ENZYMATIC HYDROLYSIS OF SWITCHGRASS AND COASTAL BERMUDA GRASS PRETREATED USING DIFFERENT CHEMICAL METHODS

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To investigate the effects of biomass feedstock and pretreatment method on the enzyme requirement during hydrolysis, swichgrass and coastal Bermuda grass pretreated using H_2SO_4, NaOH, and $Ca(OH)_2$ at the optimal conditions were subjected to enzymatic hydrolysis using two enzyme combinations: NS 50013 + NS 50010 and Cellic CTec + Cellic HTec. The enzyme loadings were optimized, and correlations between feedstock property, pretreatment strategy, and enzyme usage were evaluated. The results show that pretreatment methods resulting in greater lignin contents in the pretreated biomass were generally associated with higher enzyme requirements. More sugars could be recovered from alkaline-pretreated biomass during enzymatic hydrolysis due to the better carbohydrate preservation achieved at mild pretreatment temperatures. The cellulase enzyme, Cellic CTec, was more efficient in catalyzing the hydrolysis of coastal Bermuda grass, a feedstock more digestible than the pretreated swichgrass, following pretreatment with NaOH or $Ca(OH)_2$.

Keywords: Biomass conversion; Cellulase enzyme; Chemical pretreatment; Coastal Bermuda grass; Enzymatic hydrolysis; Ethanol; Switchgrass

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INTRODUCTION

Ethanol produced from biomass is one of the best alternative fuels to power the U.S. transportation sector. Compared with first-generation corn ethanol, cellulosic ethanol is more promising because of feedstock abundance and reduced carbon footprint. However, lignocellulose-to-ethanol conversion is much more challenging and costly than corn-based ethanol production due to the inherent recalcitrance and complex structure of lignocellulosic biomass. This necessitates a pretreatment step to break up lignocellulosic matrix and a subsequent hydrolysis step using cellulase enzymes to release sugars from the pretreated biomass for ethanol fermentation.

Different pretreatment methods, which aim at removing lignin/hemicellulose, reducing cellulose crystallinity, and increasing the porosity and surface area of the materials, have been investigated (Keshwani and Cheng 2010; Silverstein et al. 2007; Kim and Lee 2005; Kim and Holtzapple 2005; Sun and Cheng 2005; Alizadeh et al. 2005; Negro et al. 2003). Among all these techniques, acid and alkaline-based chemical pretreatments have attracted much attention because of their high efficiencies and potentially low costs. Acid pretreatments effectively solubilize hemicellulose and reduce

the crystallinity of cellulose by disrupting covalent bonds, hydrogen bonds, and van der Waals forces that hold the cellulose, hemicelloses, and lignin together in the biomass, which can result in separation of the lignin from the cellulose to some extent (Li et al. 2010; Sun and Cheng 2005). Rather than applying concentrated acid at moderate temperatures, dilute acid pretreatments at high temperatures are normally practiced to achieve improved cellulose hydrolysis (McMillan 1994). On the other hand, alkaline pretreatments result in delignification of biomass and partial degradation of hemicellulose and make the lignocellulose swollen through solvation and saponification reactions (Xu et al. 2010a; Hendriks and Zeeman 2009). Research has shown that alkaline pretreatments are effective within a wide temperature range at various chemical concentrations (Xu et al. 2010a, 2010b).

In the enzymatic hydrolysis of pretreated biomass, cellulase enzymes including cellulases and hemicellulases are used to catalyze the depolymerization of cellulose and hemicellulose. Cellulases are a mixture of three different cellulolytic enzymes: 1,4-β-D-glucan glucanohydrolase which randomly attacks and cleaves the β-1-4 glycosidic bonds of cellulose to produce cello-oligosaccharides and glucose, 1,4-β-D-glucan cellobiohydrolase which releases cellobiose from the nonreducing ends of a cellulosic substrate, and β-glucosidase which hydrolyzes cellobiose to glucose (Wang 2009; Ladisch et al. 1983). In the hydrolysis of hemicellulose, three major enzymes including endo-β-1-4-xylanase which targets β-1-4 bonds between D-xylose residues of heteroxylans and xylo-oligosaccharides, exoxylanase which releases xylobiose, and β-xylosidase which hydrolyzes xylo-oligosaccharides to xylose are involved (Saha and Bothast 1999). With the rapid development of enzyme technology, new generations of cellulase enzymes with higher activities and specificities have emerged, which would greatly improve the economic viability of lignocellulose-to-ethanol conversion.

