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Deficiency of Cellulase Activity Measurements for Enzyme Evaluation

Scott W. Pryor · Nurun Nahar

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Abstract Switchgrass was used as a model feedstock to determine the influence of pretreatment conditions and biomass quality on enzymatic hydrolysis using different enzyme products. Dilute sulfuric acid and soaking in aqueous ammonia pretreatments were used to produce biomass with varied levels of hemicellulose and lignin sheathing. Pretreated switchgrass solids were tested with simple enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) with three commercial enzyme products: Accellerase 1000 (Genencor), Spezyme CP (Genencor)/Novozyme 188 (Novozymes), and Celluclast/Novozyme 188 (Novozymes). Enzymes were loaded on a common activity basis (FPU/g cellulose and CBU/g cellulose). Despite identical enzyme loadings, glucose yields were significantly different for both acid and alkaline pretreatments but differences diminished as hydrolysis progressed for acid-pretreated biomass. Cellobiose concentrations in Accellerase treatments indicated an initial β-glucosidase limitation that became less significant over time. SSF experiments showed that differences in glucose and ethanol yields could not be attributed to enzyme product inhibition. Yield discrepancies of glucose or ethanol in acid pretreatment, alkaline pretreatment, and acid pretreatment/SSF were as much as 15%, 19%, and 5%. These results indicate that standardized protocols for measuring enzyme activity may not be adequate for assessing activity using pretreated biomass substrates.

Keywords Cellulase activity · Enzymatic hydrolysis · Pretreatment · Cellobiose · β-glucosidase

Introduction

Thermochemical pretreatment followed by enzymatic hydrolysis is a likely technology for producing fermentable sugars from biomass structural polysaccharides. Leading pretreatment technologies typically utilize elevated temperatures and highly acidic or alkaline

S. W. Pryor (✉) · N. Nahar
Department of Agricultural and Biosystems Engineering, North Dakota State University,
Dept. 7620, P.O. Box 6050, Fargo, ND 58108-6050, USA
e-mail: scott.pryor@ndsu.edu

environments [1]. These and other technologies meet the pretreatment goal of improving efficacy of enzymatic hydrolysis by altering the structure and composition of biomass. Biomass that has been effectively pretreated has an increased susceptibility to enzymatic hydrolysis, but the structure and composition of the resulting material will depend not only on the initial feedstock but on the type of pretreatment performed. Acidic pretreatment processes, including dilute acid pretreatment, compressed liquid hot water, and steam explosion, tend to function primarily through hemicellulose hydrolysis [2]. Alkaline pretreatments including soaking in aqueous ammonia, ammonia fiber expansion, and ammonia recycle percolation function primarily by swelling of cellulose molecules and lignin removal or modification [1, 3].

Acid pretreatments are also known to produce inhibitors such as acetic acid, furfural, or hydroxymethyl furfural (HMF) from excessive degradation of lignocellulosic material while these products exist at much lower concentrations after most alkaline pretreatments [4–6]. Such compounds primarily impact fermentation, but enzymatic hydrolysis has also been shown to be inhibited by acid pretreatment degradation products [7–9]. Alkaline pretreatments have also been shown to produce inhibitory lignin degradation products which have a significant impact on xylanase activity [10–12]. Inhibition of cellulase and cellobiase activity by sugars produced during hydrolysis has been well established and has led to the development of simultaneous saccharification and fermentation (SSF) to relieve such inhibition [3, 13].

Commercial cellulases are mixtures of enzymes with varying proportions of endoglucanases, exoglucanases, and β-glucosidases with supplementary noncellulolytic enzymes such as hemicellulases (e.g., xylanases and mannanases) and pectinases. The mixtures will vary based on the producing organism and substrate on which it is grown [14]. These enzymes all function in a synergistic manner to allow hydrolysis of cellulose in biomass to cellobiose and ultimately glucose. Extent of hydrolysis will depend on a number of factors including the mixtures of cellulase and supplementary enzymes as well as the substrate crystallinity, degree of polymerization, presence of lignin and hemicellulose, and accessible surface area [15–17].

Cellulase and β-glucosidase activities are typically assessed using standard assays in which sugar yields are measured upon hydrolysis of a standard substrate under standardized conditions of temperature, pH, and time. Cellulase activity assays utilize a piece of Whatman's No. 1 filter paper as substrate, and activity is expressed in filter paper units (FPU); an FPU is defined as the quantity of enzyme that releases 1 μmol of glucose-equivalents per milliliter per minute from that standard substrate under the defined assay conditions [18, 19]. Likewise, β-glucosidase activities are assessed using standard protocols using cellobiose substrate and resulting activities are expressed in cellobiase units (CBU). A CBU is defined as the enzyme amount which converts 1 μmol of cellobiose to 2 μmol of glucose per milliliter per minute. These measurements form the basis of enzyme loading rates. Development of new cellulase formulations has been an important area of commercial and academic research with the goal of lowering production costs on an enzyme activity basis [20].

