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Assessment of Xylanase Activity in Dry Storage as a Potential Method of Reducing Feedstock Cost

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Abstract Enzymatic preprocessing of lignocellulosic biomass in dry storage systems has the potential to improve feedstock characteristics and lower ethanol production costs. To assess the potential for endoxylanase activity at low water contents, endoxylanase activity was tested using a refined wheat arabinoxylan substrate and three commercial endoxylanases over the water activity range 0.21–1.0, corresponding to water contents of 5% to >60% (dry basis). Homogeneously mixed dry samples were prepared at a fixed enzyme to substrate ratio and incubated in chambers at a variety of fixed water activities. Replicates were sacrificed periodically, and endoxylanase activity was quantified as an increase in reducing sugar relative to desiccant-stored controls. Endoxylanase activity was observed at water activities over 0.91 in all enzyme preparations in less than 4 days and at a water activity of 0.59 in less than 1 week in two preparations. Endoxylanase activity after storage was confirmed for selected desiccant-stored controls by incubation at 100% relative humidity. Water content to water activity relationships were determined for three lignocellulosic substrates, and results indicate that two endoxylanase preparations retained limited activity as low as 7% to 13% water content (dry basis), which is well within the range of water contents representative of dry biomass storage. Future work will examine the effects of endoxylanase activity toward substrates such as corn stover, wheat straw, and switchgrass in low water content environments.

Keywords Endoxylanase · Water activity · Water content · Biomass · Lignocellulose · Storage · Feedstock · Preprocessing · Stability

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Introduction

A major challenge to providing for sustainable biorefineries is ensuring that feedstock supply chain costs do not outpace allowable feedstock purchase prices. This requires continuous improvement in both feedstock harvest and handling methods and increasing the value of the harvested material through intermediate processing. One method of increasing feedstock value is chemical or biological preprocessing within the supply chain to improve feedstock characteristics for downstream processing at the biorefinery.

Our work examines enzymatically catalyzed xylan hydrolysis as a means to facilitate hemicellulose degradation, a treatment that can be applied at multiple points along the biomass feedstock supply chain. Potential benefits of xylan hydrolysis include improvements in feedstock handling and densification, storage, queuing, and downstream processing. Potential application points include harvest and collection, dry storage (e.g., bales and piles), wet storage (e.g., ensiling in tubes and bunkers), and queuing (wet or dry) within the biorefinery. Our study used commercially available xylan hydrolyzing enzymes (β-1,4-endoxylanases) as a means to save material costs, permit immediate use in medium and large-scale experiments, and to make stronger economic inferences on treatment efficacy.

Limited xylan hydrolysis has the potential to improve feedstock quality. Endoxylanase catalyzes the hydrolysis of the xylan backbone of hemicellulose, which is found in association with cellulose in the plant cell walls [1, 2]. Hemicellulose is a complex heteropolymer that forms hydrogen bonds with cellulose and is covalently bound to lignin, both of which increase the strength and rigidity of the plant cell wall. Hemicellulose must be removed from cellulosic material prior to the enzymatic or chemical pretreatment of biomass for glucose formation and subsequent fermentation to ethanol [3, 4]. It must also be hydrolyzed into monomeric pentoses (xylose and arabinose) prior to C-5 sugar fermentation. The hydrolysis of xylan by endoxylanase may be partial, resulting in smaller soluble or insoluble complex oligosaccharides, or complete, resulting in xylobiose and xylotriose. Breaking these covalent bonds requires energy input in the form of mechanical shearing (e.g., grinding), heating, chemical, or enzymatic hydrolysis or a combination of the three. Breaking some portion of these bonds during storage would produce a material with reduced energy requirements for preprocessing, resulting in similar particle size and treatment efficacy for a lower energy input or a higher value material for an equal energy input. Reduced pretreatment severity may result from any one or a combination of the following impacts: reduced need for elevated temperature and pressure, decreased use of concentrated acids and bases, reduced concentrations of fermentation inhibitors such as furfural and hydroxymethyl furfural, and reduced capital costs for plant construction and operation resulting from the less severe physical and chemical conditions used in feedstock deconstruction. Changes in particle size or particle size distribution would result in better densification and less material rebound during compaction, which would decrease material shipping and handling costs. Additionally, material formatting could be improved through the decreased energy or binder requirements for pelleting or briquetting.

Many of the potential insertion points for enzymatic treatments contain substrate materials of low water content. These so-called dry storage and queuing systems have the primary benefit of limited degradation and low dry matter loss during storage; stability is imparted by the reduced microbial activity that exists in these water-limited systems. Ultimately, our research will examine endoxylanase-treated feedstocks such as corn stover, switchgrass, and wheat straw. A first step in realizing the potential of enzymatic treatment during dry storage is to assess the ability of commercial endoxylanases to function in water-

Table 1 Manufacturer's description of the soluble substrate used in this study.

Medium viscosity wheat arabinoxylan	
Manufacturer	Megazyme
MW	270,000 Da
Composition	39% arabinan, 62% xylan
Purity	~95%

limited environments. This is especially important for hydrolyses since water is also a reactant. In preparation for future studies using natural materials, we chose to use a purified soluble surrogate, wheat arabinoxylan, which allowed us to simplify analytical procedures, minimize within treatment variance, increase replication level, and eliminate some of the variability associated with natural materials. Wheat arabinoxylan was used to examine the range of water activities over which the chosen enzymes would function to provide an initial estimate of reaction rate and extent and to serve as a tool to screen a broader range of endoxylanase preparations before setting up large-scale experiments using natural materials.

