Aminopeptidase Modified Hydrolytic Enzymes to Improve the Efficiency of Sugar Production from Alkaline Pretreated Switchgrass

Debra A. Clare,[a,*] Ziyu Wang,[b] Jay J. Cheng,[b] and Dhanalekshmi Savithri [c]

Cellulases and β-glucosidases (βGSD) are enzymes commonly used in the biofuel industry. In this study, smaller-sized variants were generated with aminopeptidase such that high catalytic capabilities were retained. Under the defined experimental conditions, the degree of hydrolysis was greater using cellulase substrates, compared to βGSD, based on *ortho* phthaldialdehyde (OPA) assay data (44% *versus* 15%). Proteolysis of cellulases was also evident based on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) protein banding patterns seen after peptidase treatment. Residual cellulase activity was retained after peptidase hydrolysis (67% to 73%) based on standard filter paper assays. Peptidase treated cellulases and βGSD were then utilized for hydrolysis of alkaline-pretreated switchgrass (*Panicum virgatum)*. Interestingly, the efficiency of the reaction, defined as milligrams of sugar produced per filter paper unit, was higher using truncated cellulases for bioprocessing reactions (~14%), especially in the absence of sodium azide. Conversely, incubation of βGSD with peptidase revealed minimal proteolysis with low impact on the efficiency of hydrolysis.

Keywords: Biofuel; Panicum virgatum (switchgrass); Cellulase; Beta-glucosidase; Immobilized enzymes; Truncated enzymes; Proteases; Sodium azide

*Contact information: a: Emeritus, Department of Food, Bioprocessing, and Nutrition Sciences, NC State University, Raleigh, NC 27695-7624, USA; b: Department of Biological and Agricultural Engineering, NC State University, Raleigh NC, 27695-7625, USA; c: Department of Forest Biomaterials, NC State University, Raleigh, NC 27695-7625, USA; *Corresponding author: debra_clare@ncsu.edu*

INTRODUCTION

Cellulosic biomass continues to gain increasing attention due to its abundance, relatively low cost, and immense potential as a bio-feedstock for developing alternative fuels (Wyman 2003; Limayaem and Ricke 2012). Cellulose is the major carbohydrate constituent of plant materials (35% to 45%) that is often embedded in a matrix of other structural biopolymers such as hemicellulose and lignin, which comprise 20% to 35%, and 5% to 30%, respectively, of the dry weight matter. An important feature of the complex lies in the crystalline structure, a factor that influences both the rate and extent of enzymatic breakdown of cellulosic substrates to release sugar end products (Lynd *et al.* 2002; Hu *et al.* 2015).

The natural resistance of plant biomaterials to microbial and enzymatic deconstruction is collectively known as recalcitrance (Himmel *et al.* 2007; Abd Hamid *et al.* 2015). In nature, this trait protects the cell wall from microbial and enzymatic degradation, yet this resilience also impedes the development of an economically viable biofuel industry.

To become more cost competitive, the fees linked with supplying lignocellulosic starting materials and commercial enzyme reagents must be lowered, coupled with designing less expensive pretreatment technologies.

Oftentimes physical methods, such as mechanical disruption, pyrolysis, and steam, ammonia, or carbon dioxide fiber explosions, are used in conjunction with chemical procedures, *viz.*, ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, or organosolv processes, to overcome some of the macroscopic rigidity associated with the surface matrix and/or increase mass transport (Sun and Cheng 2002). In many cases, the surface area and/or pore size of a given biosubstrate might be altered in such a way to improve accessibility of enzyme catalysts with target sugar residues (Keshwani and Cheng 2010; Xu *et al.* 2010). Ultimately, the end goal for pretreatment technologies is centered on enriching sugar recoveries after enzymatic breakdown of cellulosic matter.

After pretreatment, a wide range of enzymes are used to degrade refractory cellulosic structures. On a commercial scale, fungal cellulases, especially those derived from the soft rot fungus *Trichoderma reesei*, remain among the most promising candidate enzymes for generating fermentable sugars from lignocellulosic substrates. This microbe produces a complete set of efficiently secreted hydrolytic enzymes, including (i) two exogluconases: [cellobiohydrolases (CBH), EC 3.2.1.91; Cel6A (CBHII), Cel7A (CBHI)], (ii) five endoglucanases [(EG), EC3.2.1.4; Cel5A (EGII), Cel7B (EGI), Cel12A (EGIII), Cel45A (EGV), Cel61A (EGIV)], and (iii) two β-glucosidases [(βGSD), EC 3.2.1.21; Cel1A (βGSD II), Cel3A (βGSD I)] to accomplish degradation (Henrissat and Bairoch 1996). Generally, the complete breakdown of cellulose to simple sugars requires synergy among the three enzyme types (Wood 1992; Irwin *et al.* 1993; Saha *et al.* 1994; Srisodsuk *et al.* 1998).

Exoglucanases, named cellobiohydrolases in fungal systems, split off cellobiose from the shortened cellulose chain while endoglucanases randomly cleave internal β-1,4-glycosidic linkages to decrease the length of the cellulose chain (Wood 1992). In separate reactions, βGSD hydrolyze cellobiose to render glucose; thus relieving the system from end product inhibition (Wood 1992; Saha *et al.* 1994; Srisodsuk *et al.* 1998; Teugjas and Väljamäe 2013).

In preliminary studies, the authors observed proteolytic activity in commercial cellulolytic enzyme reagents. In many cases, the reported activities did not correlate with experimentally measured values. These observations led to a search of early literature regarding the presence of native proteases in these cultures. Previously, several family types were identified including metallo-, serine, and aspartate proteases, along with aminopeptidases (Hagspiel *et al.* 1989; Haab *et al.* 1990; Eneyskaya *et al.* 1999). Typically, acid proteases predominate among those produced by *T. reesei* (Haab *et al.* 1990; Eneyskaya *et al.* 1999).

Independently, the impact of protease processing on cellulases with respect to altering the substrate specificity was described. Van Tilbeurgh *et al.* (1986) and Tomme *et al.* (1988) reported that papain cleaved CBHI and CBHII from *T. reesei* to yield a separate binding domain (C-terminus), responsible for adsorption onto cellulose, and a catalytic domain (N-terminus) that proved active with small, soluble substrates. After digestion with papain, these hydrolases no longer cleaved insoluble, microcrystalline cellulose.

Similarly, Chen and Grethlein (1988) treated crude *T. reesei* cellulase fractions with trypsin or chymotrypsin and reported marginally increased activity of endo-1,4 β-

glucanase with carboxymethyl cellulose. However, reactivity with hardwood or pretreated hardwood was compromised. Ultimately, these effects were attributed to a decreased adsorption onto woody biomass, due to the modification of substrate binding sites, which diminished sugar yields.

In other reports, Chen *et al.* (1992) noted the absence of smaller protein bands, typically attributable to proteolysis, in βGSD II samples prepared from *T. reesei* after storage for 2 months. Furthermore, βGSD from *Aspergillus niger* showed increased stability to chymotrypsin degradation in the presence of non-covalently attached polysaccharides (Rashid and Suddiqui 1996). Notably, long-term incubation with thermolysin produced 10 low molecular weight bands, all of which were catalytically active. However, the total loss of enzymatic activity was only 10% (Rashid and Siddiqui 1997). Seemingly, βGSD were more resistant to protease cleavage, especially in the presence of polysaccharides.

In parallel, Hill and Smith (1960) discovered that the activity of papain, a cysteine protease found in pineapple, was essentially the same after removal of nearly 60% of the amino acid content after digestion with leucine aminopeptidase. Moreover, Nylander and Malmstrom (1959) observed that the catalytic proficiencies of yeast enolase remained relatively unchanged after an 80% decrease in the amino acid content following incubation with leucine aminopeptidase or carboxypeptidase.

Taken together, the authors hypothesized that the substrate binding sites of cellulases might be minimally compromised after cleavage with aminopeptidase given their location at the carboxy-terminus end (Van Tilbeurgh *et al.* 1986; Tomme *et al.* 1988). Furthermore, a minimal loss of cellulase activity after peptidase processing was assumed based on these early literature citations (Nylander and Malstrom 1959; Hill and Smith 1960). Thus, smaller sized cellulolytic enzymes, generated after treatment with peptidase, might potentially gain better access to lignocellulosic substrates with increased sugar production.