Although different enzyme formulations will vary in activity expression (FPU/ml or FPU/g protein), the standardization of enzyme activity and dosing ideally should remove much of the variation in performance results. Accordingly, typical cellulase loadings vary between 10 and 30 FPU/g cellulose based on pretreatment method and conditions, among other factors [21–24]. Although dosing is sometimes reported on a protein or volumetric basis in enzyme product literature or elsewhere, it is still related to standard activity measurements [25]. Ethanol processors might, therefore, approach enzyme purchasing by comparing products on an economic basis of $/FPU [20]. In practice, however, equal FPU and CBU loadings with different enzyme cocktails could still lead to performance variation

because of differences in substrate accessibility, presence of lignin and hemicellulose, and response to inhibitory products either in the form of sugars, lignin, or their degradation products [7, 12, 26, 27]. In short, cellulases are quantified on an activity basis using standard substrates and conditions; equal activities under the standard conditions may not lead to equal hydrolysis rates or yields using pretreated biomass with differences in structure and noncellulosic components.

This study compared several commercial cellulase products for the hydrolysis of pretreated switchgrass. All cellulases were derived from the fungus *Trichoderma reesei*, and those deficient in β-glucosidase activity were supplemented with β-glucosidase derived from *Aspergillus niger*. The aim was to examine if conventional activity assays are sufficient to compare newly developed enzymes when considering the wide range of potential feedstocks, pretreatment methods, and hydrolysis conditions.

Materials and Methods

Raw Material

Sunburst switchgrass (*Panicum virgatum* L.) was grown at the Central Grasslands Research Center (Streeter, ND, USA) and harvested at full maturity from a 5-year plot. The biomass was air-dried and ground using a Wiley mill with a sieve diameter of 2 mm. The ground biomass (5% moisture content) was then stored in sealed plastic bags at room temperature.

The chemical composition of switchgrass was determined using the National Renewable Energy Laboratory standard laboratory procedures for biomass analysis [28]. Cellulose content of original feedstock was determined at the ARS Northern Great Plains Research Laboratory (Mandan, ND, USA) using an ANKOM 200 fiber analyzer (ANKOM Technology, Fairport, NY, USA) and manufacturer standard procedures. Biomass composition is shown in Table 1.

Pretreatment

Acid and alkaline pretreatments were conducted to produce pretreated biomass with a range of qualities in terms of presence of lignin and degree of hemicellulose hydrolysis. Ground switchgrass (80 dry g) was mixed with dilute sulfuric acid (0.75% or 1.5% (*w*/*w*)) at a solid loading rate of 10% (*w*/*w*) and pretreated for 10 or 20 min at 140 °C in a 1-l pressure vessel

Table 1 Compositional analysis results for switchgrass feedstock (dry basis).

Analyte	% (dwb)	SD
Cellulose	35.9	0.30
Extractives (95% ethanol)	9.9	0.39
Glucan	32.3	0.35
Xylan	17.0	0.34
Arabinan	2.4	0.35
Galactan	0.5	0.09
Total lignin	21.9	0.20
Ash	4.9	0.1

(4600 Series-Parr Industries; Moline, IL, USA). A summary of the dilute acid pretreatment operating conditions used is shown in Table 2. Resulting pressures for acid pretreatments were approximately 40 psi. The residence time of pretreatment was counted when the mixture of biomass and dilute sulfuric acid reached the desired reaction temperature as listed. After the specified reaction time, the reactor was immersed in an ice water bath until the pressure inside the reactor equalized with atmospheric pressure. Pretreated biomass was transferred to a Buchner funnel and was filtered under vacuum while washing with 1 l of deionized water. The total volume of wash water filtrate was recorded, and a sample was taken for individual sugar analysis via HPLC. Filtered solids were weighed and used for enzymatic hydrolysis.

Soaking in aqueous ammonia was carried out in the same pretreatment vessel noted above. The same feedstock material was pretreated with 15% ammonia at a 1:6 solid loading rate for 24 h. Temperatures of 40 °C, 60 °C, and 80 °C were tested with resulting pressures of approximatley10, 15, and 20 psi, respectively. Pretreated biomass was filtered and washed as described above.

Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out using several commercial cellulase products derived from *T. reesei*. Accellerase 1000 (Genencor International; Rochester, NY, USA), Spezyme CP (Genencor), and Celluclast 1.5L (Novozymes, Inc.; Bagsvaerd, Denmark) were all used at a cellulase loading rate of 25 FPU/g original cellulose. Novozyme 188 (Novozymes, Inc.), a β-glucosidase derived from *A. niger*, was used to supplement Spezyme and Celluclast treatments to achieve a total loading of 31.3 CBU/g original cellulose. This loading was based on the measured β-glucosidase activity of Accellerase 1000 to achieve identical FPU and CBU loading rates for each base cellulase product. Cellulase and β-glucosidase activities of enzyme products are shown in Table 3. All enzyme activities were tested multiple times through experimentation, and results were found to be consistent.

