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Electron beam pretreatment of switchgrass to enhance enzymatic hydrolysis to produce sugars for biofuels

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ABSTRACT

Conversion of lignocellulosic biomass to value added products such as ethanol and other platform chemicals is enhanced by pretreatment, which reduces the crystallinity and molecular weight of cell wall polymers, thus increasing the available reaction sites. In this study, switchgrass (*Panicum virgatum* L.) was pretreated with high energy electron beam (EB) irradiation to reduce its recalcitrance and achieve higher sugar conversion rates during treatment with cellulases and β-glucosidase. Conversion rates to sugars were compared before and after hot water (HW) extraction of EB-treated and control samples of switchgrass. Thermogravimetric analysis (TGA) was employed to determine peak degradation temperature of these EB-treated biomass samples before and after HW extraction, and near infrared spectroscopy (NIR) was used as a rapid technique to determine cellulose, hemicellulose, and lignin contents in the samples. TGA data confirm previously reported results that EB pretreatment reduces the molecular weight and crystallinity of cellulose and hemicellulose. This leaves hemicellulose more amenable to HW extraction and creates more cellulase-accessible sites, as shown by NIR and glucose yield data, respectively. Hemicellulose content was reduced from 30.2 to 16.9% after HW extraction and 1000 kGy EB treatment, and ultimate glucose yield after cellulase hydrolysis increased more than 4-fold. This study provides evidence that when EB pretreatment is utilized in combination with HW extraction, higher conversion rates and yields of glucose can be obtained from the cellulosic fraction of switchgrass.

1. Introduction

As described in the Energy Independence and Security Act of 2007, consumption of petroleum in the transportation sector needs to be replaced with at least 36 billion gallons of renewable fuel by 2022 (Biomass Research and Development Board, 2008). Primary feedstocks such as sugar cane, sugar beets, corn, potato, and wheat have been important candidates as a source of sugars for biofuel production. Significant reduction of greenhouse gas, particulate, and NO_x emissions can also be achieved through this shift from petroleum-based fuels to biofuels (Lynd, Cushman, Nichols, & Wyman, 1991). However, the 'food vs. fuel' conflict has opened up the search for new feedstocks for ethanol production. The abundance and renewability of lignocellulosic biomass have made it a potential candidate for ethanol production. However, due to inherent properties of cellulose, it is still more costly to produce cellulose-based ethanol than current grain-fermented ethanol (Paster, Pellegrino, & Carole, 2003). This issue has led to numerous research studies focusing on cost-effective pretreatment techniques to produce cellulosic ethanol that can be economically comparable to grain-derived ethanol (Mosier et al., 2005). Cellulose hydrolysis using cellulases produced by microorganisms such as *Trichoderma* spp. combined with β-glucosidases is an accepted method for the production of glucose which can then be fermented into cellulosic ethanol (Himmel et al., 2007; Yoshida et al., 2008). However, issues with cellulosic ethanol production arise because the crystallinity and insolubility of cellulose slow enzymatic conversion and reduce sugar yield with respect to starch-based processes, and the association of lignin and hemicellulose inhibits enzyme accessibility (Yoshida et al., 2008). These inherent challenges in the conversion of lignocellulosic biomass are generally termed as "recalcitrance" (Gharpuray, Lee, & Fan, 1983). Many methods have been explored to reduce recalcitrance that include treatments such as (1) mechanical, (2) dilute acid, (3) organosolv, and (4) steam explosion. All of these methods tend to decrease the crystallinity and degree of polymerization of cellulose, reduce hemicellulose and lignin content, and increase surface area and porosity of the biomass (Driscoll et al., 2009; Yoshida et al., 2008). Pretreatment of biomass results in increased sugar yield during enzymatic hydrolysis. However, the pretreatment methods

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have their own merits and demerits depending on the operational conditions (Dashtban, Maki, Leung, Mao, & Qin, 2010; Eggeman & Elander, 2005; Hall, Bansal, Lee, Realff, & Bommarius, 2010; Tao et al., 2011; Tucker, Kim, Newman, & Nguyen, 2003).

