HPLC Analysis

HPLC analysis of digestion hydrolysates was conducted on an 8 mm × 300 mm Shodex (Kawasaki, Japan) SP0810 Sugar Column at 80 °C with water running at 0.6 ml/min as the eluent.

Results and Discussion

Cloning and Expression

The gene encoding XlnD was amplified and isolated from genomic DNA of *A. niger* using the PCR and cloned into the pCR2.1-TOPO vector. The coding sequence was then subcloned into our fungal expression vector pFE2 [8] at the *BbvCI* and *XbaI* sites to allow for expression driven by the glucoamylase promoter and secretion based on the gene's native signal sequence. The DNA sequence showed that introns were not present, and there were no

Table 2 Pretreated corn stover substrates; pretreatment information and glucan, xylan and lignin content as a fraction of the pretreated material.

Pretreatment	Source	Pretreatment Information	Glucan	Xylan	Lignin
Alkalnine peroxide	NREL	1.0% hydrogen peroxide; pH 11.5; 3 h at 50 °C	0.58	0.27	0.06
Dilute acid	NREL	Low severity sulfuric acid pretreatment in Sunds at NREL	0.54	0.14	0.27
Sulfite steam explosion	CAFI2-UBC	190C; 5 min; 3% SO$_2$; 20% solids	0.60	0.11	0.01
AFEX	CAFI2-MSU	90 °C; 60% moisture; ammonia loading of 1.0 kg/KG	0.37	0.20	0.05

Table 3 Matching peptide fragments from MALDI TOF/TOF analysis of Trypsin Digested XlnD; analyzed at the Cornell Proteomics and mass spectrometry core facility at Cornell University in Ithaca, NY.

Start AA	End AA	Sequence	Observed MW
65	78	SHLICDETATPYDR	1,677.7577
79	104	AASLISFTLDELIANTGNTGLGVSR	2,633.3792
105	122	LGLPAYQVWSEALHGLDR	2,025.05
150	164	TLIHQIASIISTQGR	1,637.9381
165	171	AFNNAGR	749.4118
172	185	YGLDVYAPNINTFR	1,642.8271
172	191	YGLDVYAPNINTFRHPVWCR	2,375.1667
186	191	HPVWGR	751.4315
192	224	GQETPGEDSLAAVYAYEYITGIQGPDPESNLK	3,511.6707
231	245	HYAGYDIENWHNHSR	1,898.8374
368	376	DDIEQGVIR	1,044.5609
394	399	ANNPYR	734.4005
428	438	NSNNVLPLTEK	1,228.6628
532	546	ESIAWPGNQLDLIQK	1,711.8864
596	607	DIITGKKNPAGR	1,269.7437
608	640	LVTTQYPASYAEEFPATDMNLRPEGDNPGQTYK	3,719.7461
730	737	KWLVGWDR	1,059.6017
731	737	WLVGWDR	931.5106
738	747	LGEVKVGETR	1,087.6412
738	750	LGEVKVGETRELR	1,485.7902
748	760	ELRVPVEVGSFAR	1,458.8146
751	760	VPVEVGSFAR	1,060.6046
761	782	VNEDGDWVVFPGTFELALNLER	2,520.2102

amino acid differences between our cloned sequence and the sequence reported in GenBank for XlnD (*A. niger* CBS 120.49). Peptide sequences derived from trypsin digests followed by MALDI TOF/TOF MS analysis was used for positive identification of the purified enzyme. This analysis was performed at the Cornell Proteomics and Mass Spectrometry core facility at Cornell University in Ithaca, NY. From the analysis, 23 peptides were matched representing ~38% mass coverage of the entire amino acid sequence (Table 3).