Methods

Soluble Substrate

Medium viscosity wheat arabinoxylan was obtained from Megazyme (Megazyme International Wicklow, Ireland). A stock solution of wheat arabinoxylan (10 mg mL^{-1}), herein referred to as WAX, was made as per the manufacturer's instructions with the exception that stock solutions for lyophilized samples were made in 18 MΩ cm^{-1} water rather than buffer. All water used in this study was 18 MΩ cm^{-1} 0.2-μm-filtered, unless otherwise stated. The measured pH value for the WAX solution in water was 6.5. Stock solutions were made weekly and stored at 4 °C until use. A description of the substrate is found in Table 1.

Endoxylanase Enzymes

Three commercial enzyme preparations were used. Two research-grade preparations were obtained from Megazyme (Megazyme International Wicklow, Ireland): β-xylanases M2 and M4 (Table 2). Working stocks of these enzymes were prepared by resuspending the

Table 2 Sources of the endoxylanase enzymes used in this study plus the pH and temperature optima given by the manufacturers.

Enzyme	Manufacturer	Synonym	Source	Optima	
				pH	°C
β-Xylanase M2	Megazyme	M2	*Trichoderma longibrachiatum*	4.5	50
β-Xylanase M4	Megazyme	M4	*Aspergillus niger*	4.5	60
Pentopan Mono BG®	Novozyme	TLX	*Thermomyces lanuginosus* (expressed in *A. oryzae*)	4–6	"Up to 75"

Enzyme activity assays for TLX were performed at pH 6 and 60 °C. Novozyme product information did not include a temperature optimum, but stated that the enzyme was active "up to 75 °C"

enzymes in 0.1 M ammonium acetate buffer (pH 4.5), loading them onto a PD-10 desalting column (Amersham Biosciences Piscataway, NJ, USA) and eluting the protein with the same buffer. The stock solutions were analyzed for enzyme activity toward wheat arabinoxylan (described below). The samples were lyophilized and resuspended in water (for lyophilization) or in a suitable buffer (for activity assay). The third endoxylanase was a preparation obtained through Sigma-Aldrich (Sigma-Aldrich St. Louis, MO, USA), which was manufactured by Novo Nordisk (Novo Nordisk Ferment Ltd. Dittingen Switzerland) and is sold as Pentopan® Mono BG, herein referred to as TLX, for *Thermomyces lanuginosus* xylanase. This product is marketed for bread dough modification and was provided as a free-flowing, agglomerated powder. The enzyme was extracted (1 g solid per 50 mL water) from the powder in 25 °C water while agitating on a shaker table (100 rpm). The extract was centrifuged for 5 min at 1,000×*g*, and the supernatant was removed and stored at 4 °C for later use. Enzyme preparations were remade weekly to minimize potential effects from enzyme degradation over time. Stock solutions were made at concentrations of up to 10 U mL^{-1} for assessing enzyme saturation; however, in practice, all working solutions for sample preparation were made to a target activity of 1.4 U mL^{-1}.

Enzyme Activity and Reducing Sugar Assays

Endoxylanase activity was determined in all three enzyme preparations using a modified Nelson–Somogyi reducing sugar assay [5–7]. Activity was determined by incubating 1.25 mg substrate (WAX) in 175 μL of suitable buffer (0.1 M sodium acetate at pH 4.5 for M2 and M4, 0.1 M sodium phosphate at pH 6.0 for TLX) for 10 min at 50 °C (M2) or 60 °C (M4 and TLX). Enzymatic reactions were stopped by the addition of the copper reagent and were incubated in a boiling water bath to allow the reducing ends formed during substrate hydrolysis to react with the Cu(II) in solution. The arsenomolybdate reagent was added for final color development, and the samples were quantified against a set of xylose standards made in solutions of 10 mg mL^{-1} WAX to eliminate any substrate matrix interferences from the assay. Samples and standards were measured spectrophotometrically for absorbance at 520 nm. A copper-quenched enzyme-added blank was run for each set of enzymes to measure any potential matrix interference of the enzyme preparation. In practice, only TLX required any correction for enzyme interferences and then only during the initial enzyme saturation assay. Reducing sugar concentration was reported in terms of xylose reducing sugar equivalents (XRE). One unit of endoxylanase activity was defined as the amount of enzyme that released 1 μmol equivalent of reducing sugar equivalents of xylose per minute under the above conditions.

At higher enzymatic activities and in the higher water activity samples, a 1:5 or 1:10 dilution was required to bring sample absorbance values into range. Quantitative dilution was performed using the copper reagent in the initial step of the reducing sugar assay and accounting for the subsequent dilution in the calculation of the final reducing sugar concentration. A modified version of the reducing sugar assay was used for the lyophilized samples (described below).