Enzymatic hydrolysis was performed in 250-ml Erlenmeyer flasks using washed and filtered pretreated biomass based on 2% (original) cellulose loadings in 50 mM sodium citrate buffer (pH 4.8). Sodium azide (0.04%) was used to inhibit microbial growth during the hydrolysis. Flasks were shaken at 100 rpm and 50 °C for 72 h in a water bath shaker. Controls were conducted with nonpretreated biomass samples at the same enzyme loadings. All treatments were conducted in triplicate with the exception of controls which were done in duplicate.

Samples (1 ml) were removed from each flask at 0, 2, 18, 24, 48, and 72 h for analysis. Samples were centrifuged (1,300 rpm, 3 min) and filtered through a 0.2-μm nylon filter (Pall Corporation; West Chester, PA, USA) prior to sugar quantification via HPLC. The samples were stored at −20 °C until analysis.

Table 2 Conditions for dilute acid pretreatment of switchgrass.

Treatment	H_2SO_4 (%)	Temp (°C)	Time (min)
1	0.75	140	10
2	0.75	140	20
3	1.5	140	10
4	1.5	140	20

Table 3 Cellulase and cellobiase activities of enzyme products.

Enzyme	Cellulase Activity (FPU/ml)	β-glucosidase Activity (CBU/ml)
Accellerase 1000	67.3	84.2
Spezyme CP	55.2	15.4
Celluclast 1.5L	60.7	6.5
Novozyme 188	0.1	661

Simultaneous Saccharification and Fermentation

Simultaneous SSF of dilute acid-pretreated biomass was carried out to determine the specific impact of substrate product inhibition on the different enzyme systems. SSF methods were based on NREL protocols [29] with minor modifications. Biomass was pretreated at 0.75% H_2SO_4 and 140 °C for 20 min. Washed and filtered solid residue was then sterilized in Erlenmeyer flasks at 121 °C for 30 min. Citrate buffer (0.05 mM, pH 4.8) with 1% yeast extract, and 2% peptone was added to achieve an approximate cellulose loading rate of 3% (*w*/*v*) based on previous quantification of glucan hydrolysis during pretreatment. Cellulases were loaded at 25 FPU/g original cellulose and 31.3 CBU/g original cellulose as described for enzymatic hydrolysis. *Saccharomyces cerevisiae* inoculum was grown at 37 °C and 130 rpm for 24 h in media containing 10 g glucose/l and 5 g yeast extract/l, and this culture was inoculated into fermentation flasks at a 1% (*v*/*v*) loading. Fermentations were carried out in duplicate at 37 °C and 130 rpm for 7 days. Controls were conducted without biomass addition to account for ethanol produced from basic media components.

Analytical Methods

Biomass was oven dried at 105 °C for 24 h until constant weight was obtained for moisture content analysis. Cellulase and β-glucosidase activities were assayed as FPU and CBU, respectively, as per Ghose [19].

The sugar concentrations of the pretreatment filtrate, enzymatic hydrolyzate, and fermentation broth were determined by HPLC. The HPLC system (Waters Corporation; Milford, MA, USA) was equipped with an autosampler, an isocratic pump, and a refractive index detector (RID, model 2414, Waters Corporation). Separation was achieved using an Aminex HPX-87P (300×7.8 mm) carbohydrate analysis column with a Carbo-P micro-guard cartridge (30×4.6 mm, Bio-Rad; Hercules, CA, USA). The column was maintained at 85 °C, and the sugars were eluted using 18 mΩ NANOpure water at a flow rate of 0.6 ml/min. Cellobiose, glucose, and xylose peaks were detected by RID and quantified using a 3-point external standard curve.

Quantification of all inhibitors (furfural, HMF, acetic acid, and levulinic acid) as well as glucose and ethanol concentrations during SSF experiments was done via HPLC using an Aminex HPX-87H (300×7.8 mm) ion exclusion column (Bio-Rad) with a Cation H microguard cartridge (30×4.6 mm). The column temperature was maintained at 65 °C, and sample was eluted using a mobile phase of 5 mM sulfuric acid at a rate of 0.6 ml/min. Fermentation products were monitored with an RID and quantification was completed using 3-point external calibration curves. Quantification of delignification from soaking in aqueous ammonia pretreatment was calculated using standard procedures [28].

Statistical Analysis

Enzymatic hydrolysis of pretreated biomass was completed in triplicate, and SSF was done in duplicate with all biomass for all replicates and enzyme treatments coming from a single pretreatment batch. Statistical analysis including analysis of variance (ANOVA) was done using Minitab software (Minitab, Inc.; State College, PA, USA). Multiple comparisons of means testing was done for each hydrolysis or fermentation time point using a Tukey's test controlling the overall error rate at the 5% level. This resulted in individual comparison error rates at 2.2% and 2.5% for hydrolysis and SSF, respectively.