In pretreatment investigations, pretreatment effectiveness is normally assessed based on sugar production during enzymatic hydrolysis of pretreated biomass at excessive enzyme loadings. Although enzyme loadings were optimized in some studies (Deepak 2009; Sun and Cheng 2005; Chang et al. 1997), enzyme sources were different and hydrolysis procedures adopted were inconsistent, which made it difficult to compare the enzyme requirements resulted from various pretreatment strategies. Consequently, the discussion on the promise of a specific pretreatment technique was less conclusive, considering the currently high cost of cellulase enzymes. Moreover, besides pretreatment strategy, the property of biomass feedstock would also affect the enzyme requirement. Therefore, it is necessary to conduct a comprehensive investigation, in which biomass pretreated differently are subjected to the same hydrolysis process so that the impacts of biomass feedstock and pretreatment strategy on the enzyme requirement in hydrolysis can be evaluated.

In this study, switchgrass (*Panicum virgatum L.*) and coastal Bermuda grass (CBG) (*Cynodon dactylon L.*), two grass species showing great promise for ethanol production, were pretreated using dilute sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), and lime ($Ca(OH)_2$) under the respective optimal conditions obtained in our previous studies (Xu et al. 2010a, 2010b; Wang et al. 2010; Wang and Cheng 2011; Redding et al. 2011; Yang et al. 2009). The pretreated biomass was subjected to enzymatic hydrolysis using two enzyme combinations which were NS 50013 + NS 50010

and Cellic CTec + Cellic HTec. The enzyme loadings were investigated, with the effects of feedstock and pretreatment strategy on enzyme requirement analyzed.

EXPERIMENTAL

Biomass Preparation

Both switchgrass and CBG were obtained from Central Crops Research Station near Clayton, North Carolina. Switchgrass was oven dried at 50 ^{o}C for 72 hr, while CBG was air dried until the moisture content was lower than 10%. Different drying methods were applied because the switchgrass and CBG biomass samples used in this study were previously prepared for two different research projects. The dried plants were ground to pass a 2 mm sieve using a Thomas Wiley Laboratory Mill (Model No. 4) and then stored in sealed plastic bags at room temperature. The compositions of biomass samples were analyzed in previous studies and the results are shown in Table 1. Other undefined components include, but are not limited to, proteins, waxes, fats, resins, gums, and chlorophyll (Kuhad and Singh 1993; Sluiter et al. 2005).

Table 1. Chemical Compositions of Switchgrass and CBG (Xu et al. 2010a; Wang et al. 2010)

Component	Dry weight (%)	
	Switchgrass	CBG
Glucan	32.0	25.6
Xylan	17.9	15.9
Arabinan	1.87	1.95
Galactan	1.73	1.46
Lignin	21.4	19.3
Ash	3.77	6.60
Other	21.3	29.2

Pretreatment

The equipments and methods used for the pretreatments of swichgrass and CBG using H_2SO_4, NaOH, and $Ca(OH)_2$ were according to Xu et al. (2010a, 2010b), Wang et al. (2010), Wang and Cheng (2011), Redding et al. (2011), and Yang et al. (2009). Based on the previously obtained optimal pretreatment conditions, switchgrass was pretreated respectively using 1.5% (w/v) H_2SO_4 at 121 °C for 45 min (Yang et al. 2009), 1.0% (w/v) NaOH at 50 °C for 12 h (Xu et al. 2010a), and a $Ca(OH)_2$ loading of 0.1g/g raw biomass at 50 °C for 24 h (Xu et al. 2010b). CBG was pretreated respectively using 1.21% (w/v) H_2SO_4 at 140 °C for 30 min (Redding et al. 2011), 0.75% (w/v) NaOH at 121 °C for 15 min (Wang et al. 2010), and a $Ca(OH)_2$ loading of 0.1g/g raw biomass at 100 °C for 15 min (Wang and Cheng 2011). The compositional changes of the biomass after pretreatments were not reported in this paper and it is assumed that the results are the same as those obtained in our prior works.

Enzymatic Hydrolysis

The switchgrass and CBG samples pretreated under the optimal conditions were hydrolyzed using two enzyme combinations. One combination included cellulases

produced by *Trichoderma reesei* (NS 50013 cellulase complex) and cellobiase from *Aspergillus niger* (NS 50010 β-glucosidase). β-glucosidase was supplemented during hydrolysis to prevent cellulases inhibition caused by cellobiose accumulation due to its insufficient amount in cellulases from *T. reesei*. Xylanase supplementation was not examined since previous results show that the addition of xylanase did not significantly ($P>0.05$) enhance sugar yields from both acid- and alkaline-pretreated biomass (Wang 2009; Yang et al. 2009). Hemicellulases were identified in the *T. reesei* cellulases system by conducting 2D electrophoresis (Vinzant et al. 2001), which can probably explain the reason why xylanase addition was not necessary since NS 50013 itself is generated by *T. reesei*. The other combination included Cellic CTec (aggressive cellulases and high level of β-glucosidase) and Cellic HTec (endoxylanase). All the enzymes were obtained from Novozymes North America Inc. (Franklinton, North Carolina, USA), and the activities of enzymes are reported in Table 2.