Fig. 1 DSC scan of the purified XlnD protein; the protein was scanned from 30 to 95 °C in 20 mM acetate buffer, pH 5.0 with 100 mM NaCl, at a scanning rate of 60 °C/h. T_{max} was estimated to be 78.2 °C
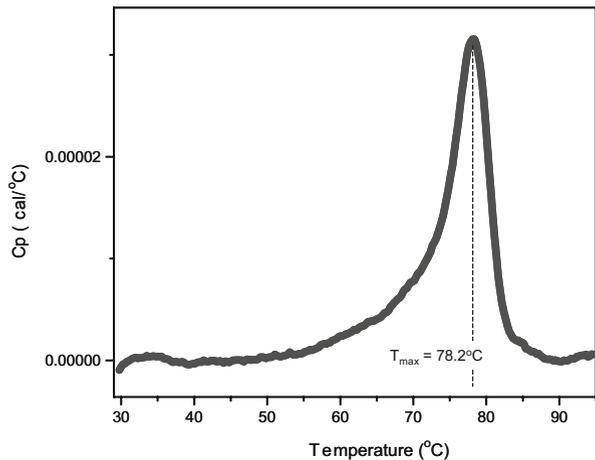
Fig. 2 Assays with the purified XlnD on *p*NP-x, run at 37 °C in 50 mM citrate buffer at pH 4.8; **a** reaction rate vs substrate concentration and **b** Michaelis–Menten plots of data from assays with varying initial D-xylose concentrations
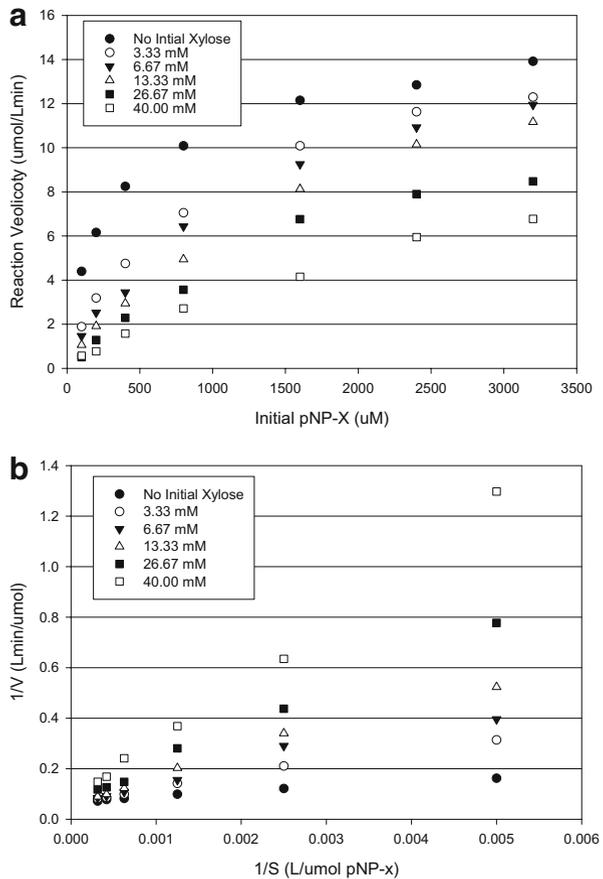
Thermal Analysis of XlnD

Analysis by DSC (Fig. 1) revealed the purified XlnD to have a high thermal denaturation point. By this method, the T_{max} of the protein was estimated to be 78.2 °C; repeat scans on the protein verified this value. In addition, a rescan (data not shown) of the deconvoluted protein showed the XlnD was unable to refold after thermal collapse. This data is in agreement with previously published temperature vs activity data indicating the thermostable nature of the *A. niger* XlnD [14, 15] and is a strong indication that our expression was successful in retaining thermostability of the protein.