Accordingly, these experiments were designed to create and characterize truncated enzyme subtypes *via* proteolysis with exogeneous immobilized proteases (trypsin, chymotrypsin, and aminopeptidase). As predicted, Peptidase-modified cellulases and βGSD (Peptidase_Cellulase, Peptidase_βGSD) were identified as the best candidates to use for hydrolysis reactions of alkaline pretreated switchgrass based on their residual enzyme activity after treatment.

All enzyme forms were characterized with respect to the relative degree of cleavage using OPA assay methods, and resultant protein banding profiles were examined after SDS-PAGE. Peptidase_Cellulase and Peptidase_βGSD catalytic activities were assessed using filter paper and cellobiose substrates, respectively.

Modified enzyme preps were then used to hydrolyze the grass. Sugar yields (per gram of biomass) were measured, and the efficiency of each reaction, defined as mg sugar generated/filter paper unit (FPU) for cellulase, or mg sugar generated/cellobiase unit (CBU) for βGSD, was compared to data obtained with non-protease treated control samples (Control 1, Ctr 1_Cellulase or Ctr 1_βGSD).

Native proteolysis was also observed, together with the effect of sodium azide on sugar production. Potentially, the results gleaned from this study offer new insights for understanding the complex yet fundamental dynamics that influence the outcome of lignocellulosic bioprocessing reactions.

EXPERIMENTAL

Materials

Chemicals and commercial enzyme resources

Cellulases (NS50013 cellulase complex, density: 1.20 g/L) produced by *Trichoderma reesei* and β-glucosidase from *Aspergillus niger* (NS50010 cellobiase, density: 1.24 g/L) were generously donated by Novozymes North America Incorporated (Franklington, NC, USA). These enzymes are used in the second generation biofuels industry. Trypsin (T1426, L-1-Tosylamide-2-phenylethyl chloromethyl ketone), (TPCK-treated, 10,000 BAEE units/mg), chymotrypsin (C 3142, Tosyl-L-lysyl-chloromethane hydrochloride (TLCK-treated, 40 to 60 units/mg), and aminopeptidase (P 7500 to 7550 units) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA)

Preparation of immobilized trypsin, immobilized chymotrypsin, immobilized aminopeptidase, and soluble peptidase

Immobilized proteases (trypsin, chymotrypsin, and aminopeptidase) were prepared using controlled pore glass beads (CPG 2000, 80 to 120 mesh), supplied by Millipore (Billerica, MA, USA), according to previous methodologies (Janolino and Swaisgood 1982; Thresher *et al.* 1989; Mateo *et al.* 2007).

Immobilized trypsin activity was measured as 150 units/g of bead using *p*-tosyl-L-arginine methyl ester (TAME) as the substrate while chymotrypsin activity was determined as 20 units/g with benzoyltyrosine ethyl ester (BTEE). Immobilized and soluble peptidase activity was assayed as 0.13 units/g of bead and 102 units/g of enzyme reagent, respectively, with the substrate L-leucine-*p*-nitroaniline (Thresher *et al.* 1989).

Cellulase and βGSD sample preparation - Protease digestion conditions

In the first set of experiments, 1 mL of commercial cellulase or βGSD stock solution was diluted to a final volume of 10 mL with 50 mM sodium phosphate buffer, pH 7.0. Then, 50 µL of immobilized protease (trypsin, chymotrypsin, or peptidase) was added to 2.0 mL of each experimental sample type and incubated for 24 h at 37 °C (Experimental results are depicted in Figs. 1A and 1B).

In later trials, a volume of 0.50 mL of either immobilized or soluble peptidase was added to 10 mL of the diluted cellulase or βGSD substrate, and the mixture was slowly rotated on a shaker platform for either 24 h or 48 h at 37 °C. A test aliquot of 0.5 mL was removed from the reaction vessel at indicated time intervals to: (1) measure the relative degree of hydrolysis (OPA assay, Table 1), (2) examine the resulting protein banding profiles (SDS-PAGE, Figs. 2A and 2B), and (3) determine the residual catalytic activity after proteolysis (Fig. 3).

Definition of sample types

After dilution, Control 1 samples (Ctr 1_Cellulase, Ctr 1_βGSD) were stored in the refrigerator prior to analysis. Control 2 samples, (Ctr 2_Cellulase, Ctr 2_ βGSD), which represented the impact of native protease activity on the system, and Peptidase_Cellulase or Peptidase_βGSD samples were held at 37 °C for defined time intervals prior to experimentation.

Methods

Cellulase and βGSD activity measurements

Standard cellulase (filter paper units, FPU) and βGSD (cellobiase units, CBU) activity assays were performed according to the procedures developed by the International Union of Pure and Applied Chemistry (IUPAC; Ghose 1987). For cellulase, filter paper was used as a model insoluble cellulose substrate (P8, Thermo Fisher Scientific, Waltham, MA, USA), while βGSD activity was determined using D +/- cellobiose (98%, Molecular Weight = 342.29; Acros Organics, Morris Plains, Morris, USA).

Cellulase activity was defined as the amount of enzyme that produces 1 µmol of glucose from substrate filter paper/min/mL protein solution, while βGSD activity was defined as the amount of enzyme that produces 2 µmol of glucose from cellobiose/min/mL protein solution.

Bicinchoninic acid protein assay (BCA) methodology

Soluble supernatants were obtained after microcentrifugation (13,600 × *g*; 5 min) at room temperature and diluted appropriately. Protein concentrations were measured in triplicate according to BCA methodologies developed by Thermo Scientific (Waltham, MA, USA).

OPA analyses to quantify the degree of proteolytic hydrolysis

Soluble supernatants were diluted as necessary. The OPA assay was then accomplished according to previously established protocols (Church *et al*. 1985; Clare and Daubert 2011). All test samples were measured in triplicate, and the final A_{340} nm readings fell with the range from 0.1 to 0.5.

SDS-PAGE and electrophoresis protocols

SDS PAGE was performed as previously described by Clare *et al.* (2007).

Switchgrass bioprocessing reactions

Biomass preparation, alkaline pretreatment, and enzymatic hydrolysis of the starting switchgrass material was accomplished according to previous methodologies (Wang *et al.* 2012). In earlier studies, Xu *et al.* (2011) noticed that *T. reesei* cellulase reagents contained insufficient amounts of βGSD for lignocellulosic bioprocessing applications. Therefore, βGSD was added during hydrolysis of the grass to prevent cellulase inhibition that was caused by an accumulation of cellobiose.

In summary, pretreatment was completed after the ground biomass (2 mm) was re-suspended in 0.5% (w/v) NaOH, followed by heating for 15 min at 121 °C prior to enzymatic hydrolysis. The wetted biomass, equivalent to 0.5 g on a dry basis, was immersed in a 0.05 M sodium citrate buffer, pH 4.8, to a total liquid volume of 15 mL. Catalysis was performed in a 50 mM sodium citrate buffer, pH 4.8, for 72 h at 50 °C (150 rpm) in the presence of 0.03% sodium azide to inhibit microbial growth. In some cases, the biocide was omitted.

Peptidase_Cellulases were delivered to the reaction vessel, together with non-modified βGSD, such that equal volumes of enzyme reagent were added to the alkaline pretreated grass. Based on prior assay measurements, Ctr 1_Cellulase loading levels were limited to approximately 8 FPU/g of biomass; whereas the residual activity of Ctr 2_Cellulase and Peptidase_Cellulase was quantified as 6.3 FPU/g and 5.8 FPU/g of

biomass, respectively. Cellobiase levels were adjusted to 70 units of activity (excess CBU, *i.e.*, cellobiase activity was not a limiting factor) across all sample types. These experiments were performed in the presence and absence of 0.03% sodium azide (Fig. 4 and Fig. 5).

In a second trial, in which Peptidase_βGSD were employed together with non-modified cellulases, the effect of limited cellobiase activity on sugar production from switchgrass was examined. In this case, Ctr 1_βGSD activity levels were adjusted to 10 CBU, together with 40 FPU (excess FPU, *i.e.*, cellulase activity was not a limiting factor), to accomplish degradation of the feedstock in the presence of 0.03% sodium azide (Fig. 6A, Fig. 6B).

Sugar analyses

After enzymatic breakdown of the grass, the amount of total reducing sugars was measured according to the dinitrosalicylic acid (DNS) method using appropriate glucose standards (Miller 1959). Ion-exchange chromatography (IC) (Dionex ICS-5000 system, Dionex Corporation, Sunnyvale, CA) was employed to measure glucose amounts in the cellobiase reaction mixtures. The IC was equipped with a pulsed electrochemical detector. The CarboPac PA1 (4 mm × 250 mm) column was operated at 18 °C with 18 mM sodium hydroxide serving as the mobile phase at a flow rate of 0.9 mL/min.