Table 2. Activities of Enzymes Examined in the Study

Activity	NS 50013	NS 50010	Cellic CTec	Cellic HTec
FPU[a]/mL	76.4[d]	N/A	115.6[d]	N/A
CBU[b]/mL	N/A	283.1[d]	281.8[d]	N/A
FXU[c]/mL	N/A	N/A	N/A	1090[e]

[a] Filter paper unit, defined as the amount of enzyme that produces 1 μmol of glucose from filter paper per minute.
[b] Cellobiase unit, defined as the amount of enzyme that produces 2 μmol of glucose from cellobiose per minute.
[c] Fungal xylanase unit.
[d] Determined experimentally according to Ghose (1987).
[e] Obtained from Novozymes product data sheet.

Enzymatic hydrolysis of the pretreated biomass was carried out in 50 mL plastic tubes in a controlled reciprocal shaking water bath (Model C76, New Brunswick Scientific). The hydrolysis temperatures were 55 °C for NS 50013 and NS 50010, and 50 °C for Cellic CTec and Cellic HTec. A total of 0.5 g (dry basis) pretreated biomass was immersed in desired volume of 0.05 M sodium citrate to reach a total volume of 15 mL and a pH of 4.8. After the addition of enzymes, sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to inhibit microbial growth. The hydrolysis was carried out at 150 rpm for 72 hr, after which the hydrolyzate was centrifuged at 8×10^3 g and the supernatant was collected and stored at -20 °C for sugar analysis at a later time. In the hydrolysis of pretreated biomass using the combination of NS 50013 and NS 50010, NS 50013 loadings of 0-40 FPU/g pretreated biomass were studied at an excessive NS 50010 loading of 70 CBU/g pretreated biomass to eliminate the impact of β-glucosidase limitation, and then NS 50010 loadings of 0, 10, 20, 30, 40, and 50 CBU/g pretreated biomass were studied based on the optimal NS 50013 loading. In the hydrolysis of pretreated biomass using the combination of Cellic CTec and Cellic HTec, Cellic CTec loadings of 0-40 FPU/g pretreated biomass were studied at an excessive Cellic HTec loading of 75 FXU/g pretreated biomass, and then Cellic HTec loadings of 0, 25, 50, 75 FXU/g pretreated biomass were studied based on the optimal Cellic CTec loading. Since in H_2SO_4 pretreatment, most hemicellulose was solubilized into the pretreatment liquor,

which was not used in the enzymatic hydrolysis, Cellic HTec loading was not studied for H_2SO_4-pretreated biomass.

Analytical Methods

The moisture content of biomass was determined by drying the samples at 105 °C in a convection oven (Isotemp, Fisher Scientific) to constant weight. Composition analysis of the biomass samples were conducted according to Xu et al. (2010a). Total reducing sugars in the hydrolyzate were determined by the DNS (3,5-dinitrosalicylic acid) method using glucose as the standard (Miller 1959). Monosaccharides (glucose and xylose) in the hydrolyzate were measured using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography (HPLC) equipped with a Bio-Rad Aminex HPX-87H column, a corresponding guard column, and a refractive index detector (Shimadzu RID-10A). The analytical column was operated at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Other monomeric sugars including galactose, arabinose, and mannose were measured but not reported due to their low concentrations. Experimental data were statistically analyzed using the GLM procedure in SAS 9.1 software (SAS Institute Inc., Cary, NC). Differences between treatments were evaluated by Tukey adjustment at a 95% confidence level. All treatments were conducted in triplicate.

RESULTS AND DISCUSSION

Switchgrass

H_2SO_4-pretreated biomass

After H_2SO_4 pretreatment of swichgrass under the optimal conditions, more than 80% of hemicellulose was solubilized, while the losses of cellulose and lignin were much less (Yang et al. 2009). With the increase of NS 50013 loading from 0 to 20 FPU/g pretreated biomass, the total reducing sugar yield was elevated by 7.6 times, reaching 199.4 mg/g raw biomass (Fig. 1).