Activity Analysis on pNP-beta-D-xylopyranoside

Initial activity analysis on *p*NP-X showed the kinetic behavior of our purified XlnD to be in agreement with previously published work regarding the β-xylosidase enzyme from *A. niger* [16, 17]. From triplicate assays at each substrate loading, the K_m and k_{cat} of the protein were estimated to be 254.8±6.8 µM and 13.7±0.7 s^{-1}, respectively (note that all deviations presented are the standard deviations between values obtained for each parameter as determined from each replicate assay set). Assays with increasing amounts

Fig. 3 HPLC chromatograms from 0 to 2 h digestions of a mixed solution of xylobiose, xylotriose, xylotetraose, and xylohexose; 0 and 2 h sampling times are shown
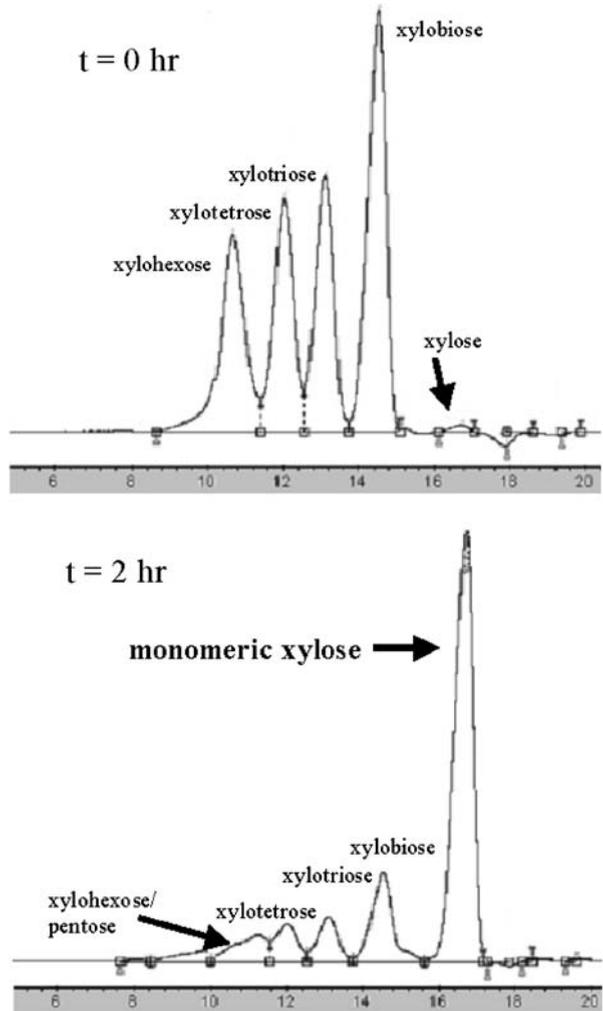
of initial D-xylose (Fig. 2a) confirmed the end-product inhibition of the enzyme by D-xylose as described by Tavobilov and coworkers [16, 17]. Michaelis–Menten plots (Fig. 2b) of initial reaction rate and substrate data for each initial D-xylose loading show the slopes of each plot to have very similar y-intercepts (0.0073 ± 0.0004 min/μM), indicating that the inhibition by D-xylose is most likely competitive. Based on the assumption that the inhibition is competitive, the K_i was estimated from the data to be 3.35 ± 0.78 mM. Overall, the strong agreement between our analysis and published kinetic data for the *A. niger* β-xylosidase proves we have successfully expressed an active and useful form of this enzyme using the *A. awamori* system.

Efficacy in Converting Xylo-Oligomers

Digestions of prepared xylo-oligomers with the XlnD demonstrated the enzymes ability to quickly hydrolyze small oligomers. The 5 mg/g oligomer loading of the XlnD was observed to