Statistical analysis

All sample types were assayed in triplicate. An analysis of variance (ANOVA) analysis was performed using the statistical program SAS 9.1 (SAS Institute Inc., Cary NC, USA) and Tukey's multiple means comparison test ($P \leq 0.05$). Different letters, as depicted in all figures and Table 1, reflect significant differences at a 95% confidence level ($P \leq 0.05$). The same lettering associated with the mean represents no significant differences.

RESULTS AND DISCUSSION

Effect of Protease Treatment on Cellulase and βGSD Activity (24 h, 37 °C)

Cleavage of cellulase preps with either immobilized trypsin or chymotrypsin after a 24 h treatment period at 37 °C resulted in significant loss of cellulolytic capacity. However, digestion with peptidase showed approximately 86% residual functionality after proteolysis, compared to non-treated Ctr 1_Cellulase (Fig. 1A).

Similarly, incubation of βGSD with either immobilized trypsin or chymotrypsin (24 h, 37 °C) considerably decreased the amount of glucose generated from cellobiose, whereas approximately 67% of the catalytic potential was retained after cleavage with peptidase. However, in this case, a 30% loss of cellobiase activity also occurred in the Control 2 sample type, devoid of the added immobilized protease (Fig. 1B). Notably, decreased cellobiase activity in Ctr 2_βGSD samples was essentially the same as measurements made after peptidase processing.

Given these findings, the next experiments were designed to characterize only the effects of peptidase processing on cellulolytic functionalities. Initially, a time course study was performed whereby diluted cellulase and βGSD stock reagents were incubated for 24 h, 32 h, 48 h, and 72 h at 37 °C, and the ratio of immobilized peptidase to the substrate (Cellulase or βGSD) was increased two-fold. Ultimately, it was established that

a 48 h treatment regime served to deliver optimal performance, *i.e.*, maximal cleavage with the highest residual enzymatic activity (data not shown).

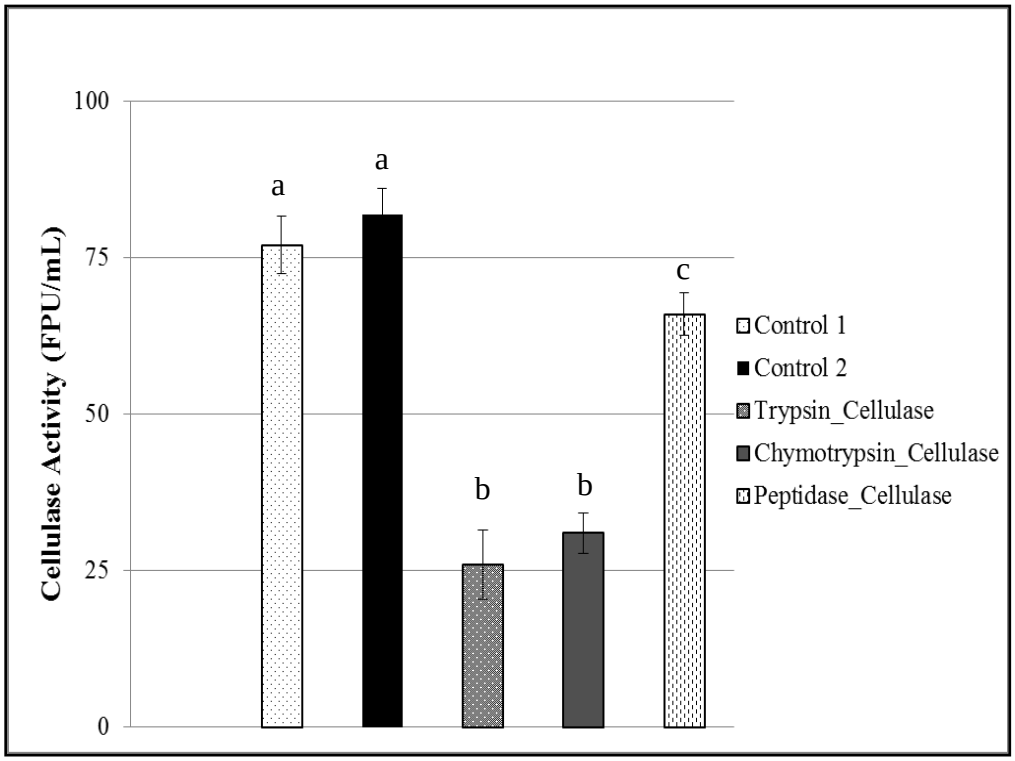

Fig. 1A. Effect of immobilized protease treatment on cellulase activity; a to c: Different letters associated with the mean represent significant differences.

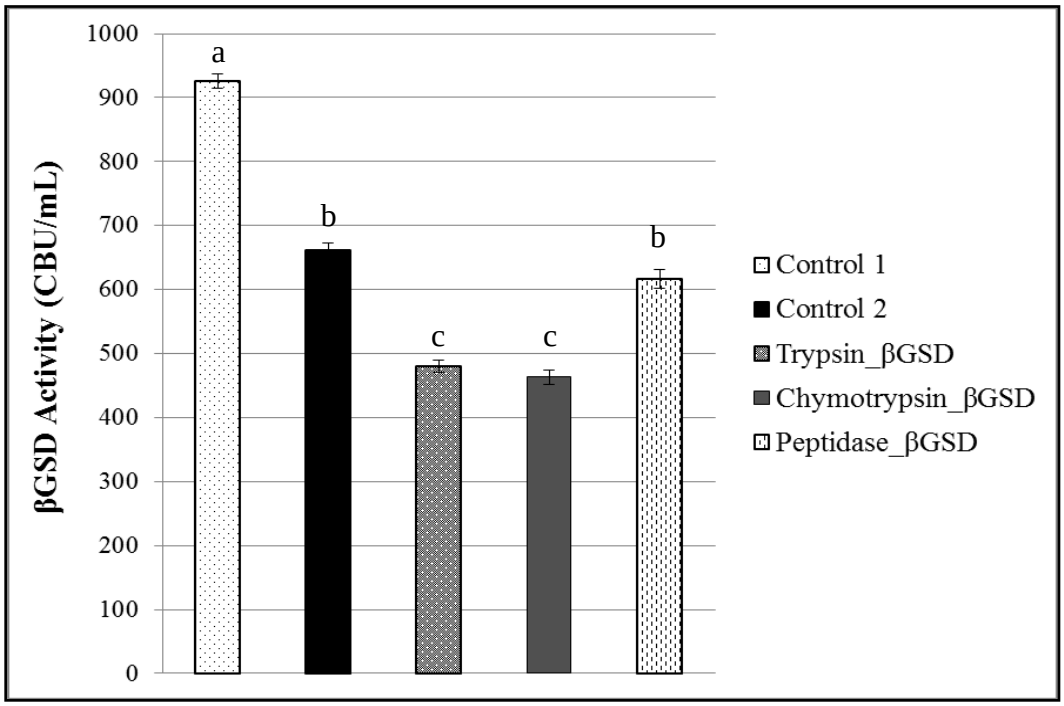

Fig. 1B. The effect of immobilized protease treatment on βGSD activity; a to c: Different letters associated with the mean represent significant differences.

Degree of Hydrolysis After Proteolytic Cleavage

The OPA reagent reacted with newly released free amino groups that were formed after proteolysis of protein substrates. All readings (A_{340} nm) were made using soluble supernatant fractions, obtained after microcentrifugation.

At time zero, Ctr 1_Cellulase and Ctr 1_βGSD samples were removed from the refrigerator, diluted, and the A_{340} nm values assigned a value of zero. At 48 h, the degree of hydrolysis, attributable to native protease activity, was calculated as approximately 19% based on A_{340} nm data collected for Ctr 2_Cellulase *versus* Ctr 1_Cellulase samples (Table 1). By comparison, cellulase samples treated with immobilized peptidase exhibited an approximate 34% increase in the concentration of soluble free amino groups compared to the non-hydrolyzed control (Ctr 1_Cellulase). Moreover, incubation with soluble peptidase, delivering higher levels of protease activity, also showed significant cleavage, whereby OPA values increased approximately 44% compared to Ctr 1_Cellulase preps. However, this data was not statistically different from the results obtained with immobilized peptidase (Table 1). In peptidase treated sample types, the total percent hydrolysis presumably represented the outcome of both endogeneous (Ctr 2_native) and exogeneous (*e.g.*, Peptidase_Cellulase) protease events.