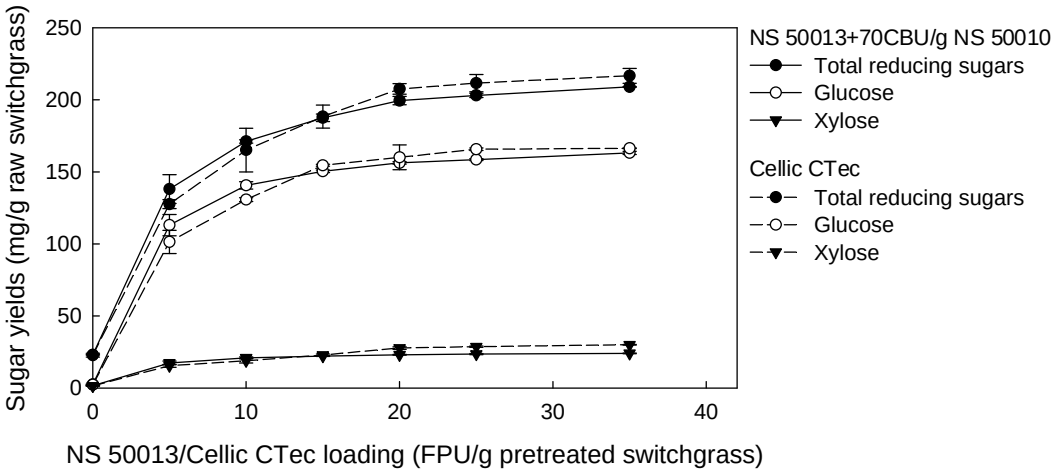

Fig. 1. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from switchgrass pretreated using 1.5% (w/v) H_2SO_4 at 121 °C for 45 min.

Further increasing NS 50013 loading did not favor the improvement of sugar production. Similar trends were observed for both glucose and xylose yields. NS 50010 loadings were studied based on the optimal NS 50013 loading of 20 FPU/g pretreated biomass. Supplementing NS 50010 significantly ($P<0.05$) increased sugar yields, and a NS 50010 loading of 10 CBU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 188.7 mg/g raw biomass, 23.4% higher than that obtained without NS 50010 supplementation. A Cellic CTec loading of 20 FPU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 207.5 mg/g raw biomass. The results also show that, in catalyzing the hydrolysis of H_2SO_4-pretreated switchgrass, the effectivenesses of using NS 50013 + NS 50010 and Cellic CTec alone were comparable at different enzyme loadings.

NaOH-pretreated biomass

According to Xu et al. (2010a), after NaOH pretreatment, more than 55% of the lignin in the raw biomass was removed, resulting in a 24% increase in carbohydrate content (cellulose and hemicellulose) in the biomass. As shown in Fig. 2, the optimal NS 50013 loading was 15 FPU/g pretreated biomass. At this NS 50013 loading, a NS 50010 loading of 20 CBU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 431.4 mg/g raw biomass, 19.5% higher than that obtained without NS 50010 supplementation. The reduced NS 50013 requirement is probably due to the reduced lignin content in the alkaline-pretreated biomass. The lignin content of H_2SO_4-pretreated biomass was 30.9%, while that of NaOH-pretreated biomass was 9.6%. Lignin limits enzyme access to carbohydrates not only through posing physical barrier, but also by causing unproductive binding of enzymes (Palonen 2004). The increase in NS 50010 requirement can be the result of the reduced β-glucosidase dosage with the decreased usage of NS 50013. Since there was substantial hemicellulose present in the pretreated biomass, Cellic HTec was supplemented in the hydrolysis, and the results were compared with those without HTec addition. It was found that adding Cellic HTec not only increased the yields of xylose, the dominant building block of hemicellulose, but also improved glucose yields (Fig. 2). A plausible explanation is that the improved hydrolysis of hemicellulose caused by Cellic HTec addition significantly increased the accessibility of cellulose by Cellic CTec, thus resulting in higher glucose yields. The optimal loading of Cellic CTec was found to be 15 FPU/g pretreated biomass. At this Cellic CTec loading, a Cellic HTec loading of 25 FXU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 399.1 mg/g raw biomass, 9.0% higher than that obtained without Cellic HTec addition. The reduced Cellic CTec requirement, compared with that applied on H_2SO_4-pretreated biomass, can also be the result of the decreased lignin content in the alkaline-pretreated biomass. Both enzyme combinations were effective in recovering sugars from the NaOH-pretreated biomass. The maximum total reducing sugar yields achieved (431.4 and 399.1 mg/g raw biomass for NS 50013 + NS 50010 and Cellic CTec + Cellic HTec, respectively) were about twice as high as those obtained from H_2SO_4-pretreated biomass, which is apparently due to the better carbohydrate preservation at a much lower pretreatment temperature (50 ^{o}C). Alkaline pretreatments are effective even at moderate

temperatures, which is an important advantage over acid pretreatments. After pretreatment at milder conditions, more carbohydrates remained in the residual solids for the subsequent hydrolysis rather than being solubilized into pretreatment liquor and mixed with inhibitory degradation byproducts normally generated in high-temperature acid pretreatments.