Enzyme Saturation Assay

The abovementioned methods were used to determine a satisfactory enzyme concentration for the quantity of substrate used in our assays. While enzyme saturation was not strictly necessary for our experiments, a near-saturation concentration was used to provide a basis for relative comparison between different preparations and to minimize time for experiment

completion. An enzyme dilution series was used that spanned 1.0 U per reaction to 0.01 U per reaction using TLX in water at 25 °C. Enzyme activity was stopped at 5 min by the addition of the copper reagent, and samples were analyzed for reducing sugar concentration.

Lyophilized Sample Preparation

The viscous stock solution of substrate was reverse-pipetted into a 1.5 mL cryo-vial (for lyophilization) or 1.5 mL micro-centrifuge tube (for activity assay and standards). Final substrate mass in each tube was 1.25 mg or 125 μL 10 mg mL^{-1} WAX plus 50 μL enzyme preparation in each 175 μL reaction.

Samples were lyophilized to decrease starting water activity and to allow samples to reach equilibrium water activity under controlled conditions by absorbing rather than losing water. Additionally, this method of preparation brings the substrate and enzyme into close contact, optimizing enzyme kinetics in low water content environments where diffusion of the substrate to the enzyme is limited [8]. Note that the substrate (WAX) was also the matrix in these experiments and that water (as a limiting reactant) diffused to the matrix/substrate-bound enzyme. All samples for lyophilization were prepared using deionized (18 MΩ cm^{-1}) water to minimize any potential impacts of solute concentration during drying. Substrate (125 μL 10 mg mL^{-1} WAX) and enzyme (50 μL 1.4 U mL^{-1}) were cooled to 4 °C and stored on ice during sample preparation. Enzyme and substrate were added to 1.5 mL cryo-vials and vortexed for 3 to 5 s, capped, and plunged immediately into a dewar of liquid nitrogen. Frozen samples were transferred to a −80 °C freezer for storage or loaded directly into a lyophilizer (FTS Flexi-Dry MP, Stone Ridge, NY, USA) after loosening the caps. Samples remained on the lyophilizer a minimum of 16 h at 44 to 57 mT and −88 to −91 °C [9]. After lyophilization, samples were capped and placed into either a −80 °C freezer for storage or into a 25 °C desiccator (to thaw after freezing) to prevent water condensation on the samples. Samples remained sealed until exposure in the fixed humidity chambers, described below.

Fixed Humidity Chambers

Glass desiccators were placed into a 35 °C environmental chamber during sample incubation. Relative humidities were fixed by incubation in these sealed chambers containing various saturated salt solutions [10]. Desiccators were partially filled with sand, and one half of a 10-cm petri plate was placed into the desiccator to hold saturated salt slurry. Saturated salt mixtures were made from ACS grade or better reagents (Fisher Science Pittsburgh, PA, USA) in distilled water and equilibrated overnight at 35 °C before use. The presence of insoluble salts and salt solutions insured saturation. Salts and target water activities are shown in Table 3. The water activity for potassium acetate at 35 °C was determined by nonlinear regression of water activity data from 10 to 30 °C from Greenspan [10]. A subset of the samples was incubated in a desiccant-filled desiccator to assess changes in enzyme activity over time and to serve as an additional point of comparison (beyond the initial time point) to measure relative enzymatic activity in the samples. A digital temperature and relative humidity meter (Fisherbrand Certified Traceable Digital Hygrometer/Thermometer, Fisher Science Pittsburgh, PA, USA) was placed into each desiccator in order to allow periodic inspection of the environmental conditions without removing the desiccator lid. At the start of each experiment, samples were randomly assigned to a specific water activity treatment and were uncapped and placed into their respective incubator. Sample vials were allowed to incubate for up to 7 weeks and were

Table 3 Water activities, relative humidities, and the salts used in the saturated slurries to maintain those conditions within each of the treatments used.

a_w 35 °C	Salt (RH)
0.00	DriRite® (0%)
0.20	K-Oac (20%)[a]
0.43	K_2CO_3 (43%)
0.50	$Mg(NO_3)_2$ (50%)
0.59	$CoCl_2$ (59%)
0.72	$NaNO_3$ (72%)
0.80	$(NH_4)_2SO_4$ (80%)
0.83	KCl (83%)
0.91	KNO_3 (91%)
0.97	K_2SO_4 (97%)
1.00	DI H_2O (100%)

[a] Extrapolated to 35 °C

periodically sacrificially sampled to measure enzyme activity via reducing sugar concentration. Two to five samples within each treatment were randomly selected for analysis at each time point.

Sorption Isotherms

Relationship between water activity and water content in WAX was determined using sorption isotherms. Working isotherms were generated by alternatively drying and rewetting samples of wheat arabinoxylan in either a conventional gravity oven at 45 °C or a sealed chamber (desiccator) at 35 °C and 100% relative humidity [11]. Total sample mass was determined in closed containers before and after each individual water activity determination. Sample mass is reported as the average of the two values. Water activity was determined using an AquaLab 3TE (Decagon, Inc. Pullman, WA, USA) water activity meter in normal sampling mode at 35 °C.

Wheat straw, corn stover, and switchgrass were analyzed for full isotherms using the dynamic dewpoint isotherm (DDI) method at 25 °C [12]. Isotherms were conducted in the AquaSorp Isotherm Generator (Decagon Devices, Inc.), which utilizes the DDI method at a flow rate of 300 ml min^{-1} with a minimum water activity setting of 0.10 a_w and a maximum water activity of 0.90 a_w.