The Ctr 2_βGSD samples exhibited a minor increase in A_{340} nm values (approximately 4.6%) compared to Ctr 1_βGSD solutions at 48 h. Moreover, the degree of hydrolysis measured for immobilized Peptidase_βGSD (13%) and soluble Peptidase_βGSD (15%) was not statistically different from Ctr 2_βGSD samples (Table 1). Overall, peptidase cleavage appeared to be more limited in βGSD sample types compared to the test cellulase solutions.

Table 1. Degree of Hydrolysis

Test Samples	Soluble, Free Amino groups[1] (μ mol.L^{-1})	Hydrolysis After 48 h at 37 °C (%)
Ctr 1_Cellulase	52.46 ± 0.93^{c}	0
Ctr 2_Cellulase	62.34 ± 1.26^{b}	19
Immobilized Peptidase_Cellulase	70.53 ± 4.83^{a}	34
Soluble Peptidase_Cellulase	75.53 ± 3.16^{a}	44
Ctr 1_βGSD	61.25 ± 0.68$^{b'}$	0
Ctr 2_βGSD	64.04 ± 0.77$^{a'}$	4.6
Immobilized Peptidase_βGSD	69.41 ± 0.39$^{a'}$	13
Soluble Peptidase_βGSD	70.53 ± 2.92$^{a'}$	15

1. Concentration of reactive amino groups (μ mol.L^{-1}) based on the OPA assay method;
a to c: Different letters associated with the mean represent significant differences between cellulase sample types; and a' and b': Different letters associated with the mean represent significant differences between βGSD sample types.

SDS-PAGE Profile after Treatment of Cellulase and βGSD Samples with Peptidase

Digestion of cellulases with either immobilized or soluble peptidase was also confirmed using SDS-PAGE techniques (Fig. 2A). The thickness of the major protein band (lane 2), which essentially corresponded to the reported molecular weight of *T. reesei* cellobiohydrolase I (Worthington Enzyme Manual 1993), was marginally diminished after incubation with immobilized peptidase for 24 h at 37 °C (lane 4), and to a greater extent when soluble peptidase was used for hydrolysis (lane 5). Most notably, peptidyl fragments of small size, ≤ 10 kDa, were observed in soluble Peptidase_Cellulase preps after a 24 h and 48 h incubation period (lanes 5 and 8), while they were visualized in immobilized Peptidase_Cellulase samples only after 48 h (lane 7). This finding was explained by the higher peptidase activity delivered with the soluble enzyme preparation.

The density of the primary band in Ctr 2_Cellulase samples, held at 37 °C for 48 h in the absence of exogenous peptidase, was slightly reduced (lane 6). Additionally, the appearance of minor bands, with molecular weights between 10 kDa to 17 kDa, was observed. However, this banding profile was not identical to the one observed after peptidase treatment given the absence of smaller peptidyl fragments, ≤ 10 kDa (lanes 7 and 8).

In contrast, the staining patterns observed after hydrolysis of βGSD stock solutions with either immobilized or soluble peptidase revealed essentially no changes in the soluble protein content after a 24 h or 48 h treatment period (Fig. 2B). However, electrophoretic analysis of Ctr 2_βGSD and immobilized or soluble Peptidase_βGSD pelleted fractions revealed numerous low molecular weight precipitated end products (data not shown).

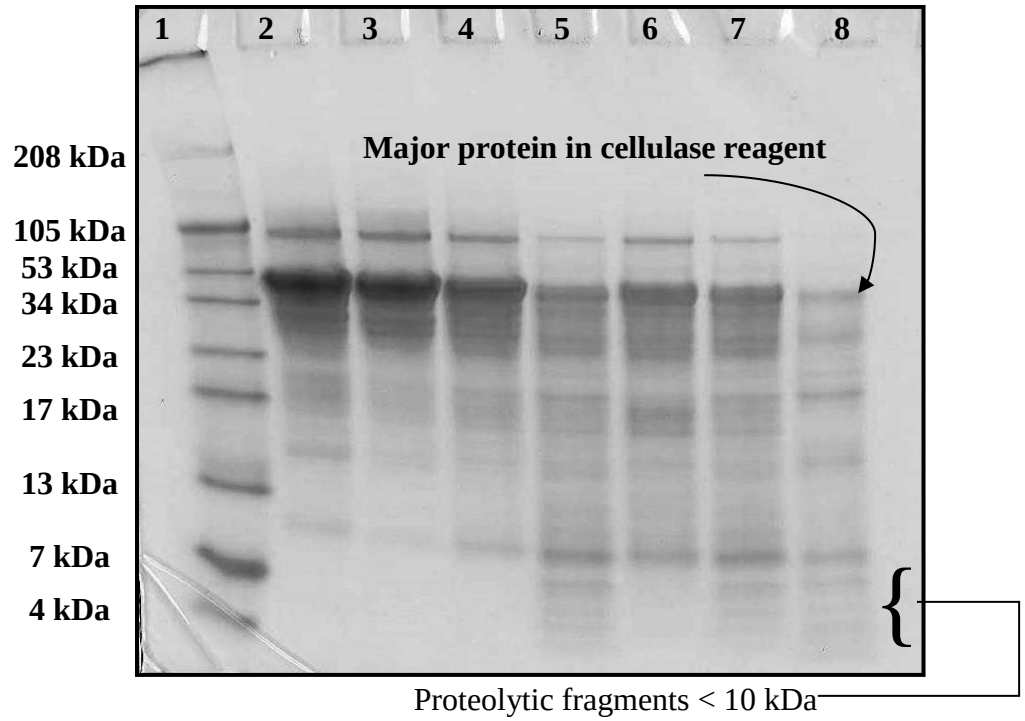

Fig. 2A. The SDS-PAGE protein banding pattern of test cellulase samples held under the defined experimental conditions. Lane 1: Marker; Lane 2: Ctr 1_Cellulase (0 h, 4 °C); Lane 3: Ctr 2_Cellulase (24 h, 37 °C); Lane 4: Immobilized Peptidase_Cellulase (24 h, 37 °C); Lane 5: Soluble Peptidase_Cellulase (24 h, 37 °C); Lane 6: Ctr 2_Cellulase (48 h, 37 °C); Lane 7: Immobilized Peptidase_Cellulase (48 h, 37 °C); and Lane 8: Soluble Peptidase_Cellulase (48 h, 37 °C)

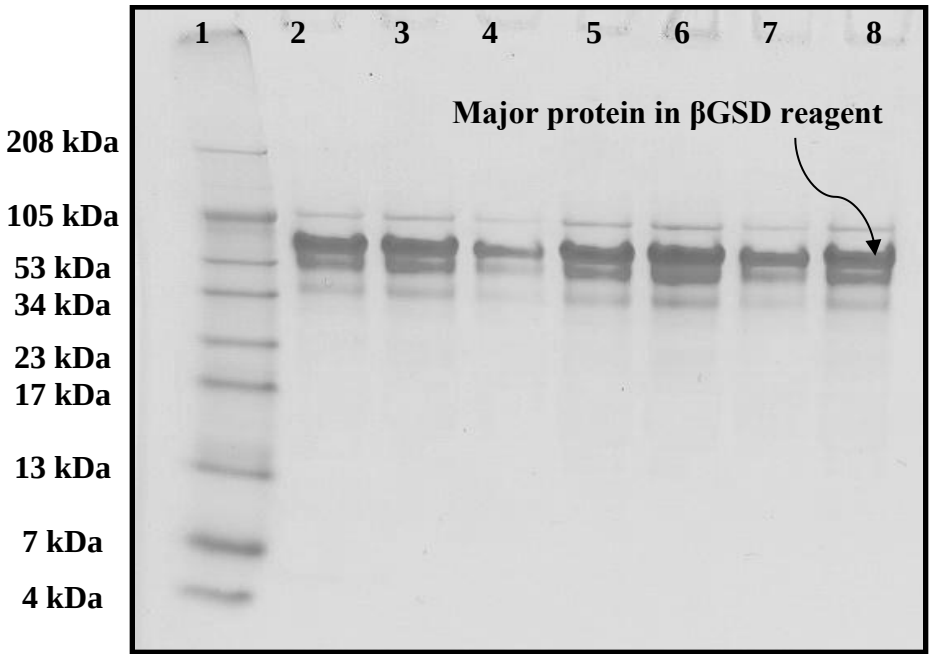

Fig. 2B. The SDS-PAGE protein banding pattern of test βGSD samples held under the defined experimental conditions. Lane 1: Marker; Lane 2: Ctr 1_βGSD (0 h, 4 °C); Lane 3: Ctr 2_ βGSD (24 h, 37 °C); Lane 4: Immobilized Peptidase_βGSD (24 h, 37 °C); Lane 5: Soluble Peptidase_ βGSD (24 h, 37 °C); Lane 6: Ctr 2_βGSD (48 h, 37 °C); Lane 7: Immobilized Peptidase_ βGSD (48 h, 37 °C); and Lane 8: Soluble Peptidase_ βGSD (48 h, 37 °C)