Fig. 4 Twenty-four-hour diges-
tions of oat spelt xylan with
varying loadings of XynA pre-
sented as the percent of xylan
converted to xylotetrose, xylo-
triose, xylobiose, and xylose. All
error bars represent the SD repli-
cate assays
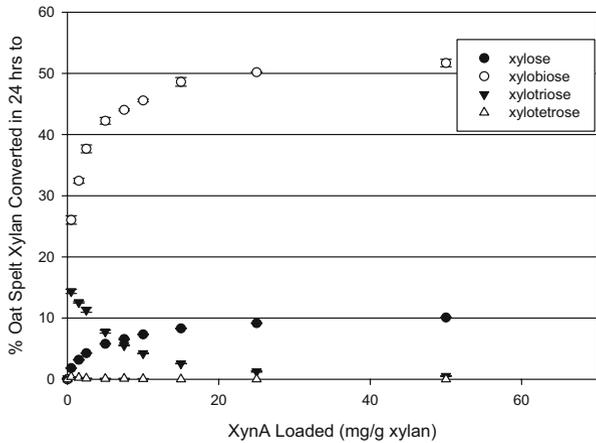
completely hydrolyze 1.5 mg/ml solutions of individual xylo-oligomers up to xylohexose within 2 to 3 h (data not shown). This enzyme showed no cross-reactivity with cellobiose, even for extended periods (24 h). Figure 3 depicts the rapid hydrolysis of a mixed solution of xylo-oligomers over a 2-h period. The negligible effect of this enzyme on cellobiose will make this enzyme a useful tool for reducing xylan degradation to monomeric xylose release, without directly hydrolyzing sugars released by cellulose hydrolysis. This enzyme will be of particular use when studying the effects of specific hemicellulases on the ability of cellobiohydrolases to release cellobiose from lignocellulosic materials. In addition, the ability of the enzyme to rapidly hydrolyze low molecular weight oligomers may prove useful in the treatment of high molecular weight xylo-oligomer containing hydrolysates from pretreatments of lignocellulose.

Digestions of oat spelt xylan with purified XynA and the XlnD further show the utility of the β-xylosidase activity in a real lignocellulosic system. For example, in Fig. 4, we show how the endoxylanase primarily degrades the xylan to xylobiose and xylotriose; and to a lesser extent, xylose. Shown in Fig. 5, xylan converted by XynA in the presence of small quantities of XlnD is entirely converted to monomeric xylose during the 24-h incubation

Fig. 5 Twenty-four-hour diges-
tions of oat spelt xylan with a
nominal XynA loading of 2.5 mg/g
xylan and varying loadings of the
purified XlnD enzyme. The data is
presented as the percent of xylan
converted to xylotetrose, xylo-
triose, xylobiose, and xylose. All
error bars represent the SD be-
tween replicate assays
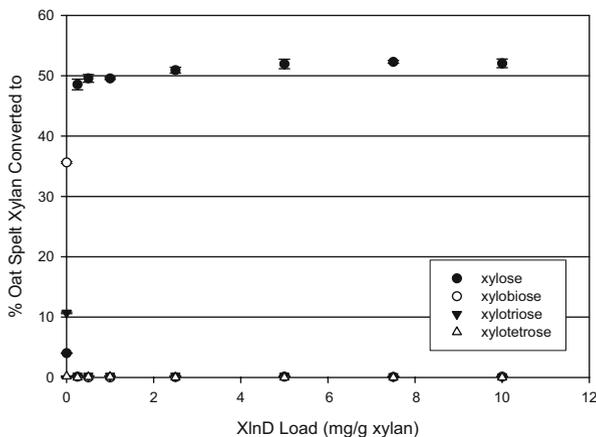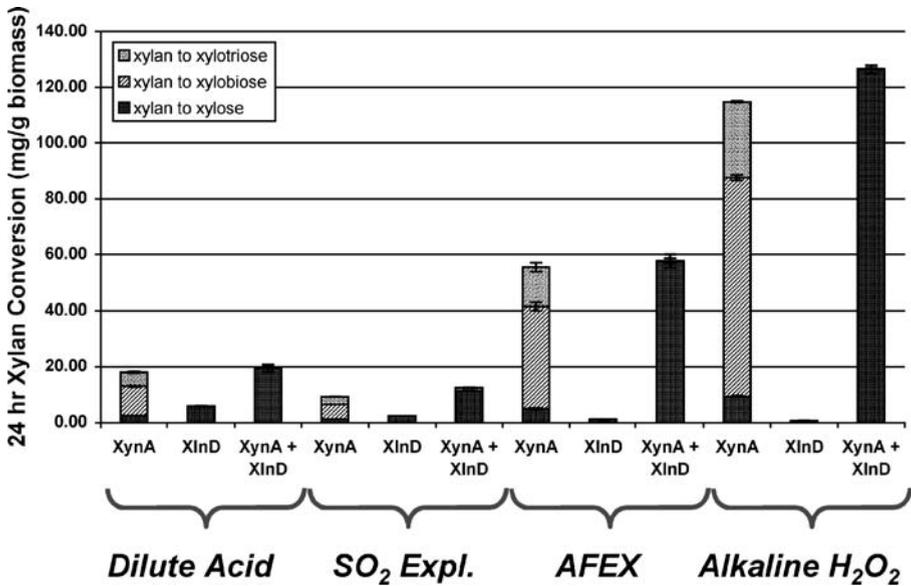
Fig. 6 Twenty-four-hour digestions of pretreated corn stover with individual and combined loadings of XynA and XlnD. XynA and XlnD were loaded at 2.5 and 1.0 mg/g xylan, respectively. All *error bars* represent the SD between replicate assays