Results and Discussion

This work uses equilibrium water activity as a means to balance enzyme activity—a desirable reaction—and microbial activity—an undesirable loss of substrate—to improve the quality of biomass feedstocks for the purpose of producing fuel ethanol more efficiently. Water activity, rather than water content, was chosen as the variable because water content–water activity relationships vary with material properties, and focusing on equilibrium water activity allows inferences that apply to a broad range of substrates under similar conditions.

The minimum water content required for enzymatic and microbial activities depends a great deal upon the solid matrix because the true determinant of water's function is water activity (a_w), which is directly related to water potential or matrix potential (φ) [13]:

$$\phi = \frac{RT}{V_w} \ln a_w$$

where R is the universal gas constant, T is in Kelvin, a_w is the water activity, and V_w is the partial molar volume of water. In general, molds will be active at values of a_w above about 0.70, yeasts above about 0.88, and bacteria above about 0.90 [14]. These ranges are not always accurate for specific groups of microbes; for example, the high metabolic rates of Gram-negative bacteria occur over a relatively small range of a_w (0.970–0.995), which corresponds to a range of φ of about −0.2 to −8.5 bar [15]. Enzymes are active at values of a_w as low as 0.10–0.20 [14]. The relationship between a_w and water content for a given material can be experimentally determined by plotting a_w versus percent moisture.

A simplified, more practical definition of water activity is the ratio of the equilibrium water vapor pressure (P) over a material to that over "pure" water (P_0) [16, 17]:

$$a_w = \frac{P}{P_0}.$$

Multiplication of the water activity by 100 gives the equilibrium relative humidity of the atmosphere over any moist material in a sealed system. Standard salt solutions were used to fix the relative humidity within our incubators [10], and total vapor-phase water concentration was fixed by holding the materials and incubators at a fixed temperature [18].

Experiments covered a range of water activities corresponding to the water contents typically observed in dry storage conditions (5% to 15% moisture; a_w 0.2 to a_w 0.8). To choose the experimental water activity ranges, isothermal water activities for wheat arabinoxylan (Fig. 1) and for three lignocellulosic feedstocks (Fig. 2) were determined. Working isotherms (WAX, Fig. 1) and adsorption isotherms (bottom curves in Fig. 2) for the natural substrates displayed similar slopes within the range of a_w 0.2 to 0.8, meaning that they increased in moisture content equivalently as water activity increased. However, wheat straw and WAX both had higher water contents than either corn stover or switchgrass at any measured water activity within this range. Wheat straw and switchgrass' water affinity was greater than that of stover and switchgrass, but all four materials adsorbed water at an equivalent rate as water activity increased.

In measured hydrolysis rates, the saturating enzyme concentration was determined to be between 0.05 and 0.1 U per reaction (175 μL 10 mg mL^{-1} WAX) for TLX (Fig. 3) in deionized water at 25 °C for 5 min. Enzyme loading rate was fixed for all subsequent testing to allow relative comparison between enzymes at a fixed enzyme to substrate ratio under a common set of conditions. Based upon this result, an enzyme loading of 0.1 U per

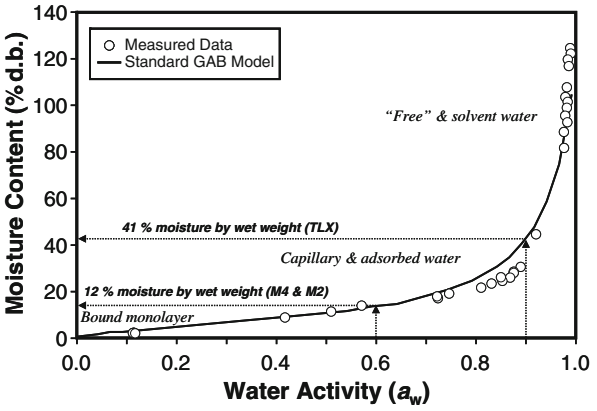

Fig. 1 Water content on a dry basis (*d.b.*) by water activity for medium viscosity wheat arabinoxylan (Megazyme)