Results and Discussion

Acid Pretreatment

Table 4 shows concentrations of glucose, xylose, and sugar degradation products present in the dilute acid pretreatment filtrate under varying pretreatment conditions. Higher severity pretreatments led to increases in formation of degradation products from hydrolyzed glucan and xylan. The formation of such products would not be desirable for actual processing but the resulting solids have varying qualities of biomass in terms of carbohydrate composition and structure. Estimates of hemicellulose hydrolysis (based on xylan recovery in filtrate) were between 62% and 105% (Table 4); actual hemicellulose hydrolysis is assumed near complete for all treatments because the treatments with lower xylan recoveries had correspondingly higher concentrations of degradation products.

Figure 1 shows glucose concentrations following enzymatic hydrolysis with the individual enzyme products under varied dilute acid pretreatment conditions. A one-way ANOVA was completed on data from each of the pretreatment conditions. Results showed a rejection of the null hypothesis of equal means ($p<0.05$) between enzyme treatments under each of the four pretreatment conditions. Additional pretreatments at high temperatures resulted in excessive glucan and xylan hydrolysis, and degradation in pretreatment but enzymatic hydrolysis of remaining solids showed similar results (data not shown). Multiple comparison of means showed that glucose concentrations following hydrolysis with Accellerase were significantly lower ($p<0.05$) than for the other two enzyme systems for all pretreatment conditions. Final sugar concentrations following hydrolysis with Spezyme and Celluclast were not distinguishable from each other at the 0.05 overall confidence level for any treatments. Final sugar yield following hydrolysis with Accellerase had a mean value 15% less than those produced with the other enzyme combinations across all acidic

Table 4 Sugar and inhibitor concentrations in dilute acid pretreatment filtrate.

Pretreatment conditions	Glucose (g/l)	Xylose (g/l)	Formic acid (g/l)	Acetic acid (g/l)	Levulinic acid (g/l)	HMF (g/l)	Furfural (g/l)
0.75%, 140 °C, 10 min	4.18 (25%)	10.89 (98%)	–	–	–	–	–
0.75%, 140 °C, 20 min	4.52 (27%)	11.63 (105%)	–	0.80	0.09	–	–
1.5%, 140 °C, 10 min	4.35 (25%)	10.05 (86%)	0.11	1.02	0.35	0.43	0.46
1.5%, 140 °C, 20 min	2.87 (16%)	7.28 (62%)	0.14	0.96	0.43	0.37	0.48

Glucose and xylose numbers in parentheses represent recovered monomers as a percentage of original glucan and xylan equivalents

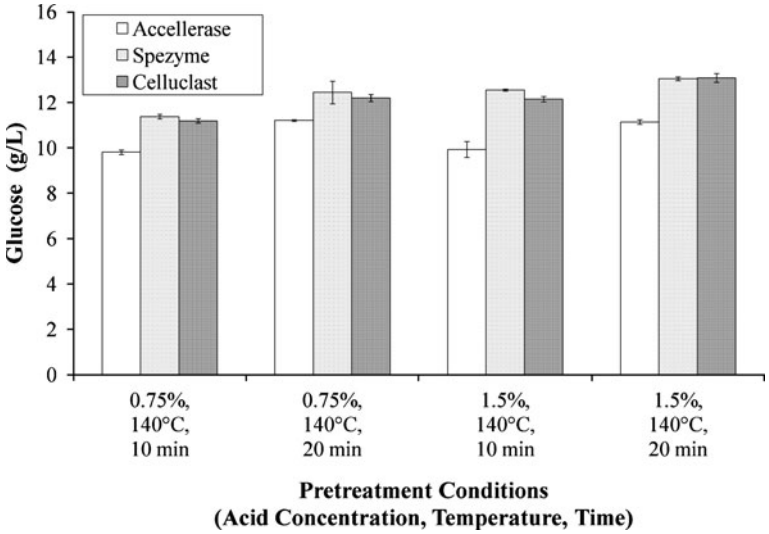

Fig. 1 Concentrations of glucose following 72-h enzymatic hydrolysis of washed biomass solids following pretreatment under varied conditions

pretreatment conditions despite loading with the same FPU and CBU levels. Similar results comparing Celluclast and Accellerase have been reported using steam-pretreated spruce with equal cellulase loadings but without adjustment for β-glucosidase activity [30].