time. Results from a 1-h incubation (data not shown) with both XynA and XlnD are nearly identical to the 24-h digest (lower overall yields), with only trace amounts of xylobiose present in solution at the lowest XlnD loadings. This result is a good indication that the XlnD is fairly capable of keeping pace with the release of xylo-oligomers by the endoxylanase.
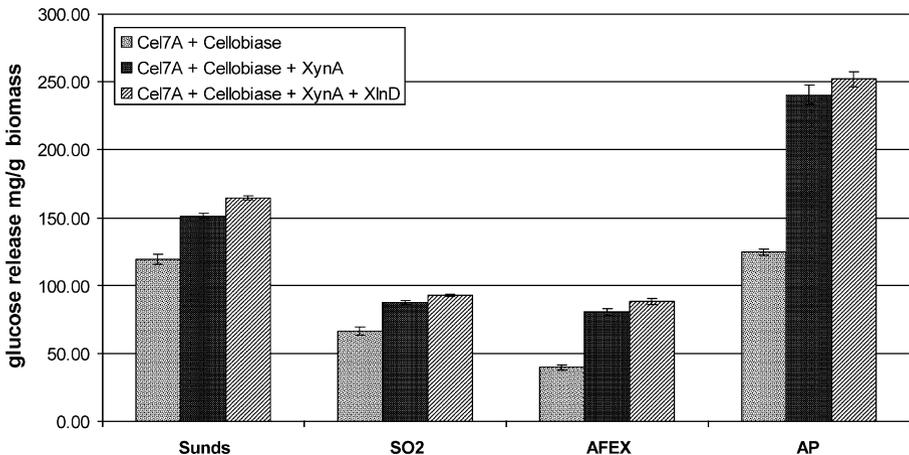
Fig. 7 Twenty-four-hour digestions of pretreated corn stover with a nominal mix of purified Cel7A and cellobiase (50 and 5 mg/g cellulose, respectively) with individual and combined loadings of XynA and XlnD. XynA and XlnD were loaded at 2.5 and 1.0 mg/g xylan, respectively. All *error bars* represent the SD between replicate assays

Effects on the Saccharification of Pretreated Corn Stover

In Fig. 6, we show that the addition of XlnD contributes to the degradation of xylan present in pretreated corn stover substrates. In this work, the XlnD has a significant impact on the quantity of monomeric xylose that can be recovered from the pretreated substrates, while also allowing for mild improvements in the total amount of xylan converted (i.e., compared to digestions with only XynA). In Fig. 7, we show how enzymatic xylan removal can have a significant and synergistic impact on the conversion of cellulose by the simplified cellulase system: Cel7A and a cellobiase. In this work, the mild enhancement in xylan conversion with the addition of XlnD correlates to slightly higher recoveries of glucose from the pretreated biomass. The overall synergy we have observed between these simplified xylanolytic and cellulolytic systems is in agreement with previously published work showing synergism between unpurified cellulase and xylanase systems on lignocellulosic materials [18, 19]. From these data (Fig. 7), we show that the enhancement of cellulose conversion by simultaneous enzymatic xylan conversion is most significant for substrates that have higher post-pretreatment xylan content (i.e., AFEX and alkaline peroxide stover).