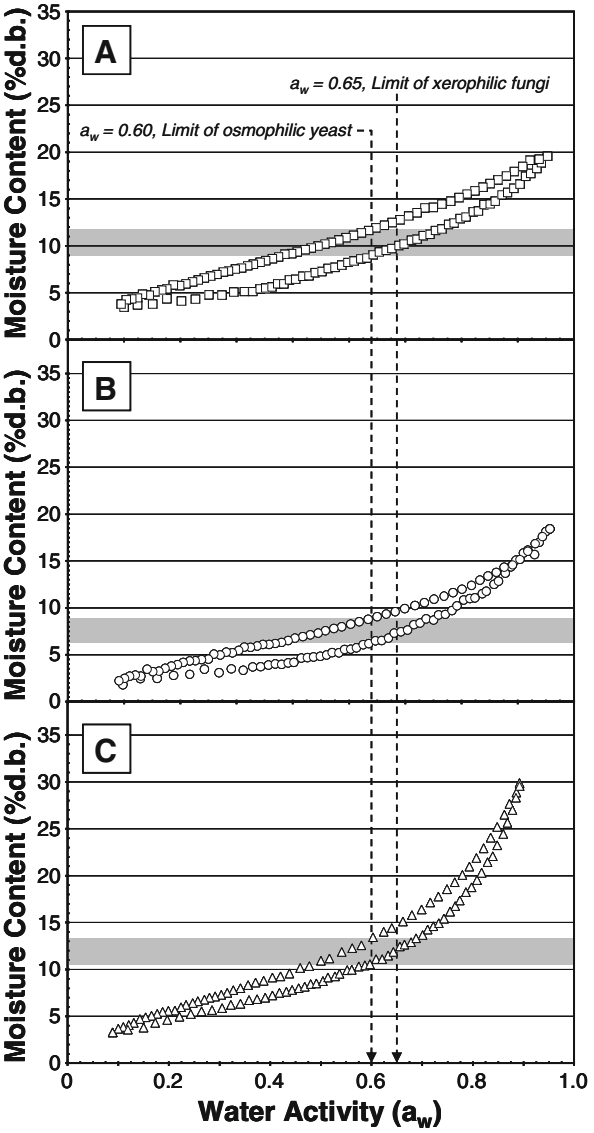

Fig. 2 Water content on a dry basis (*d.b.*) by water activity for three selected cellulosic biomass feedstocks: **a** corn stover, **b** switchgrass, and **c** wheat straw. In each panel, the *top curve* is the drying (desorption) curve, and the *bottom curve* is the wetting (adsorption) curve. The *shaded areas in each panel* show the range of observed moisture contents at a_w=0.60 and indicate the corresponding moisture content range of about 7–13% water content for the three materials

reaction was chosen as the target for all three enzymes described herein (Table 2). Variability in the weekly enzyme stock solutions resulted in a measured activity of 0.07 U per reaction (or 56 U per gram WAX)—slightly less than the initial target but still acceptable for these experimental purposes. After a suitable enzyme concentration was determined and selected, we examined the effects of lyophilization and resuspension on enzyme activity toward WAX (1.25 mg) using TLX (0.1 U) in our 175 μL reactions. No detectable difference in enzyme activity was noted between lyophilized and resuspended and nonlyophilized samples (data not shown) incubated in deionized water at 25 °C for 5 min. Results confirmed that lyophilization neither reduced enzyme activity nor increased substrate recalcitrance.

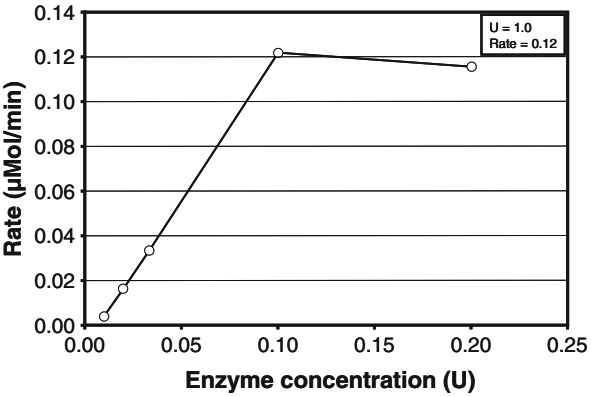

Fig. 3 Enzyme saturation assay for Pentopan Mono BG (TLX) in deionized water after incubation for 5 min with 1.25 mg wheat arabinoxylan. Data for 1.0 U per reaction was truncated from the plot to display the region between 0.01 and 0.1 U clearly

All three enzymes (Table 2) demonstrated activity toward WAX at a_w=1.0. Results confirmed that vapor-phase transport of water to the enzyme–substrate mixture was sufficient to support enzyme activity (Figs. 4, 5, 6, and 7) in our experimental system. XRE increased in the a_w 1.0 samples relative to both the enzyme-inactivated controls at T_0 and the desiccant-stored samples across all time points. The model substrate was rapidly hydrolyzed under saturating conditions within 1 day. However, measured XRE decreased in M4 after 5 days, either as a result of the nonsterile conditions used or due to analytical error.

Two of the tested enzymes—M2 and M4—demonstrated endoxylanase activity down to a_w=0.59 within 2 and 11 days, respectively (Figs. 4 and 5) relative to the controls. The desiccant is shown as a_w=0.06 in the figures; this was the value reported by the relative humidity gauge within the desiccator throughout the experiment. Reactions at a_w=0.72 (M2 and M4) and 0.59 (M4) proceeded to greater extent than reactions at higher water activity, presumably due to the longer incubation time of the lower water activity samples. Endoxylanase activity was proportional to water activity in all tested enzymes (Figs. 4, 5, and 7); however, there are insufficient data to suggest that this was a result of differences in rates alone or of differences in extents of reaction.