Residual Cellulase and βGSD Activity After Peptidase Cleavage

Cellulase activity was measured after treatment with either immobilized or soluble peptidase (48 h, 37 °C). The residual catalytic potentials were assessed accordingly: Ctr 1_Cellulase (100% by definition) > Ctr 2_Cellulase (80% of Ctr 1) > immobilized Peptidase_Cellulase (73% of Ctr 1) > soluble Peptidase_Cellulase (67% of Ctr 1), as summarized in Fig. 3. This trend correlated with the degree of hydrolysis (Table 1), *i.e.*, greater cleavage reduced enzymatic function. Conversely, incubation of βGSD with immobilized or soluble peptidase did not significantly lower cellobiase activity compared to the Ctr 2_βGSD samples (data not shown).

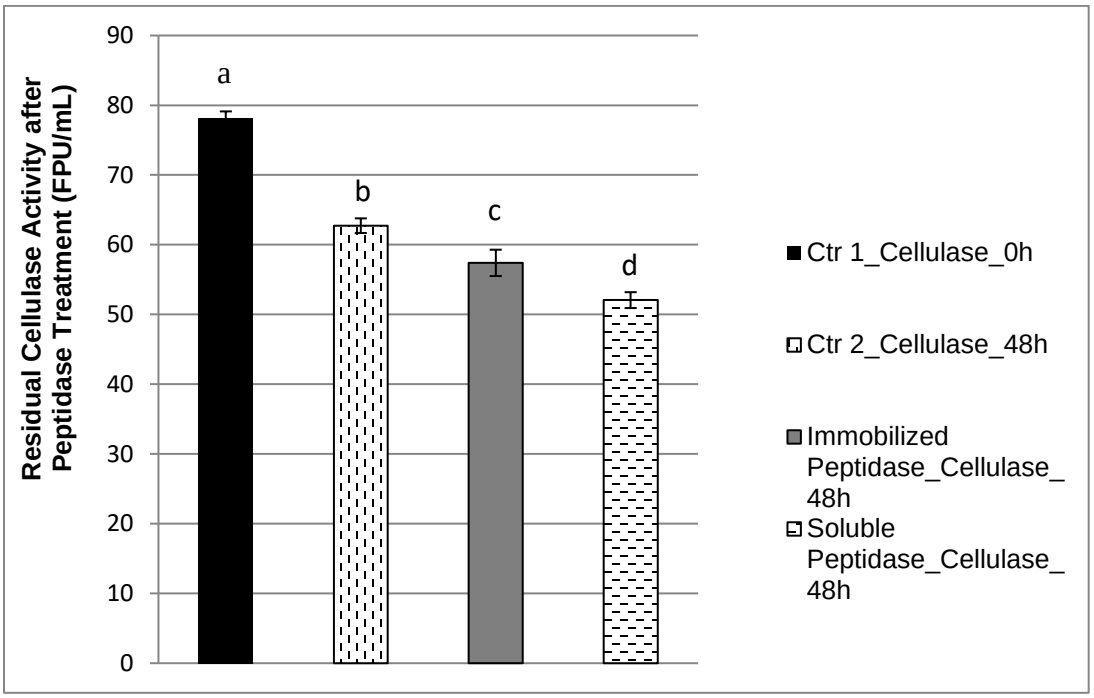

Fig. 3. Residual cellulase activity after digestion with immobilized and soluble peptidase (FPU/mL); a to d: Different letters associated with the mean represent significant differences.

Total Sugar Yields After Hydrolysis of Switchgrass using Truncated Cellulolytic Enzymes in the Presence and Absence of Sodium Azide

All test samples, including both control samples (Ctr_1 and Ctr_2) and Peptidase_Cellulase or Peptidase_βGSD, were then used to hydrolyze alkaline-pretreated switchgrass. Immobilized peptidase was used to prepare these enzyme forms because the glass beads, with covalently attached peptidase, can be physically removed from cellulolytic enzyme preps prior to digestion of the grass.

In the first trial, equal volumes of enzyme reagent were added to the alkaline grass. Based on prior assay measurements, Ctr 1_Cellulase loading levels were limited to approximately 8 FPU/g of biomass; whereas, the residual activity of Ctr 2_Cellulase and Peptidase_Cellulase was quantified as 6.3 FPU/g and 5.8 FPU/g of biomass, respectively. Cellobiase levels were adjusted to 70 units of activity (excess CBU) across all sample types. Then, digestion of the feedstock was performed in the presence of sodium azide, which served to inhibit microbial growth during this extended treatment period.

Under these conditions, the (sugar yield/g biomass) did not appreciably differ between Ctr 1_Cellulase, Ctr 2_Cellulase, and Peptidase_Cellulase enzyme preps (Fig.

4). However, it was important to realize that equivalent "total" sugar production was achieved at lesser catalytic levels when either Ctr 2_Cellulase (6.3 units/g) or Peptidase_Cellulase (5.8 units/g) was used for switchgrass processing compared to Ctr 1_Cellulase (8.0 units/g).

Somewhat surprisingly, the (sugar yield/g biomass) significantly increased when hydrolysis was accomplished in the absence of sodium azide, and this outcome appeared to be independent of enzyme type (Fig. 4). Again, protease effects (native and/or peptidase) did not hinder sugar production while omission of an antimicrobial agent during breakdown of the biomass appreciably improved the total sugar amounts.

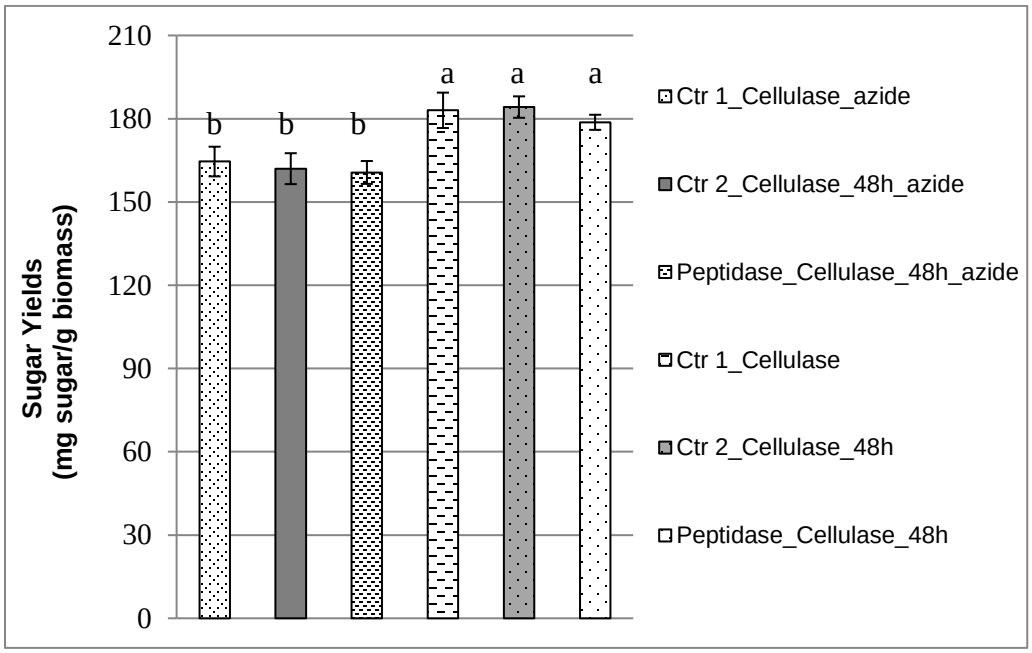

Fig. 4. Total reducing sugar yields/g biomass using Ctr 1_Cellulase (8 FPU), Ctr 2_Cellulase (6.3 FPU), and Peptidase_Cellulase (5.8 FPU) enzyme preps for hydrolysis (72 h, 55 °C); a to b: Different letters associated with the mean represent significant differences.