Differences in glucose yields between enzyme treatments can be explained partially by cellobiose concentrations during hydrolysis. All treatments were loaded with identical β-glucosidase loadings; Accellerase has inherent β-glucosidase activity while the other treatments were supplemented with Novozyme 188 to achieve the same FPU–CBU ratio as found in Accellerase. Accellerase treatments had the lowest glucose concentrations throughout hydrolysis, and the concentration differences were greatest early in hydrolysis and the discrepancy continued to diminish with time (see Fig. 2). The Accellerase treatments had approximately two times the cellobiose concentrations compared with Celluclast and Spezyme treatments during the earliest stage of hydrolysis (see Fig. 3). The high initial cellobiose concentrations for Accellerase treatments indicate an initial β-glucosidase limitation, and this agrees with the corresponding low glucose concentrations. An initial β-glucosidase limitation would be expected to decrease as hydrolysis continues and available substrate decreases. Accellerase treatment yields do increase over time relative to the other enzyme products, further supporting an initial β-glucosidase limitation. Although all enzyme treatments were adjusted to the same β-glucosidase loading, both Spezyme and Celluclast were supplemented with cellobiases derived from *A. niger* while Accellerase β-glucosidases were produced concurrently with cellulase using *T. reesei*. Others have shown that β-glucosidase activity can be impacted by low concentrations of hydrolyzate or fermentation components and that activity variation is also dependent on the source of the enzyme [31, 32].

Although the discrepancy in apparent β-glucosidase activities in the different treatments is notable, it cannot fully explain the glucose concentration differences. Hydrolysis of 1 g of cellobiose yields 1.05 g of glucose and the sum of this with the actual glucose concentrations shows that cellobiose formation (cellulose hydrolysis) is also occurring at a slower rate. The fact that cellobiose is a strong inhibitor of cellulases exacerbates the impact of a β-glucosidase limitation [33]. Such a cellulase deficiency could be attributed to

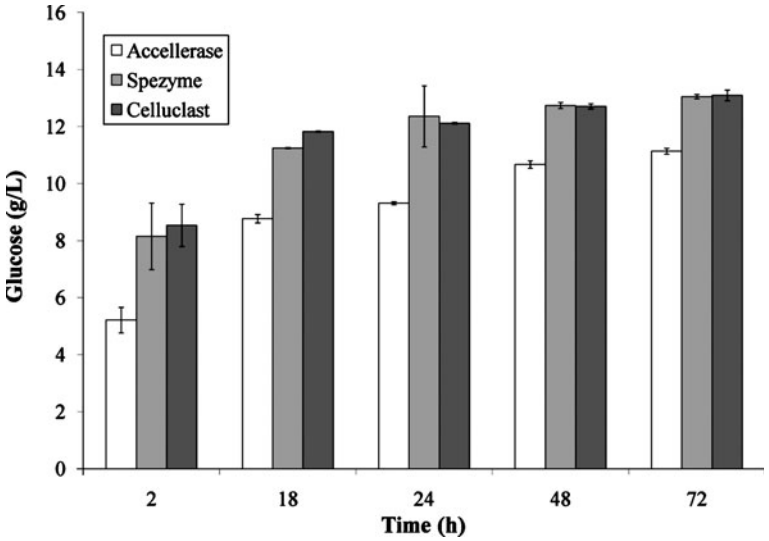

Fig. 2 Glucose concentrations during enzymatic hydrolysis following dilute acid pretreatment (1.5% H_2SO_4, 140 °C for 20 min)

binding limitations in the cellulases themselves when acting on pretreated biomass [26]. Lack of supplementary enzymes such as hemicellulases or pectinases, which are not accounted for in standard cellulase activity assays, could impact hydrolysis but are not likely given the high yields of xylan in pretreatment filtrate [25, 34, 35].

Enzymatic hydrolysis following dilute acid pretreatment was repeated using moderate pretreatment conditions (0.75% H_2SO_4, 140 °C, 20 min) and enzyme loadings of 15, 20, 25, and 30 FPU/g cellulose (and the same FPU:CBU ratio) to determine if variability in activity measurements could be causing the discrepancy. Results for hydrolysis following these experiments are shown in Fig. 4. Sugar yields with the use of Accellerase were again the lowest for each enzyme loading rate and Spezyme yielded the highest concentrations at each loading. These differences were statistically significant at all loading rates. Increasing Accellerase loading rates produced an apparent increase in sugar yields to 25 FPU but those differences were not significant between 20, 25, and 30 FPU/g cellulose. Yields following hydrolysis with Spezyme increased up to 25 FPU/g cellulose with no significant difference at higher loadings. These results indicate that differences between enzymes were not likely attributable to variability in activity measurements. Because there was no significant increase in yields for Accellerase treatments above 20 FPU/g and these yields were less than those from other enzymes, any β-glucosidase limitation appears to be a limitation of function rather than quantity.

Alkaline Pretreatment

Table 5 shows the impact of soaking in aqueous ammonia on delignification of switchgrass prior to enzymatic hydrolysis. Significant loss of Klason lignin was observed under all temperatures with increasing delignification between 40 °C and 60 °C. No increase in delignification was observed by increasing the pretreatment temperature to 80 °C. Hemicellulose hydrolysis during alkaline pretreatment was found to be negligible with less than 1% of original xylan recovered as xylose in pretreatment filtrate.