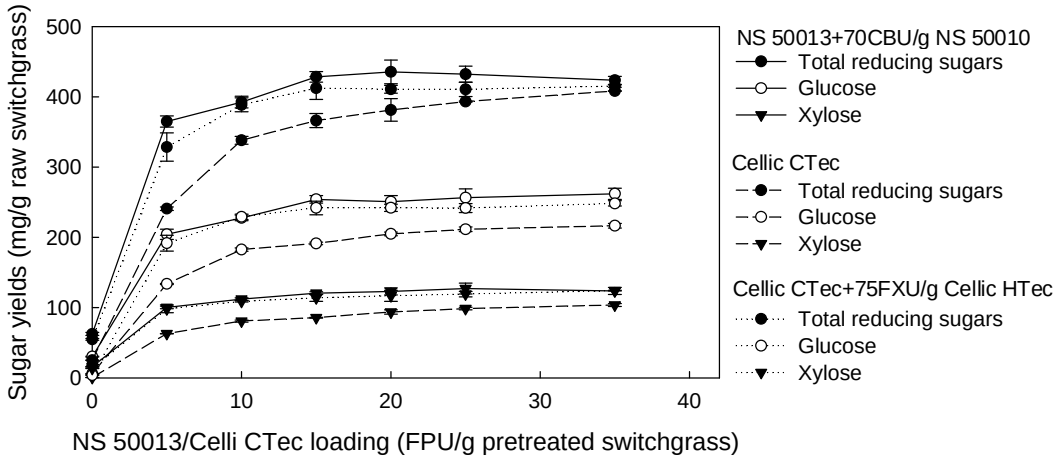

Fig. 2. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from switchgrass pretreated using 1.0% (w/v) NaOH at 50 °C for 12 h.

$Ca(OH)_2$-pretreated biomass

Although $Ca(OH)_2$ pretreatment caused substantial increase in the enzymatic digestibility of biomass by effectively breaking up the lignocellulosic structure, the composition of biomass did not change much after the pretreatment due to the bonding of Ca^{2+} to various lignocellulose components, especially lignin (Xu et al. 2010b; Xu et al. 2011). Calcium ions from $Ca(OH)_2$ dissociation, each carrying two positive charges, tend to crosslink lignin molecules, which are negatively charged under alkaline conditions, thus preventing intensive lignin solubilization during pretreatment (Xu et al. 2010b; Torre et al. 1992). As shown in Fig. 3, the optimal NS 50013 loading was 20 FPU/g pretreated biomass. At this NS 50013 loading, a NS 50010 loading of 20 CBU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 433.0 mg/g raw biomass, 11.1% higher than that obtained without NS 50010 supplementation. Compared with NaOH-pretreated biomass, more NS 50013 was needed by $Ca(OH)_2$-pretreated biomass to maximize sugar yields. This is probably due to the greater lignin content in the pretreated biomass. The lignin content of NaOH-pretreated biomass was 9.6%, while that of $Ca(OH)_2$-pretreated biomass was 14.3%. Although a previous study showed that the presence of a high lignin content in the $Ca(OH)_2$-pretreated biomass did not limit the access of enzymes to carbohydrates at excessive enzyme loadings (Xu et al. 2010b), the unproductive binding of enzymes to lignin could still result in an increased enzyme requirement. Cellic HTec supplementation was required to achieve improved sugar yields from $Ca(OH)_2$-pretreated biomass (Fig. 3). The optimal loading of Cellic CTec was found to be 20 FPU/g pretreated biomass. At this Cellic CTec loading, a Cellic HTec loading of 25 FXU/g pretreated biomass was sufficient to maximize sugar yields, at

which the total reducing sugar yield was 412.3 ma/g raw biomass, 10.9% higher than that obtained without Cellic HTec addition. Similarly, the higher Cellic CTec requirement can also be attributed to the increased lignin content in the biomass. It seems that, although $Ca(OH)_2$ pretreatment is considered as a potentially less costly chemical pretreatment technique than NaOH pretreatment (Xu et al. 2011; Kaar and Holtzapple 2000; Chang et al. 1997), the increased enzyme requirement due to the greater lignin content in the pretreated biomass might compromise the cost-effectiveness of the overall biomass conversion. Both enzyme combinations were effective in recovering sugars from $Ca(OH)_2$-pretreated switchgrass and the maximum total reducing sugar yields achieved (433.0 and 412.3 mg/g raw biomass for NS 50013 + NS 50010 and Cellic CTec + Cellic HTec, respectively) were comparable with those from NaOH-pretreated biomass.