Efficiency of Catalysis

In the presence of azide, the yield of reducing (sugar/FPU), defined herein as the efficiency of the reaction, did not significantly vary between Ctr 1_Cellulase and Ctr 2_Cellulase catalysts. However, when hydrolysis of the grass was performed with truncated Peptidase_Cellulase, an improved efficiency of nearly 14% was seen compared to Ctr 1_Cellulase under limited cellulase loading conditions (Fig. 5).

Interestingly, the efficiency was boosted approximately 29% when Ctr 2_Cellulase was used to degrade switchgrass in the absence of azide, compared to a 37% increase upon utilization of Peptidase_Cellulase variants under these conditions (Fig. 5).

In these studies, native protease activity was detected in Ctr 2_samples, prepared from the commercial cellulase reagent (NS50013), devoid of azide (Table 1, Fig. 2A). Thus, additional proteolytic events (non-peptidase) may have also occurred during enzymatic processing of the grass. In earlier work, Sheir-Neiss and Montenecourt (1984) showed that at least two acid proteases were co-produced with secreted cellulases of *T. reesei*, both of which were active in the buffering system used for switchgrass degradation (50 mM citrate buffer, pH 4.8). Notably, the inclusion or exclusion of azide

during treatment of the biomass did not impact the efficiency of Ctr 1_Cellulase enzyme forms (Fig. 5).

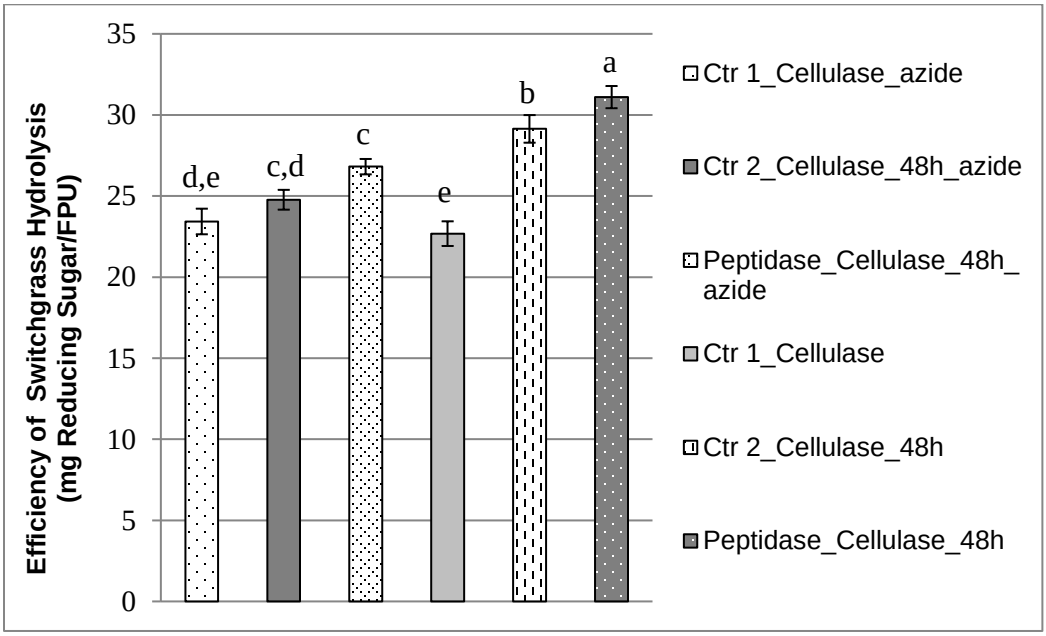

Fig. 5. The efficiency of switchgrass hydrolysis in the presence and absence of 0.03% sodium azide (72 h, 55 °C); a to e: Different letters associated with the mean represent significant differences

In a second trial, the effect of limited cellobiase activity on sugar production from switchgrass was examined. In this case, Ctr 1_βGSD activity levels were adjusted to approximately 10 CBU, together with 40 FPU (excess FPU), to accomplish degradation of the feedstock in the presence of the biocide. Under these conditions, the (total sugar yield/g of biomass) was essentially the same using either Ctr 1_βGSD, Ctr 2_βGSD, or Peptidase_βGSD for bioprocessing (Fig. 6A). As predicted, the efficiency of the reaction (mg reducing sugar/CBU) also proved very similar among all three of the cellobiase sample types (Fig. 6B). Likely, this finding can be attributed to minimal impact on βGSD activity after proteolysis (native protease or peptidase).

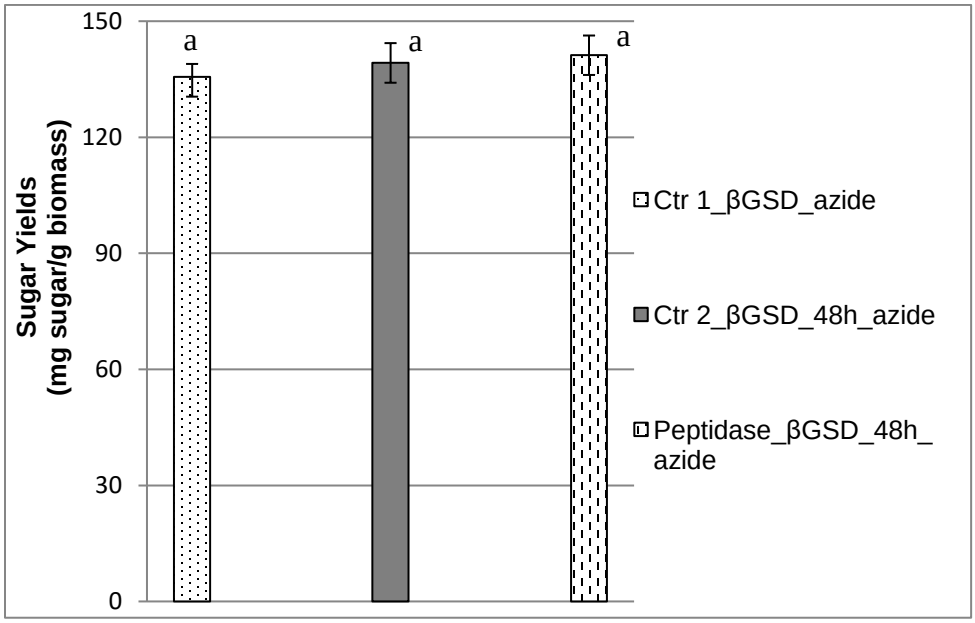

Fig. 6A. Total reducing sugar yields/g of switchgrass using Ctr 1_βGSD, Ctr 2_βGSD, and Peptidase_βGSD enzyme preps for hydrolysis of alkaline-pretreated switchgrass (72 h, 55 °C); a: Same lettering associated with the mean represents no significant differences.

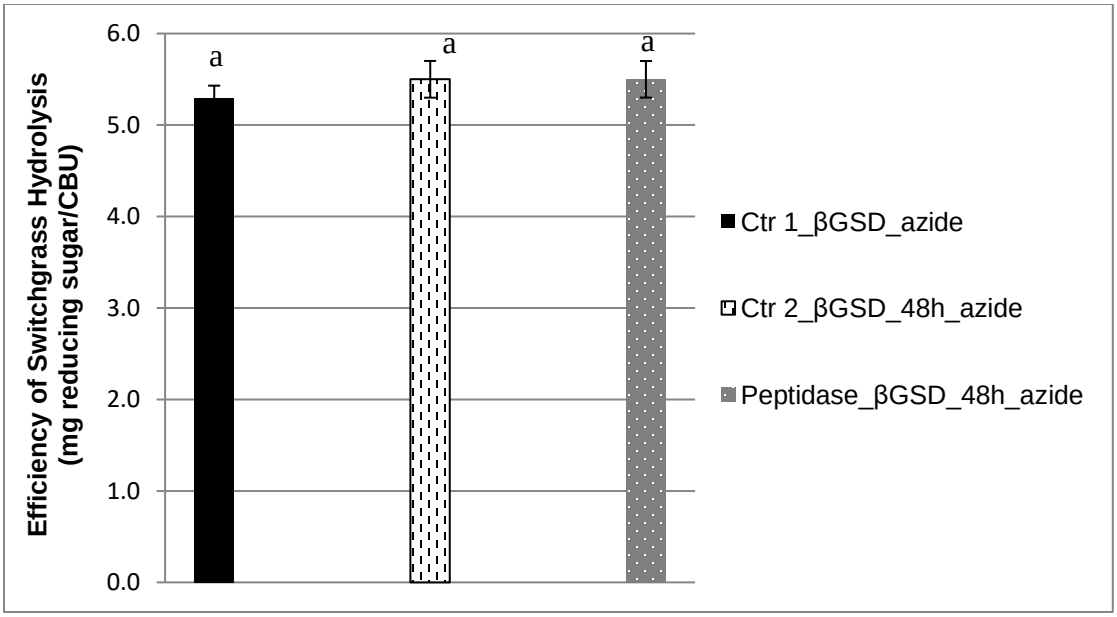

Fig. 6B. The efficiency of switchgrass hydrolysis in the presence of 0.03% sodium azide (72 h, 55 °C); a: Same lettering associated with the mean represents no significant differences.