This study is focused on the pretreatment of switchgrass using high-energy EB generated by a commercially available, industrial scale accelerator. Switchgrass was chosen as the feedstock for this study because its tolerance to cold, disease, and insects makes it a regionally appropriate energy crop in New York State (Kasi & Ragauskas, 2010). EB treatment has a wide range of industrial applications including sterilization, curing of coatings and inks, crosslinking of plastic and rubber, plastics recycling, hydrogels, and carbon nanostructure engineering (Banhart, 1999; Clough, 2001). The advantages of EB pretreatment of biomass are that it (1) is an industrially proven technology at commercial scale, (2) creates very few compounds toxic to fermentative organisms, and (3) is environmentally benign in terms of byproducts generated. EB pretreatment decreases the crystallinity of cellulose, resulting in higher sugar yields after enzyme treatment (Driscoll et al., 2009; Shin & Sung, 2008).

A previous study revealed that enzymatic hydrolysis of hybrid poplar after pretreatment by EB resulted in improved sugar yield (Shin & Sung, 2008). In another study by the same authors, it was found that hydrolysis of hemicelluloses were enhanced by EB irradiation (Shin & Sung, 2010). Our laboratory studied the effect of radiation on molecular weight of microcrystalline cellulose (MCC). The molecular weight was reduced from 82,000 to 2200 Da after 1000 kGy (kiloGray) irradiation, and crystallinity of MCC was reduced from 87 to 45% (Driscoll et al., 2009).

In this study, HW extraction, cellulase hydrolysis, NIR using chemometric models, and TGA were used to characterize the effect of EB pretreatment on the composition and accessibility of cellulose, hemicellulose, and lignin in switchgrass.

2. Materials and methods

2.1. Materials

Panicum virgatum L., Cave-In-Rock switchgrass used in this study was obtained from Professors Hilary Mayton and Julie Lynn Hansen's laboratory at Cornell University. Samples were dried at room temperature ($23 \pm 2\,^{\circ}C$) for a week and further ground to smaller size (40 mesh) using a Wiley mill (Thomas Scientific).

2.2. Electron beam pretreatment

The switchgrass samples were packed in polyethylene plastic pouches in five batches and subjected to EB irradiation. A 3.0 MeV, 250 kW Dynamitron Accelerator from IBA Industrial Inc. (formerly Radiation Dynamics Inc.), Edgewood, NY, was used for this purpose. Each 100 g batch of switchgrass sample was subjected to different dosages of radiation. A uniform irradiation was applied to the samples on a conveyor moving at 3 m/min such that each sample received a dose of 125 kGy per scan, and the samples were scanned multiple times to achieve 250, 500, 750, and 1000 kGy total dosages of exposure. (*Note*. 1 kGy = 1 kJ/kg.)

2.3. Hot water extraction

0.5 g of EB-treated switchgrass samples were HW extracted at 150 °C for 3 h in 10 mL of distilled water using a sealed screw-cap Chemglass pressure vial. The samples were filtered and further dried at 105 °C. The amount of biomass dissolved in water was calculated by mass loss, and the soluble fraction was qualitatively verified to be hemicellulose by a simple liquid NIR chemometric model developed using dilutions of HW extracts of xylan from oat spelts (Sigma, CAS 9014-63-5).

2.4. Near infrared spectroscopy (NIR)

NIR spectroscopy was carried out using a Bruker MPA Multi-Purpose FT-NIR Analyzer with an Integrating Sphere and run through OPUS v6.5 software. For solid biomass, each sample was placed into a 15 mm diameter non NIR-absorbing glass vial (Bruker) with sample depth of ~3 mm. The vials were placed directly onto the integrating sphere window, and the spectra were collected in diffuse reflectance mode over a range of 12,500–3600 cm^{-1} with a resolution of 8 cm^{-1}. A total of 32 scans were co-added for each sample, and the resulting absorbance spectra were improved using the straylight and baseline correction with the OPUS software.