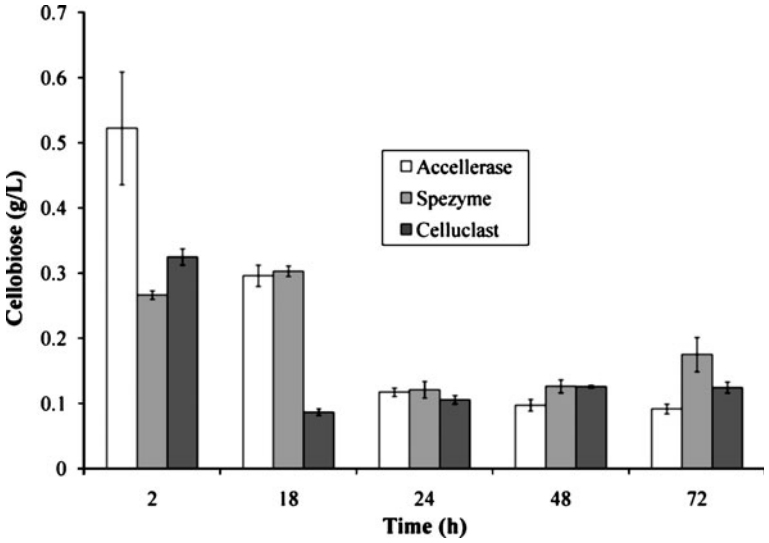

Fig. 3 Cellobiose concentrations during enzymatic hydrolysis following dilute acid pretreatment (1.5% H_2SO_4, 140 °C for 20 min)

Results of enzymatic hydrolysis using the three enzyme systems after soaking in aqueous ammonia pretreatment are shown in Fig. 5. These results agree with those following hydrolysis of dilute acid-pretreated biomass. Accellerase again produced the lowest sugar yields, while there was no significant difference between Spezyme and Celluclast treatments. Sugar yields were highest for the 60 °C treatment for all enzymes.

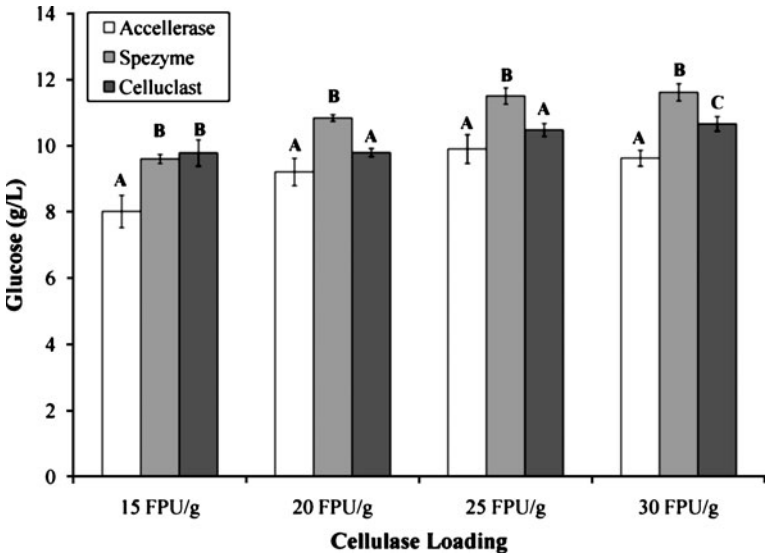

Fig. 4 Glucose concentrations after dilute acid pretreatment and 72-h enzymatic hydrolysis at various cellulase loading rates. *Letters* indicate significant differences at each loading rate

Table 5 Effect of temperature on delignification during soaking in aqueous ammonia pretreatment of switchgrass.

	Raw Switchgrass	Pretreatment temperature		
		40 °C	60 °C	80 °C
Total solids (%)	96.7 (0.1)			
Ash (%)	4.9 (0.1)	1.9 (0.1)	1.6 (0.05)	2.0 (0.09)
Extractives: 95% Ethanol (%)	9.9 (0.4)	2.9	2.7	2.6
Acid soluble lignin (ASL)[a]	3.2 (0.1)	1.9 (0.02)	1.2 (0.01)	1.2 (0.01)
Klason lignin[a] (%)	18.7 (0.3)	14.2 (0.6)	10.8 (0.3)	11.0 (0.6)
Total lignin[a] (%)	21.9 (0.2)	16.1 (0.6)	12.0 (0.3)	12.2 (0.5)
Delignification (%)	–	26.5	45.2	44.3

Sample standard deviations are in parentheses

[a] Expressed on an extractive free basis

Differences between sugar yields from the various enzymes do not appear to be attributable solely to the presence of lignin. Accellerase sugar yields were 20%, 19%, and 31% lower than the other enzymes following soaking in aqueous ammonia pretreatment at 40 °C, 60 °C, and 80 °C, respectively; extent of delignification was 27%, 45%, and 44% for these treatments.