Cellulases remain industrially relevant due to their capabilities for hydrolyzing cellulosic biomass to produce bioethanol and other chemical products. However, there are limitations, some of which may be attributed to low specific activity under standard processing conditions, such as high pH and temperature (Merino and Cherry 2007). Furthermore, commercial enzyme reagents are often quite expensive. Therefore, if the industry is to become more economically feasible, appropriate feedstocks must become

less costly, coupled with the development of more efficient practices for generating fermentable sugars.

Potentially, these goals could be achieved by designing improved thermochemical pretreatment methodologies (Alvira *et al.* 2010). Chemical and/or physical pretreatments typically alter the structural features of a given biomass in an attempt to increase liquid penetration and/or enzyme accessibility to target sites. In a separate approach, novel enzyme technologies might be devised to better enhance bioprocessing efficiencies (Qin *et al.* 2008; Qian *et al.* 2017).

With the exception of papain, there were only a few scientific reports that describe the effects of treating cellulases and βGSD samples with exogenous proteases. Notably, upon cleavage with papain, the catalytic domains of cellulases were separated from the binding domains with an overall loss in sugar production upon digestion of lignocellulosic materials (Van Tilbeurgh *et al.* 1986; Chen and Grethlein 1988; Tomme *et al.* 1988).

In another report, Chen and Grethlein (1988) showed that trypsin and chymotrypsin also altered cellulolytic competencies. In their studies, β-1-4 endoglucanase activity was increased using carboxymethyl cellulose as a substrate. Proteolysis with thermolysin, subtilisin, pepsin, or papain produced similar results. Conversely, in this study, decreased enzyme activity was observed after trypsin or chymotrypsin cleavage of cellulases and βGSD based on filter paper and cellobiose assays, respectively.

In a separate vein, the experimental findings of Hill and Smith (1960), together with Nylander and Malmstrom (1959), were of considerable relevance. As mentioned before, they established that the residual activity of papain and yeast enolase remained high after significant loss of amino acid content (60% and 80%, respectively) after treatment with peptidase.

Therefore, the authors reasoned that peptidase digestion of cellulase might create truncated enzymatic forms with high residual catalytic capabilities. Furthermore, this strategy could prove effective given that there is a conservation of essential substrate binding affinities. To the authors' knowledge, aminopeptidase modification of cellulolytic enzymes had not been previously attempted. As projected, peptidase cleavage produced smaller, active cellulase derivatives that hydrolyzed both filter paper and biomass substrates.

Although these results did not establish improved total sugar yields per gram of biomass when Peptidase_Cellulase reagents were employed for switchgrass breakdown, the efficiency of the reaction was improved. In other words, equivalent sugar yields were achieved at lower activity levels. In contrast, mostly insoluble cleavage products were detected in Peptidase_βGSD samples. Furthermore, the impact of peptidase processing on βGSD catalytic function was minimal. Apparently, βGSD was more resistant to peptidase action.

There was also evidence to suggest the possible role of native proteases in this system. Enzyme activity was lower in Ctr 2 samples (Cellulase, βGSD) compared to Ctr 1 (Cellulase, βGSD) preps. Furthermore, the electrophoretic patterns of Ctr 2 fractions (Cellulase, βGSD) differed from Ctr 1 or Peptidase modified cellulase or βGSD samples.

Perhaps the role of proteolysis in general remains an avenue for future exploration. Given the tentative huge importance of *T. reesei* and *A. niger* as host organisms for large-scale commercial production of cellulases and βGSD, respectively, there remains a critical need to understand the impact of both endogenous (native) and/or

exogenous proteolytic events to optimize cellulosic bioprocessing operations. To the authors' knowledge, protease inhibitors are not routinely added to all commercial enzyme reagents.

Potentially, the design of novel bioprocessing regimens might be realized by making relatively minor adjustments to existing methodologies. In this study, a simple elimination of azide during switchgrass hydrolysis appreciably improved (sugar yields/g biomass).

Theoretically, smaller-sized hydrolases might gain better access to lignocellulosic binding sites to boost sugar production. Thus, in future work, genetic engineering technologies could be employed, such as those of Qian *et al.* (2017), to create more efficient enzyme systems.

Ultimately, it becomes important to specifically characterize each bioprocessing system with respect to basic functional parameters, such as protein content, cellulase and βGSD catalytic efficiencies, native protease effectors, and/or the utilization of biocides during hydrolysis of various feedstocks. A recent report suggested that the cellulose structure itself (surface area and roughness) caused a loss of cellulase activity during the course of lignocellulosic bioprocessing reactions (Olsen *et al.* 2016). Ultimately, this type of information should guide the evolution of novel and more cost effective schemes for advancement of the cellulosic industry.

CONCLUSIONS

1. The efficiency of the reaction, defined as (mg sugar/unit of enzyme activity), increased after treatment of cellulases (*Trichoderma reesei*) with aminopeptidase.
2. β-glucosidase (*Aspergillus niger*) was more resistant to peptidase cleavage.
3. Native proteases likely play a role in the amount of sugar produced during treatment of lignocellulosic substrates to produce sugar end products.
4. Sugar outputs were higher in the absence of sodium azide after a 48 h treatment of alkaline pretreated switchgrass using peptidase modified cellulases for hydrolysis.

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REFERENCES CITED

Abd Hamid, S. B., Islam, M. M., and Das, R. (2015). "Cellulase biocatalysis: Key influencing factors and mode of action," *Cellulose* 22(4), 2157-2182. DOI: 10.1007/s10570-015-0672-5

Alvira, P., Tomas–pejo, E., Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Bioresour. Technol.* 101, 4851-4861. DOI: 10.1016/j.biortech.2009.11.093

Chen, H. C., and Grethlein, H. E. (1988). "Effect of cellulase size reduction on activity and accessibility," *Biotechnol. Lett.* 10(12), 913-918. DOI: 10.1007/BF01027005

Chen, H., Hayn, M., and Esterbauer, H. (1992). "Purification and characterization of two extracellular β-glucosidases from *Trichoderma reesei*," *Biochim. Biophys. Acta* 1121(1-2), 54-60. DOI: 10.1016/0167-4838(92)90336-C

Church, F. C., Porter, D. H., Catignani, G. L., and Swaisgood, H. E. (1985). "An *o*-phthalaldehyde spectrophotometric assay for proteinases," *Anal. Biochem.* 146(2), 343-348. DOI: 10.1016/0003-2697(85)90549-4

Clare, D. A., and Daubert, C. R. (2011). "Expanded functionality of modified whey protein dispersions after transglutaminase catalysis," *J. Food Sci.* 76(4), C576-C584. DOI: 10.1111/j.1750-3841.2011.02128.x

Clare, D.A., Gharst, G., and Sanders, T.H. (2007) "Transglutaminase polymerization of peanut proteins," *J. Agric. Food Chem.* 55 (2), 432-438. DOI: 10.1021/jf062309t

Eneyskaya, E. V., Kulminskaya, A. A., Savelev, A. N., Saveleva, N. V., Shabalin, K. A., and Neustroev, K. N. (1999). "Acid protease from *Trichoderma reesei*: Limited proteolysis of fungal carbohydrases," *Appl. Microbiol. Biot.* 52(2), 226–231. DOI: 10.1007/s002530051513