The third enzyme—TLX—did not initially demonstrate any indication of activity below saturating conditions (a_w=1.0) after nearly 7 weeks of incubation (Fig. 6). To determine if

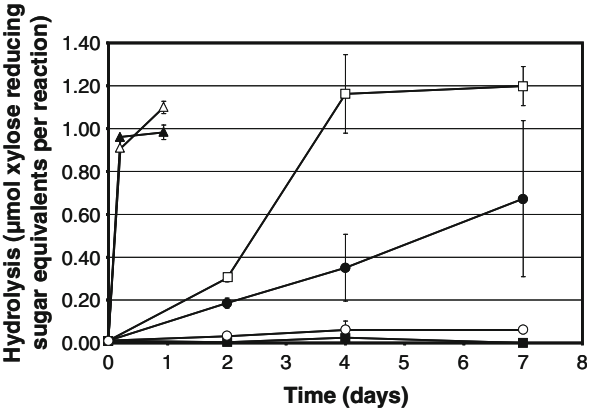

Fig. 4 Substrate hydrolysis over time: WAX and M2 at water activities from 0.5 to 1.0 over 1 week. *Open triangles* a_w=1.00, *closed triangles* a_w=0.91, *open squares* a_w=0.72, *closed circles* a_w=0.59, *open circles* a_w=0.50, and *closed squares* DriRite®

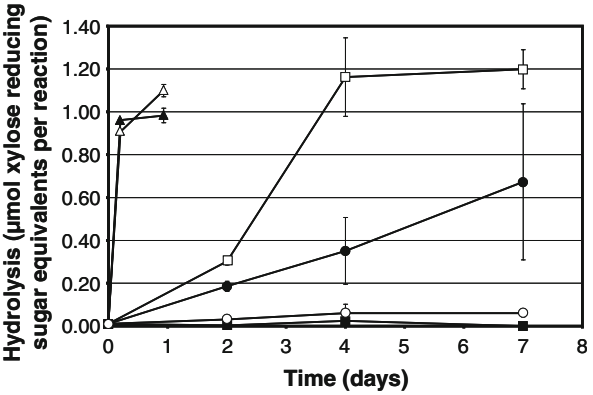

Fig. 5 Substrate hydrolysis over time: WAX and M4 at water activities from 0.5 to 1.0 over 3 weeks. *Open triangles* a_w= 1.00, *closed triangles* a_w=0.91, *open squares* a_w=0.72, *closed circles* a_w=0.59, *open circles* a_w=0.50, and *closed squares* DriRite®

there was a lower-than-saturating water activity at which this enzyme would be active, a fourth trial was begun using samples recovered from the a_w=0.2 and 0.4 chambers after 7 weeks. Samples were split between three chambers at a_w=0.97, 0.91, and 0.83 and were incubated for approximately 1 week. Activity was seen at a_w=0.97 and 0.91, but not at 0.83 (Fig. 7); the water activity limit for this enzyme preparation on WAX was between 0.91 and 0.83. Additionally, this experiment demonstrated that enzyme activity was preserved in these samples after 7 weeks of incubation at low water activity and that enzyme activity was reversible upon rehydration.

Water content by water activity on dried foods [8] and proteins [16] has been described as a sigmoidal curve with breaks in slope at approximately a_w=0.2 and a_w=0.8. The slope changes represent the transitions between a monolayer of adsorbed water molecules and multimolecular water (0.2) and between multimolecular water and condensed water within capillaries (0.8). Results for both WAX and the natural substrates show the second inflection in the range of 0.8 to 0.95 (Figs. 1 and 2) but not the first inflection, perhaps more as an artifact of not sampling at water activities around 0.2 for WAX and conducting full rather than working isotherms for the natural substrates. Several isotherm models have been developed that mathematically describe the relationship between water activity and water content [19], among the more commonly cited is the Guggenheim, Anderson, de Boer (GAB) equation. The GAB equation was used to fit the results of the adsorption

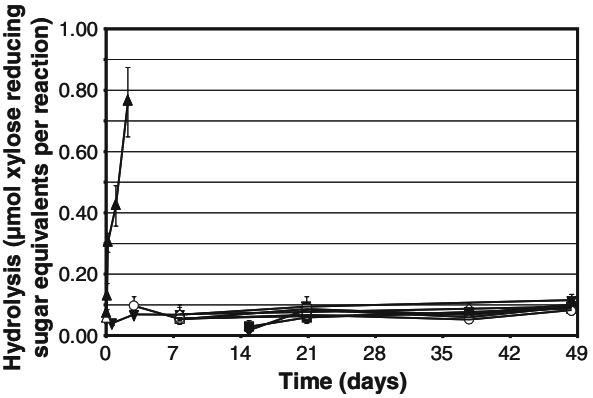

Fig. 6 Substrate hydrolysis over time: WAX and TLX at water activities from 0.2 to 1.0 over 7 weeks. *Open triangles* unexposed, *closed triangles* a_w=1.00, *open circles* a_w=0.80, *closed circles* a_w=0.72, *open squares* a_w=0.59, *open circles* a_w=0.50, *open diamonds* a_w=0.43, *closed squares* a_w=0.20, and *inverted closed triangles* DriRite®