Patterns of cellobiose and glucose formation during hydrolysis after the alkaline pretreatment were different than those following acid pretreatment. Glucose yields from acid-pretreated biomass lagged initially for Accellerase treatments but the yield deficiency decreased both in magnitude and as a relative fraction of yields from the remaining treatments. Accellerase glucose yields from alkaline-pretreated biomass, however, were also low initially but that glucose deficiency was not as great as seen after dilute acid pretreatment. Glucose

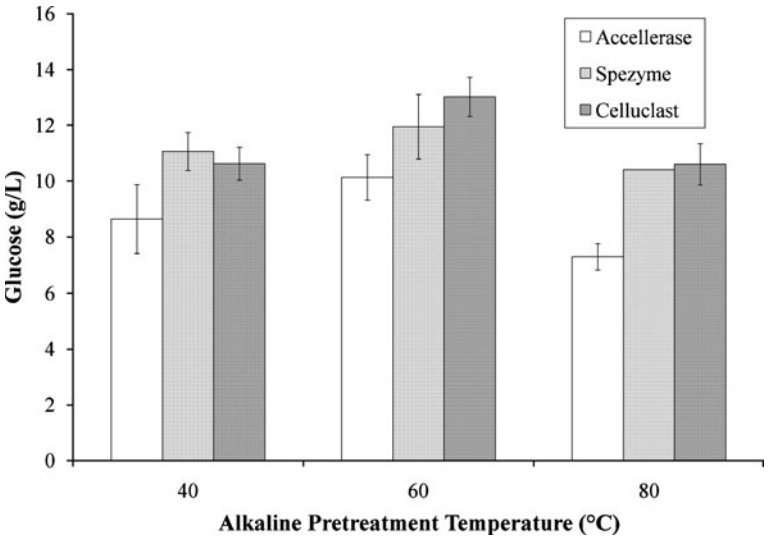

Fig. 5 Glucose concentrations after 72-h enzymatic hydrolysis following soaking in aqueous ammonia pretreatment

concentrations for Accellerase treatments were approximately 1 g/l less at 2 h into hydrolysis and that deficiency varied to some degree but generally remained constant or increased with time (see Fig. 6). Cellobiose concentrations were also not appreciably different across enzyme systems for alkaline-pretreated biomass (see Fig. 7).

Unlike results following dilute acid pretreatment, these results suggest a limitation in cellulase but not β-glucosidase. Cellulases from different sources have been shown to vary considerably in their response to inhibitory compounds [36]. Even though all cellulases used in this study are derived from *T. reesei*, variation in inhibition susceptibility have been noted between different strains of a single species and *T. reesei* is known to produce at least two exoglucanases and five endoglucanases [37–39]. Further, pretreatment technology will have an impact on the composition of pretreated biomass as well as the adsorption and desorption characteristics of cellulases, thus demonstrating that hydrolysis limitations may be pretreatment-dependent [25, 40].

Another potential reason for an apparent greater cellulase limitation in Accellerase may actually be a deficiency in hemicellulases as the importance of these accessory enzymes have been noted elsewhere [25, 34, 40]. Accellerase, Spezyme, and Celluclast are all enzyme products derived from *T. reesei*. If the Accellerase-producing strain has been modified to produce more β-glucosidase than other strains, overproduction could come at the expense of hemicellulase production. Such deficiency would not be apparent when using acid-pretreated biomass with little hemicellulose, but would be when using high-hemicellulose alkaline-pretreated biomass.

Simultaneous Saccharification and Fermentation

Results of experiments using dilute acid pretreatment followed by SSF are shown in Fig. 8. Ethanol production patterns mirrored those of glucose yields during enzymatic hydrolysis of acid-pretreated biomass. Ethanol concentrations for flasks treated with Accellerase were lower than those for either of the other enzyme products tested. Those treatments were not

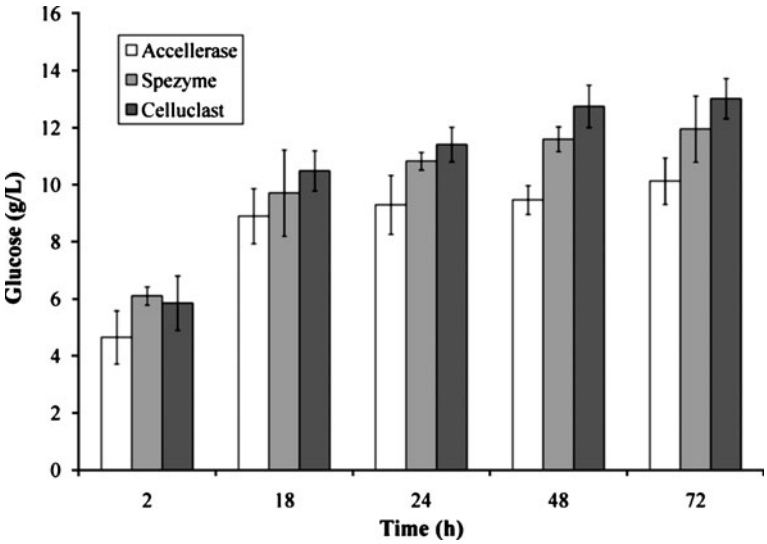

Fig. 6 Glucose concentrations during enzymatic hydrolysis following soaking in aqueous ammonia pretreatment at 60 °C