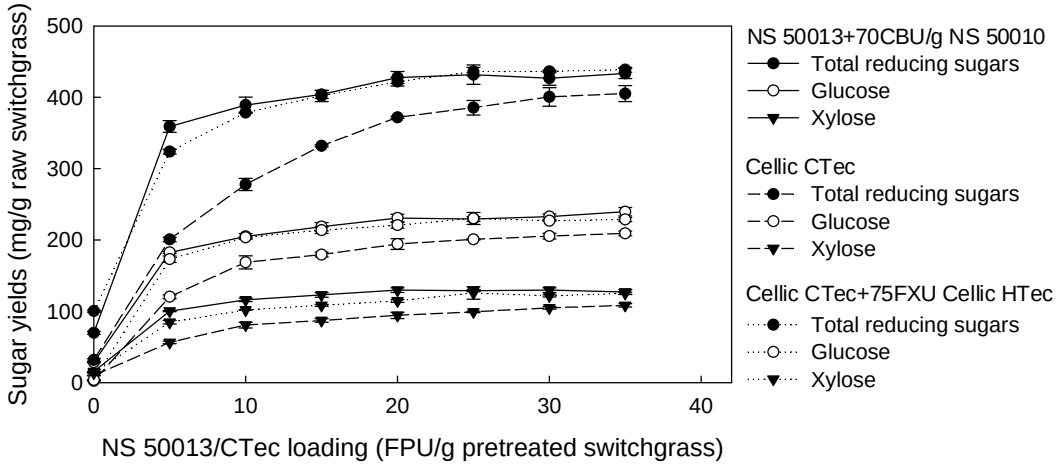

Fig. 3. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from switchgrass pretreated using 0.1 g $Ca(OH)_2$/g raw biomass at 50 °C for 24 h

Coastal Bermuda Grass

H_2SO_4-pretreated biomass

Although the carbohydrate content of CBG is significantly ($P<0.05$) lower than that of switchgrass, it is more susceptible to chemical pretreatments based on our previous studies. The effects of enzyme loading and combination on sugar yields were similar for both H_2SO_4-pretreated CBG and H_2SO_4-pretreated switchgrass. A NS 50013 loading of 20 FPU/g pretreated biomass provided optimal sugar yields, at which the total reducing sugar yield was 13.0 times higher than that obtained without enzyme addition (Fig. 4). At the optimal NS 50013 loading, a NS 50010 loading of 10 CBU/g pretreated biomass was sufficient to maximize sugar yields, at which the total reducing sugar yield was 232.1 mg/g raw biomass, 12.6% higher than that obtained without NS 50010 supplementation. The maximum glucose yield from CBG was much higher than that from switchgrass, while the maximum xylose yield was much lower. This is because since CBG was more susceptible to chemical attack than switchgrass, under similar pretreatment conditions, more cellulose was disrupted in CBG, resulting in higher glucose yields in enzymatic hydrolysis. In contrast, hemicellulose is a carbohydrate with low molecular weight. It has amorphous, heterogeneous, and branched structure, thus

having low crystallinity. At high pretreatment temperatures, hemicellulose in the susceptible CBG was more readily solubilized into pretreatment liquor, thus resulting in reduced xylose yields during the subsequent hydrolysis. Cellic HTec supplementation was not recommended in the hydrolysis of H_2SO_4-pretreated CBG, either. A Cellic CTec loading of 20 FPU/g pretreated biomass was sufficient to achieve maximum total reducing sugar yield of 242.1 mg/g raw biomass, which is comparable with those from using the combination of NS 50013 and NS 50010.

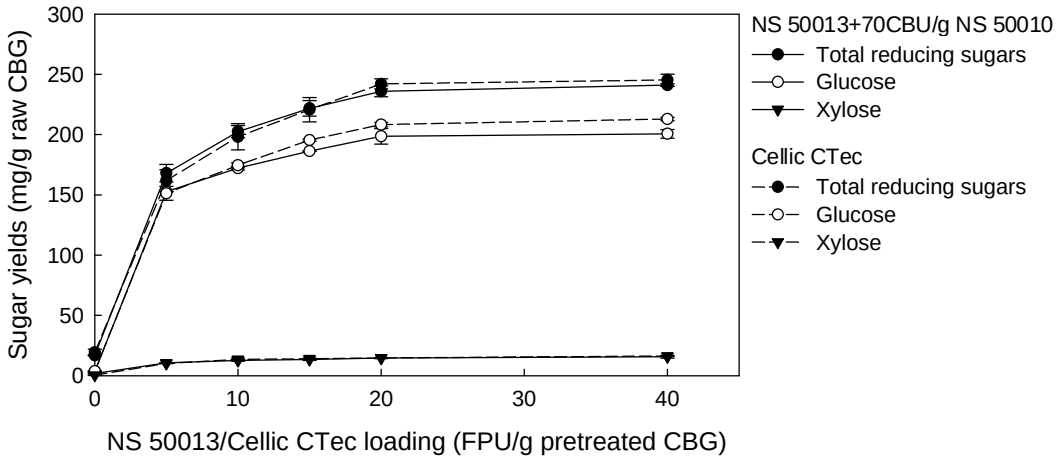

Fig. 4. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from CBG pretreated using 1.21% (w/v) H_2SO_4 at 140 °C for 30 min.