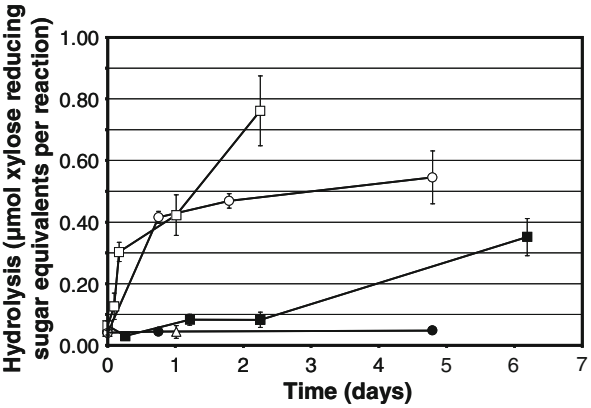

Fig. 7 Substrate hydrolysis over time: WAX and TLX at water activities from 0.83 to 1.0 over 1 week. *Open squares* a_w=1.00, *open circles* a_w=0.97, *closed squares* a_w=0.91, *closed circles* a_w=0.83, and *open triangles* DriRite®. Data for a_w=1.0 was previously presented in Fig. 6, but was shown here for comparison

curves for the combined isotherm from the WAX. The GAB equation is usually presented in the form:

$$w = \frac{w_m C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)}$$

where w is the moisture content (dry basis), a_w is the water activity, and w_m, K, and C are the matrix-dependent sorption parameters. In this form, w_m describes the moisture content corresponding to the complete sorption of a monomolecular layer of water on the materials' available surface [19]. The observed w_m for WAX was low (0.063), but within the range of natural substrates such as native wheat starch—0.05 to 0.01 [19].

The presence of water affects an enzyme's catalytic activity in a number of ways [16]. It affects protein stability and conformation, provides a reaction medium for substrate and enzyme mobility [8], and is a reactant (for example in hydrolyses). Hydrolysis of xylan by an endoxylanase consumes water during each single hydrolytic event [2] and is represented in the reaction:

$$H(C_5H_8O_4)_nOH + H_2O \Leftrightarrow H(C_5H_8O_4)_{n-p}OH + H(C_5H_8O_4)_pOH$$

Hydrolyses are equilibrium reactions; when a reactant is removed (in this instance water), the reaction shifts to the left; in nonequilibrium reactions, when water is removed, the reaction stops [16]. In enzymatically catalyzed reactions, unless catalytic activity is lost after water removal, the reaction may resume upon rehydration [20]. In these reactions, temporal water limitations result in a decrease in the observed rates [8]. Both kinetic limitations (Figs. 4 and 5) at lower water activities and resumption of enzymatic hydrolysis upon rewetting (as in TLX, discussed above) were observed in this study.

In contrast to previous work [8, 16, 20, 21] in which Avicel (FMC Biopolymers Philadelphia, PA, USA) was used as a solid insoluble substrate and a soluble reactant was used as the enzyme substrate, WAX was both the physical and enzymatic substrate. As a result, the enzyme resided within a matrix of substrate, and the role of water as a diffusion medium was minimized. Diffusion within the solid matrix would have been limited to the products (smaller arabinoxylan oligomers) or the enzyme. Solubility of—and as a result diffusion of—WAX would be expected only at higher water activities where droplets were observed (a_w>0.91) in this experimental system.

Water activity has been demonstrated to control the extent of reaction [8, 20] in "solvent-limited" systems where enzyme and substrate/products are effectively separated by absence of a diffusion medium. Hydrolysis at varying levels of water activity tended towards different levels of reaction completion. Upon exposure to increasing water activities, the levels increased to a new elevated level. Schwimmer [20] described water's role in these systems as a "vehicle for substrate transport" and concluded that the "size, mobility, and physical state of the substrate become increasingly important as water is removed". In contrast, researchers have questioned the effect of water activity on extent of reaction and imply that only the kinetics change [21]. Our data do not cover a sufficient time scale to shed light on this contradiction.

Experimental results in most cases indicated a similar level of hydrolysis, albeit somewhat less than the maximum calculated for the substrate. Assuming complete enzymatic hydrolysis of the xylan backbone of WAX to equal molar concentrations of xylobiose and xylotriose—the two predicted end products of endoxylanase activity—the final maximum xylose reducing sugar equivalent concentration in each reaction was calculated to be 2.3 μmol. In practice, the maximum xylose reducing sugar concentration observed was 1.8 μmol. Reasons may include differences in the saturating enzyme concentrations, the enzyme to substrate ratios, or the nature of the contact between enzyme and substrate used in this study compared to other work [8, 20]. The noted less-than-maximal hydrolysis was most likely due to the highly substituted nature of WAX and the lack of accessory enzymes (α-L-arabinofuranosidases) to debranch the substrate [22, 23].

One of the three enzyme preparations—TLX—did not exhibit endoxylanase activity below a_w=0.91. TLX was the only enzyme preparation that contained a potential inhibitor, starch. According to the manufacturer's literature, the enzyme was supplied in a granular form to be used for the modification of bread dough. Aqueous extraction methods resulted in carryover of soluble starch, which may have inhibited enzyme activity (e.g., via competitive binding) or competed for water at a_w<0.91. Experiments performed in our laboratory using another commercial preparation of *T. lanuginosus* endoxylanase (Ronozyme WX(L), DSM Nutritional Products, Parsippany, NJ, USA) indicate that the purified enzyme exhibits the same enzyme activity by water activity relationship observed for TLX (data not shown). This suggests that starch did not play an active role inhibiting endoxylanase activity and further indicates that the *T. lanuginosus* endoxylanase may be of limited efficacy under dry conditions.