Conclusions

We have successfully expressed an *A. niger* β-xylosidase, XlnD, in *A. awamori*. The expressed XlnD was purified and successfully identified by MALDI-TOF/TOF analysis to be the correct protein. Activity analysis on *p*NP-X showed the XlnD to behave in accordance with previously published work on the *A. niger* β-xylosidase indicating that this enzyme was successfully expressed in this heterologous system. In addition, we have shown that the purified XlnD has high thermal stability and is effective in converting low molecular weight xylo-oligomers to monomeric xylose. When working in concert with a purified endoxylanase, the presence of the XlnD permits complete recovery of the hydrolyzed xylan to monomers, and thus, provides some enhancement in total xylan conversion. This enhancement in xylan conversion translates to additional improvements in the performance of a simplified cellobiohydrolase/cellobiase system over the significant gains observed with the addition of the endoxylanase XynA. Overall, our data suggests that activities, such as XlnD, are crucial to the biomass conversion process and that discovery of new, and perhaps, even more active versions of these enzymes is thus warranted.

References

1. Collins, T., Gerday, C., & Feller, G. (2005). *Fems Microbiology Reviews*, *29*(1), 3–23.
2. Christov, L. P., & Prior, B. A. (1993). *Enzyme and Microbial Technololgy*, *15*(6), 460–475.
3. Jeffries, T. W., & Jin, Y. S. (2000). *Advances in Applied Microbiology*, *47*, 221–268.
4. Schlacher, A., et al. (1996). *Journal of Biotechnology*, *49*(1–3), 211–218.
5. Ghosh, M., & Nanda, G. (1994). *Applied and Environmental Microbiology*, *60*(12), 4620–4623.
6. Beg, Q. K., et al. (2001). *Applied Microbiology and Biotechnology*, *56*(3–4), 326–338.

7. Christov, L. P., Akhtar, M., & Prior, B. A. (1996). *Holzforschung, 50*(6), 579–582.
8. Adney, W. S., et al. (2003). *In applications of enzymes to lignocellulosics* pp. 403–437. Washington: American Chemical Society.
9. Decker, S. R., & Selig, M. J. (2006). D Milestone: enzyme enhanced conversion of low severity pretreated biomass, DOE, Editor. NREL. p. 1–31.
10. Himmel, M. E., et al. (1993). *Applied Biochemistry and Biotechnology, 39*, 213–225.
11. Nelson, D. L., & Cox, M. M. (2000). *Lehninger principles of biochemistry* (3rd ed.). New York, NY: Worth Publishers.
12. Wyman, C. E., et al. (2005). *Bioresource Technology, 96*(18), 2026–2032.
13. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D.(2004). Determination of structural carbohydrates and lignin in biomass, DOE, Editor. National Renewable Energy Laboratory.
14. Pedersen, M., et al. (2007). *Biotechnology Letters, 29*(5), 743–748.
15. Uchida, H., et al. (1992). *Journal of Fermentation and Bioengineering, 74*(3), 153–158.
16. Tavobilov, I., Rodionova, N., & Bezborodov, A. (1983). Prikladnaaà biohimiaà i mikrobiologia, *19*(2), 232–239.
17. Rodionova, N. A., Tavobilov, I. M., & Bezborodov, A. M. (1983). *Journal of Applied Biochemistry, 5*, 300–312.
18. Yu, P. Q., et al. (2003). *Journal of Agricultural and Food Chemistry, 51*(1), 218–223.
19. Murashima, K., Kosugi, A., & Doi, R. H. (2003). *Journal of Bacteriology, 185*(5), 1518–1524.