NaOH-pretreated biomass

The required enzyme loadings and the maximum sugar yields achieved were comparable between NaOH-pretreated CBG and switchgrass using the combination of NS 50013 and NS 50010. The optimal NS 50013 loading was 15 FPU/g pretreated biomass (Fig. 5), at which a NS 50010 loading of 10 CBU/g pretreated biomass was sufficient to achieve the maximum total reducing sugar yield of 396.0 mg/g raw biomass, 5.8% higher than that obtained without NS 50010 supplementation. Cellic CTec and Cellic HTec, however, performed better on CBG. Although the maximum total reducing sugar yields were comparable (405.2 and 410.3 mg/g raw biomass for NS 50013 + NS 50010 and Cellic CTec + Cellic HTec, respectively) at the excessive loading of Cellic HTec, Cellic CTec was much more efficient on NaOH-pretreated CBG at enzyme loadings lower than 15 FPU/g pretreated biomass. This could be the result of the interactions between biomass properties, pretreatment conditions applied, and enzyme characteristics. After pretreatment at a high temperature (121 ^{o}C), the enzymatic digestibility of the more susceptible CBG was much higher than that of the less susceptible switchgrass pretreated at a mild temperature (50 ^{o}C). It seems that Cellic CTec and Cellic HTec performed better in catalyzing the hydrolysis of more digestible biomass. The optimal loading of Cellic CTec was found to be 10 FPU/g pretreated biomass (Fig. 5), at which a Cellic HTec loading of 25 FXU/g pretreated biomass was sufficient to achieve the maximum total reducing sugar yield of 379.5 mg/g raw biomass, 21.0% higher than that obtained without Cellic HTec addition. Based on the information

obtained from the Novozymes product data sheet, the newly developed Cellic CTec contains not only aggressive cellulases and high level β-glucosidase, but also a unique propriety performance booster. More investigations, however, are required to better evaluate the performance of Cellic CTec and Cellic HTec on different biomass feedstocks.

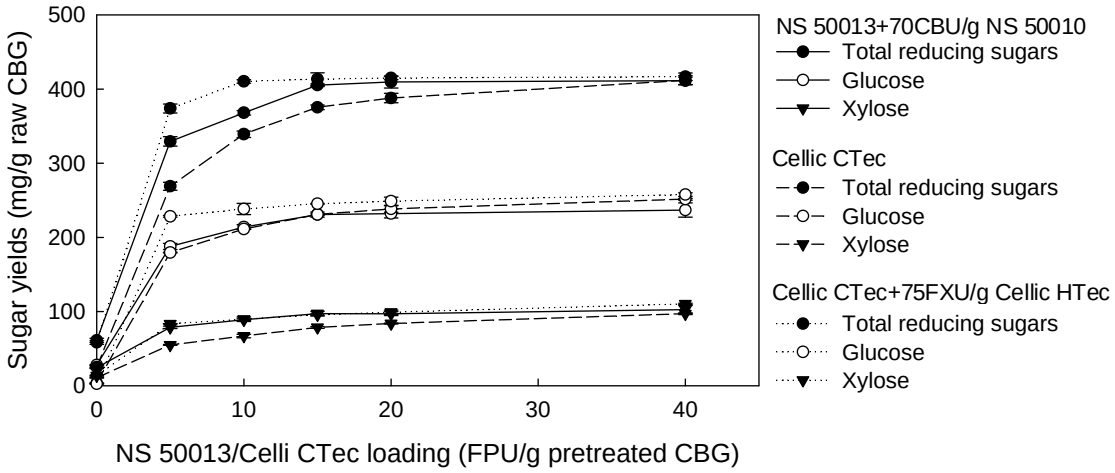

Fig. 5. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from CBG pretreated using 0.75% (w/v) NaOH at 121 °C for 15 min

$Ca(OH)_2$-pretreated biomass

The required enzyme loadings and the maximum sugar yields achieved were similar for $Ca(OH)_2$-pretreated CBG and switchgrass using the combination of NS 50013 and NS 50010. The optimal NS 50013 loading was 20 FPU/g pretreated biomass (Fig. 6), at which a NS 50010 loading of 10 CBU/g pretreated biomass was sufficient to achieve the maximum total reducing sugar yield of 426.4 mg/g raw biomass, 17.4% higher than that obtained without NS 50010 supplementation.