2.5. Chemometric modeling

The models for cellulose, hemicellulose, and lignin content analysis of the powdered biomass samples used in this study were selected from a database of Partial Least Squares chemometric models for solid woody biomass analysis developed in our laboratory. To choose the most appropriate models for analysis of the herbaceous switchgrass, each model in the catalog was evaluated using reference materials that included 8491 (sugarcane bagasse), 8492 (populus deltoides), and 8493 (monterey pine) from the National Institutes of Standards & Technology for Whole Biomass Feedstocks (Report of Investigation, 2001). The models which had the most accurate and precise agreement with the cellulose, hemicellulose, and lignin content among this range of various types of lignocellulosic biomass were selected and used for analysis of the irradiated and control samples of switchgrass before and after HW extraction to determine the component percentages. The three selected models consisted of 228, 228, and 215 calibration samples, had R^2 values of 96.8, 94.6, and 94.6, and had root mean square error of cross validations of 2.0, 1.4, and 2.6 for cellulose, hemicellulose, and lignin, respectively.

2.6. Thermogravimetric analysis

Thermal properties were compared between HW extracted and non-extracted control and EB-treated switchgrass using a TGA Q5000 series Thermogravimetric Analyzer (TA Instruments, USA). The temperature ramping rate was 20 °C/min up to 650 °C in a nitrogen (flow rate 10 mL/min) environment in high-resolution dynamic mode. The results were analyzed using TA Universal V4.7A thermal analysis software.

2.7. Hydrolysis of irradiated biomass

Cellulases (exo- and endo-glucanases) from *Trichoderma reesei* ATCC 26921 (Sigma, CAS 9012-54-8) and β-glucosidase from almonds (Sigma, CAS 9001-22-3) at 7.80 U/mg were used in combination for achieving enzymatic hydrolysis. Cellulase activities for the enzyme were calculated using LAP 006 from the National Renewable Energy Laboratory (Adney & Baker, 1996).

EB-treated samples of switchgrass were HW extracted and dried at 105 °C. A working solution of cellulase was made by diluting 1 mL of cellulase in 20 mL of 0.1 M citrate buffer (pH 4.8). Later 0.2 g of β-glucosidase was added to the working solution and mixed well. In a 20 mL glass Pyrex test tube with cap, 0.1 g equivalent of cellulose content of switchgrass was combined with 5 mL citrate buffer (0.1 M, pH 4.8). Tetracycline (0.03 mL; 10 mg/mL in water) and cyclohexamide (0.04 mL; 10 mg/mL in 70% ethanol) were added to prevent growth of any microorganisms. Deionized water was added to make the volume up to 9.8 mL. The tubes were placed in a rotary incubator at 50 °C for 1 h. The vials were removed, and

Table 1
Percentage (% (w/w), oven dry) of lignocellulosic components in EB-treated switchgrass (a) before and (b) after HW extraction. Standard deviations of 0.16, 0.09, and 0.24 for cellulose, hemicellulose, and lignin, respectively, were determined by evaluating the model with 5 repetitions of the same sample and averaging.

	Control	250 kGy	500 kGy	750 kGy	1000 kGy
(a) Non-extracted					
Cellulose (%, w/w)	35.4	37.1	36.3	35.2	35.2
Hemicellulose (%, w/w)	31.0	30.2	30.3	30.4	30.2
Lignin (%, w/w)	18.4	20.3	19.0	19.5	19.5
(b) HW extracted					
Cellulose (%, w/w)	38.0	37.2	36.1	33.8	33.9
Hemicellulose (%, w/w)	26.7	21.9	20.2	18.0	16.9
Lignin (%, w/w)	16.6	17.0	17.1	16.8	17.5

0.2 mL of working enzyme (35 FPU/g cellulose) were added to the tubes. The tubes were incubated at 50 °C for six days, and 0.05 mL of the solution were removed every 24 h. The removed aliquots were analyzed using a GlucCell glucometer (range: 1.6–33.3 mM) to determine the amount of sugar released from the samples. The percentage of hydrolysis was calculated using the following equations:

$$\%\text{Hydrolysis} = \frac{100 \times (\text{mass of glucose released in vial})}{(\text{mass of glucose expected})} \quad (1)$$

$$\text{Mass of glucose released (mg)} = [G] \times V\ell \quad (2)$$

$$\text{Mass of glucose expected (mg)} = M_c \times \frac{180}{162} \quad (3)$$

$$\text{Mass of cellulose } (M_c) = \text{mass of sample} \times P_c \quad (4)$$

where $V\ell$ is the volume of liquid in reaction vessel (dL); $[G]$ the glucose concentration measured with glucose analyzer (mg/dL); M_c the mass of oven dried cellulose (mg); P_c is the percent of cellulose in biomass.