Experimental conditions used fixed water activity to examine enzyme activity in a surrogate substrate. Endoxylanase activity produces lower molecular weight oligomers from a large molecular weight substrate—arabinoxylan. In general, increasing carbohydrate concentration in a solution held at a fixed water activity results in increasing water content in solution (under equilibrium conditions with headspace water) [24]. While the sample masses were not measured over the course of the experiment, sample masses were expected to have increased during WAX hydrolysis. This has implications for hydrolytic enzyme application in storage. If water contents of stored biomass are fixed (e.g., wrapped bales), then water activity would decrease during hydrolysis both from the consumption of water in hydrolytic reactions and by the production of smaller reaction products that would act as humectants. Conversely, if water activities are fixed (e.g., consistently high humidity), then water contents will increase during hydrolysis depending on the relative amounts of water absorbed from the vapor phase and the amount consumed due to the hydrolysis reaction. Hence, modulation of water activity via hydrolytic enzymatic activity may provide a means to control the extent of hydrolytic reaction and impart further material stability in storage.

A further implication of our results is that environmentally controlled wetting and drying over time of stored biomass can continue to change the material's composition even after the water activity moves below 0.60. Even when the biomass is stored dry, if there is significant biological activity that occurs before storage, then there is the potential to maintain active enzymes in storage that can alter the composition of the material even in the absence of biological activity (i.e., below a_w of 0.60).

The primary goal of these experiments was to screen several available endoxylanase preparations for use under varying degrees of water activity anticipated in biomass feedstock storage applications. Enzyme concentration in this study was high relative to the intended application rate in storage; experiments were intended to clearly demonstrate enzymatic activity (or lack thereof) in fewer than 30 days under the lowest water activities. Experimental loading rates were 56 U per gram wheat arabinoxylan; equivalent loading rates in natural feedstocks with 20% to 25% (*w*/*w*) hemicellulose content would range from 11 to 14 U per gram dry matter. With a commercially available endoxylanase preparation, Ronozyme WX(L) at an estimated cost of $6.50 per liter and an activity of 650 U mL^{-1}, the price per dry matter tonne to treat a feedstock at the experimental level ranges from $110 to $140—well above the value of the final feedstock. However, when used as an animal feed supplement, the manufacturer suggests an application rate of 300 U per kilogram fresh feed. Assuming the feed material has 20% hemicellulose and 15% water content (wet basis), this results in an enzyme loading rate of 1.4 U per gram hemicellulose. Estimated costs for the manufacturer's lower loading rate range from $2.80 to $3.50 per dry matter tonne depending upon the hemicellulose content in the feedstock. Experiments are in progress at INL using corn stover and switchgrass feedstocks to determine the extent that pretreatment in storage can affect energy requirements for grinding and densification, flow characteristics, and water activity that are critical to aerobic stability. Changes to feedstock properties resulting from $3.50 per tonne pretreatment in storage may be cost effective in the face of preprocessing costs for grinding and densification that may exceed $18 per tonne.

We focused on dry storage conditions due to the inherent resistance to microbial and fungal degradation that subsequently results in dry matter loss and change in material composition. Dry conditions inhibit microbial and fungal growth and activity, making enzyme production by either naturally occurring or recombinant microbes impractical. Additionally, dry storage brings with it a great deal of existing technology for amendment application and material storage. Forage amendment delivery systems exist that apply soluble nutrients, buffered acids, and suspended bacteria directly to harvested crops during baling. Soluble enzymes may be added in this manner without modification to existing equipment. Storage formats exist for these materials; baling and loafing systems for the storage of various animal feeds can be used for cellulosic feedstocks. Even using existing technology, much remains to be determined: (1) How do these amendments perform within the existing storage formats, (2) what benefits do they impart to storage, formatting, transportation, and deconstruction at the biorefinery, and (3) are the costs for enzymes and application methods offset by the observed improvements?

Conclusions and Future Directions

Endoxylanase activity was demonstrated on wheat arabinoxylan at or below the minimum water activities needed to support microbial or fungal activity. Water contents for corn stover, switchgrass, and wheat straw at equivalent water activities where enzymatic activity

was observed were within the range typically used to describe dry storage conditions. Results indicate that enzymatic transformations are possible under dry storage conditions, where low water activity enhances product stability, specifically that hydrolytic enzymes function and that the enzymatic hydrolysis products would be preserved by inhibition of microbial or fungal activity at low water activity. However, enzyme activity varied by enzyme source; thus, it is important to screen potential enzymes in this manner before application to more complex substrates.

The poor performance in dry conditions of a widely commercially available endoxylanase suggests that there may be value in development of new endoxylanase preparations for large-scale commercial use. If endoxylanase treatment in dry storage provides promise as a means to improve feedstock value to the biorefinery, then enzyme producers will have strong incentive to explore work with more xerotolerant enzymes such as M2 and M4.

Ongoing feasibility testing includes application of endoxylanase in wet systems such as ensiled biomass and under dry storage conditions such as occurs in bales, loafs, and piles. These feedstocks will be treated at relatively high enzyme loadings and tested for changes in material composition and quality, fermentable sugar recalcitrance and pretreatment severity, and physical handling characteristics to assess the range of effects that can be expected in proposed endoxylanase-treated storage systems. Once the primary effects of enzymatic treatment have been identified, future studies can explore the application rates that achieve the optimal cost-benefit balance.

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