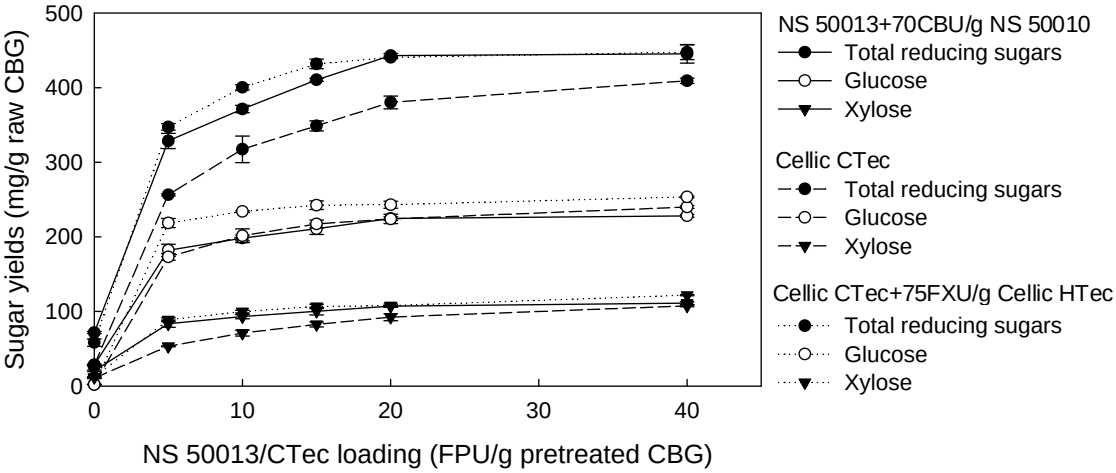

Fig. 6. Effects of the loadings of NS 50013 and Cellic CTec on glucose, xylose, and total reducing sugar yields from CBG pretreated using 0.1 g $Ca(OH)_2$/g raw biomass at 100 °C for 15 min.

Since the more susceptible CBG was also pretreated at a higher temperature (100 ^{o}C) than switchgrass (50 ^{o}C), Cellic CTec was more efficient on $Ca(OH)_2$-pretreated CBG at enzyme loadings lower than 20 FPU/g pretreated biomass. The optimal loading of Cellic CTec was found to be 15 FPU/g pretreated biomass (Fig. 6), at which a Cellic HTec loading of 50 FXU/g pretreated biomass was sufficient to achieve the maximum sugar yield of 429.7 mg/g raw biomass, 36.0% higher than that obtained without Cellic HTec addition. Although the requirement for Cellic HTec was substantially increased compared with that used in the hydrolysis of NaOH-pretreated CBG, the total reducing sugar yield at 50 FXU/g pretreated biomass was just 8% higher than that at 25 FXU/g pretreated biomass. Primary economic analysis, therefore, is required to justify the doubling of Cellic HTec loading. Table 3 summarizes the optimal enzyme loadings for the hydrolysis of switchgrass and CBG.

Table 3. Optimal Enzyme Loadings for the Hydrolysis of Switchgrass and CBG

Feedstock	Pretreatment	Lignin content of pretreated biomass, %	Enzyme Combination 1		Sugar Yield, mg/g raw biomass	Enzyme Combination 2		Sugar Yield, mg/g raw biomass
			NS 50013, FPU/g	NS 50010, CBU/g		Cellic CTec, FPU/g	Cellic HTec, FXU/g	
Switchgrass	H_2SO_4	30.9	20	10	188.7	20	-	207.5
	NaOH	9.60	15	20	431.4	15	25	399.1
	$Ca(OH)_2$	14.3	20	20	433.0	20	25	412.3
CBG	H_2SO_4	28.2	20	10	232.3	20	-	242.1
	NaOH	15.9	15	10	396.0	10	25	379.5
	$Ca(OH)_2$	20.2	20	10	426.4	15	50	429.7

CONCLUSIONS

1. The pretreatment methods that resulted in greater lignin contents in the pretreated biomass generally led to higher enzyme requirements in the subsequent hydrolysis due to the reduced enzyme access to carbohydrates caused by lignin barrier and the unproductive binding of enzymes to lignin molecules.
2. Alkaline pretreatments resulted in 56.8 to 129% higher sugar yields than acid pretreatments because alkaline pretreatments are effective even at mild temperatures, at which more carbohydrates remained in residual solids for further hydrolysis rather than being solubilized into pretreatment liquor and mixed with inhibitory degradation byproducts normally generated in high-temperature acid pretreatments.
3. Compared with using NS 50013 + NS 50010, applying Cellic CTec resulted in 25.0 to 33.3% reductions in enzyme requirement in catalyzing the hydrolysis of CBG pretreated using NaOH or $Ca(OH)_2$ at high temperatures, which is a more digestible hydrolysis feedstock.
4. Cellic HTec supplementation not only improved hemicellulose hydrolysis, but also increased glucose yield due to the better removal of hemicellulose barrier that reduces the access of cellulases to cellulose.
5. Although both enzyme combinations, NS 50013 + NS 50010 and Cellic CTec + Cellic HTec, were effective in catalyzing the hydrolysis of H_2SO_4, NaOH, and

$Ca(OH)_2$ pretreated switchgrass and CBG, using Cellic CTec + Cellic HTec might be preferred if the two enzyme combinations have the same unit cost ($/unit volume). This is because that the enzyme combination of Cellic CTec + Cellic HTec has a higher enzyme activity, which would substantially reduce the enzyme volume required.

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