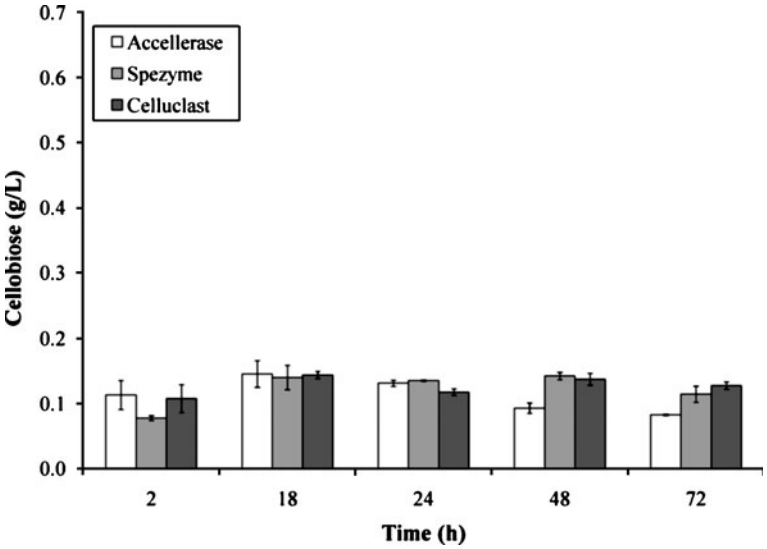

Fig. 7 Cellobiose concentrations during enzymatic hydrolysis following soaking in aqueous ammonia pretreatment at 60 °C

appreciably different from each other. Like the results from hydrolysis without fermentation (Fig. 2), the difference between Accellerase and Spezyme treatments was largest at 2 h and decreased with time. Differences between treatments were statistically significant between 24 and 96 h but not at 168 h. Glucose concentrations for the Accellerase treatment were 90% of those for the Spezyme treatment at the end of hydrolysis (72 h). Similarly, Accellerase ethanol concentrations were 89% of Spezyme treatment ethanol concentrations at 72 h and that ratio increased to 94% at the end of the SSF (168 h). The similar yield patterns for hydrolysis and SSF experiments could be still attributed to limitations in β-glucosidase and accessory enzymes in Accellerase with no indication of a significant effect of enzyme product inhibition.

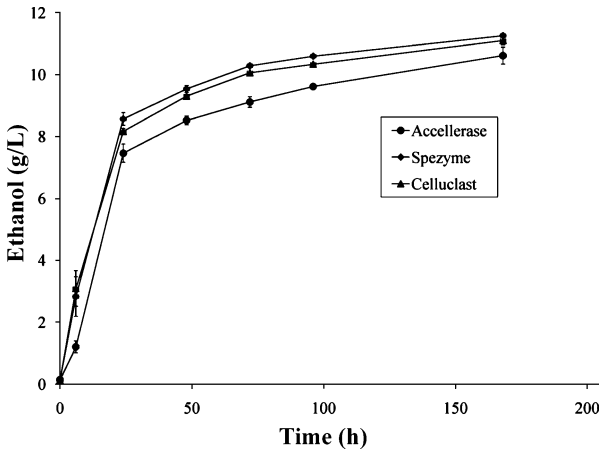

Fig. 8 Ethanol concentrations during SSF following dilute acid pretreatment

Conclusions

The differing patterns of cellobiose and glucose released during enzymatic hydrolysis of pretreated biomass indicate that the efficacy of the enzyme products is related to the presence of inhibitors, residual hemicellulose and lignin, and inherent xylanase or pectinase activity. Further, individual enzyme products will be impacted to a different degree by these factors. Limitations on enzyme efficacy will therefore differ with pretreatment methods that utilize different modes of action.

Standard enzyme activity testing is necessary for enzyme comparison and proper dosing for effective and economical biomass hydrolysis. Cellulase enzyme development is an active area of research in both academia and industry. These results underscore that standardized protocols for measuring enzyme activity are not adequate for assessing activity using pretreated biomass substrates. As such, equal cellulase loading rates should not be assumed to result in equivalent sugar yields when using different enzyme products. Such discrepancy could be caused by differing binding characteristics based on biomass quality, presence of supplementary noncellulolytic enzymes such as xylanases or pectinases, or variation in inhibitory response to sugars or their degradation products. Such differences and limitations will also change depending on the mechanism and severity of pretreatment. In this study, yields of glucose or ethanol in acid pretreatment, alkaline pretreatment, and acid pretreatment/SSF using Accellerase-treated biomass were 15%, 19%, and 5% less, respectively, than when using identical biomass treated with different enzyme products. These results were found despite starting with biomass from a single pretreatment reactor for all hydrolysis reactions and using identical cellulase and cellobiase enzyme loadings.

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