Ghose, T. K. (1987). "Measurement of cellulases activities," *Pure Appl. Chem.* 59(2), 257-268. DOI: 10.1351/pac198759020257

Haab, D., Hagspiel, K., Szakmary, K., and Kubicek, C. P. (1990). "Formation of the extracellular proteases from *Trichoderma reesei* QM9414 involved in cellulase degradation," *J. Biotechnol.* 16(3-4), 187–188. DOI: 10.1016/0168-1656(90)90035-A

Hagspiel, K., Haab, D., and Kubicek, C. (1989). "Protease activity and proteolytic modification of cellulases from a *Trichoderma reesei* QM 9414 selectant," *Appl. Microbiol. Biot.* 32(1), 61-67. DOI: 10.1007/BF00164824

Henrissat, B., and Bairoch, A. (1996). "Updating the sequence-based classification of glycosyl hydrolases," *Biochem. J.* 316(2), 695-696. DOI: 10.1042/bj3160695

Hill, R. L., and Smith, E. L. (1960). "Isolation and characterization of an enzymatically active fragment of papain," *J. Biol. Chem.* 235(8), 2332-2338.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007). "Biomass recalcitrance: Engineering plants and enzymes for biofuels production," *Science* 315, 804-807. DOI: 10.1126/science.1137016

Hu, G., Heitmann, J. A, Zhong, B., Lucia, L. A., and Argyropoulos, D. S. (2015). "Quantitative study of the interfacial adsorption of cellulase to cellulose," *J. Phys. Chem. C* 119(25), 14160-14166. DOI: 10.1021/acs.jpcc.5b02011

Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B. (1993). "Activity studies of eight purified cellulases: Specificity, synergism, and binding domain effects," *Biotechnol. Bioeng.* 42(8)1002-1013. DOI: 10.1002/bit.260420811

Janolino, V. G., and Swaisgood, H. E. (1982). "Analysis and optimization of methods using water-soluble carbodiimide for immobilization of biochemicals to porous glass," *Biotechnol. Bioeng.* 24(5) 1069-1080. DOI: 10.1002/bit.260240504

Keshwani, D. R., and Cheng, J. J. (2010). "Microwave-based alkali pretreatment of switchgrass and coastal Bermuda grass for bioethanol production," *Biotechnol. Progr.* 26(3), 644-652. DOI: 10.1002/btpr.371

Limayaem, A., and Ricke, S. C. (2012). "Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects," *Progress in Energy and Combustion Science* 38, 449-467. DOI:10.1016/j.pecs.2012.03.002

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., and Pretorius, I. S. (2002). "Microbial cellulose utilization: Fundamentals and biotechnology," *Microbiol. Mol. Biol. R.* 66(3), 506-577. DOI: 10.1128/MMBR.66.3.506–577.2002

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., and Fernandez-Lafuente, R. (2007). "Improvement of enzyme activity, stability and selectivity via immobilization techniques," *Enzyme Microb. Technol.* 40 (6), 1451-1463. DOI: 10.1016/j.enzmictec.2007.01.018

Merino, S. T., and Cherry, J. (2007). "Progress and challenges in enzyme development for biomass utilization," in: *Biofuels*, L. Olsson (ed.), Springer, Berlin, Heidelberg, Germany, pp. 95-120. DOI: 10.1007/10_2007_066

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31(3), 426-428. DOI: 10.1021/ac60147a030

Nylander, O., and Malmstrom, G. (1959). "The degradation of yeast enolase with leucine aminopeptidase and carboxypeptidase without change in enzymic activity: N- and C-terminal residues of the enzyme," *Biochim. Biophys. Acta* 34(1), 196-202. DOI: 10.1016/0006-3002(59)90247-1

Olsen, J. P., Donohoe, B. S., Borch, K., Westh, P., and Resch, M. G. (2016). "Interrelationships between cellulase activity and cellulose particle morphology," *Cellulose* 23(4) 2349-2361. DOI: 10.1007/s10570-016-0979-x

Qian, Y., Zhong, L., Gao, J., Sun, N., Wang, Y., Sun, G., Qu, Y., and Zhong, Y. (2017). "Production of highly efficient cellulase mixtures by genetically exploiting the potentials of *Trichoderma reesei* endogenous cellulases for hydrolysis of corncob residues," *Microb. Cell Fact.* 16, 207. DOI: 10.1186/s12934-017-0825-3

Qin, Y., Wei, X., Song, X., and Qu, Y. (2008). "Engineering endoglucanase II from *Trichoderma reesei* to improve the catalytic efficiency at a higher pH optimum," *J. Biotechnol.* 135, 190-195. DOI: 10.1016/j.jbiotec.2008.03.016

Rashid, M. H., and Siddiqui, K. S. (1996). "The stability of extracellular beta-glucosidase from *Aspergillus niger* is significantly enhanced by non-covalently attached polysaccharides," *Folia Microbiol.* 41(4), 341-346. DOI: 10.1007/BF02814712

Rashid, M. H., and Siddiqui, K. S. (1997). "Purification and characterization of a β-glucosidase from *Aspergillus niger*," *Folia Microbiol.* 42, 544-550. DOI: 10.1007/BF02815462

Saha, B. C., Freer, S. N., and Bothast, R. J. (1994). "Production, purification, and properties of a thermostable β-glucosidase from a color variant strain of *Aureobasidium pullulans*," *Appl. Environ. Microb.* 60(10), 3774-3780.

Sheir-Neiss, G., and Montenecourt, B. S. (1984). "Characterization of secreted cellulases of *Trichoderma reesei* wild type mutants during controlled fermentations," *Appl. Microbiol. Biot.* 20(1), 46-53. DOI: 10.1007/BF00254645

Srisodsuk, M., Kleman-Leyer, K., Keränen, S., Kirk, T. K., and Teeri, T. T. (1998). "Modes of action on cotton and bacterial cellulose of a homologous endoglucanase-exoglucanase pair from *Trichoderma reesei*," *Eur. J. Biochem.* 251(3), 885-892. DOI: 10.1046/j.1432-1327.1998.2510885.x

Sun, Y., and Cheng, J. Y. (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review," *Bioresour. Technol.* 83(1), 1-11. DOI: 10.1016/S0960-8524(01)00212-7

Teugjas, H., and Väljamäe, P. (2013). "Selecting β-glucosidases to support cellulases in cellulose saccharification," *Biotechnol. Biofuels* 6, 105. DOI: 10.1186/1754-6834-6-105

Thresher, W. C., Swaisgood, H. E., and Catignani, G. L. (1989). "Digestibilities of the protein in various foods as determined *in vitro* by an immobilized digestive enzyme assay (IDEA)," *Plant Food Hum. Nutr.* 39(1), 59–65. DOI: 10.1007/BF01092402

Tomme, P. H., Van Tilbeurgh, G., Petterson, J., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988). "Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis," *Eur. J. Biochem.* 170(3), 575-581. DOI: 10.1111/j.1432-1033.1988.tb13736.x

Van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R., and Pettersson, G. (1986). "Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*: Separation of functional domains," *FEBS. Lett.* 204(2), 223-227. DOI: 10.1016/0014-5793(86)80816-X

Wang, Z., Xu, J., Pandey, P., Cheng, J., Li, R., and Qu, R. (2012). "Improvement of sugar production from transgenic switchgrass with low-temperature alkaline pretreatment," *Energy Fuels* 26(5), 3054-3061. DOI: 10.1021/ef3004575

Wood, T. M. (1992). "Fungal cellulases," *Biochem. Soc. T.* 20(1), 46-53. DOI: 10.1042/bst0200046

Worthington Enzyme Manual (1993). "Cellulase," Worthington Biochemical Corporation, (http://www.worthington-biochem.com/cel/default.html), Accessed 15 Jan 2015.

Wyman, C. E. (2003). "Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power," *Biotechnol. Progr.* 19(2), 254-262. DOI: 10.1021/bp025654l

Xu, J., Cheng, J. J., Sharma-Shivappa, R. R., and Burns, J. C. (2010). "Lime pretreatment of switchgrass at mild temperatures for ethanol production," *Bioresour. Technol.* 101(8), 2900-2903. DOI: 10.1016/j.biortech.2009.12.015

Xu, J., Wang, Z., and Cheng, J. J. (2011). "Bermuda grass as feedstock for biofuel production: A review," *Bioresour. Technol.* 102(17), 7613–7620. DOI: 10.1016/j.biortech.2011.05.070

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