3. Results and discussion

3.1. NIR analysis

The use of NIR spectroscopy to provide quantitative biomass composition has become an increasingly more mainstream technique for rapid cellulose, hemicellulose, and lignin content determination in many types of lignocellulosic biomass (Baum, Agger, Meyer, Egebo, & Mikkelsen, 2012; Hames, Thomas, Sluiter, Roth, & Templeton, 2003; Hou & Li, 2011; Maranan & Laborie, 2007; Smith-Moritz et al., 2011). The small sample requirements and fast, nondestructive analysis offered by NIR make it an excellent technique for cell wall component analysis (Hames et al., 2003). Because the NIR region of the vibrational spectrum is cluttered with combination and overtone bands that make direct spectral interpretation very difficult, chemometric models are commonly used to correlate subtle changes in the spectra with compositional changes in the samples. Through partial least squares (PLS) algorithms, robust and predictive chemometric models can be developed and implemented with NIR spectroscopy from calibration sets of known values (Conzen, 2006; Geladi & Kowalski, 1986). In this study, NIR was used along with chemometric models for cellulose, hemicellulose, and lignin content to determine the changes in each of these components before and after HW extraction of control and EB-treated switchgrass. The NIR spectrum of each sample was analyzed with each of the models, and the results are shown in Table 1. Because the chemometric models used in this study were selected for their applicability to a wide range of biomass types rather than optimized specifically for switchgrass, the actual percentages reported should not be considered absolute, but the qualitative trends clearly show the changes in cell wall components before and after HW extraction. The observed percentage composition values for cellulose, hemicellulose, and lignin do, however, fall within reasonably expected percentages for these components in switchgrass (Kasi & Ragauskas, 2010).

It can be seen that the cellulose, hemicellulose, and lignin contents have only slight fluctuations between all 5 EB dosages before HW extraction (Table 1a). This is not unexpected since the depolymerization and decrystallization associated with EB irradiation typically do not yield low molecular weight products that readily volatilize. The post-HW extraction NIR data (Table 1b), however, show a decrease in the amount of hemicellulose remaining in the samples with increasing EB treatment. Some hemicellulose is lost in the non-irradiated control sample, which is in agreement with previously reported HW extraction of biomass (Barber, 2007), but nearly half of the initial hemicellulose is dissolved in the 1000 kGy sample. This loss of hemicellulose indicates that the hemicellulose has been partially depolymerized and cleaved from association with cellulose and lignin and, thus, more easily solubilized in water. The NIR data also shows a small amount of lignin removed in all switchgrass samples after HW extraction, and this is likely the result of the lignin in switchgrass being less condensed than in woody biomass (Adler, 1977; Boudet, Lapierre, & Grima-Pettenati, 1995) and therefore more amenable to HW extraction, given that water has acidic properties at elevated temperature and pressure (Lu et al., 2012). The cellulose content is relatively unaffected by the HW extraction even for the highest-dosage EB treatment, which is due to cellulose's insolubility in water even at high temperatures (Bergenstråhle, Wohlert, Himmel, & Brady, 2010).

The chemometric modeling based on NIR shows that EB pretreatment results in a disruption of the lignocellulosic components in switchgrass as substantiated by the large hemicellulose removal after HW extraction for higher-dosage samples. The NIR results also clearly demonstrate that hemicellulose is the primary component lost upon HW extraction.

3.2. TGA analysis

Because NIR chemometric modeling is still a relatively new technique for analyzing biomass content and is based on mathematical models rather than direct interpretation of the spectra, further analysis was required to confirm the results from the NIR models. TGA under nitrogen was employed to monitor the thermal degradation profiles of the switchgrass samples and analyze the lignocellulosic component content while also yielding some information on the crystallinity and molecular weight of the cell wall polymers. The thermogravimetric (TG; % mass loss vs temperature) and differential thermogravimetric analysis (DTG; first derivative of TG curve) curves for the EB pretreated switchgrass samples before and after HW extraction are shown in Fig. 1.

The results from TGA confirm the removal of hemicellulose upon HW extraction that was observed with NIR. It is widely accepted that the initial mass loss between 200 and 300 °C that appears on a

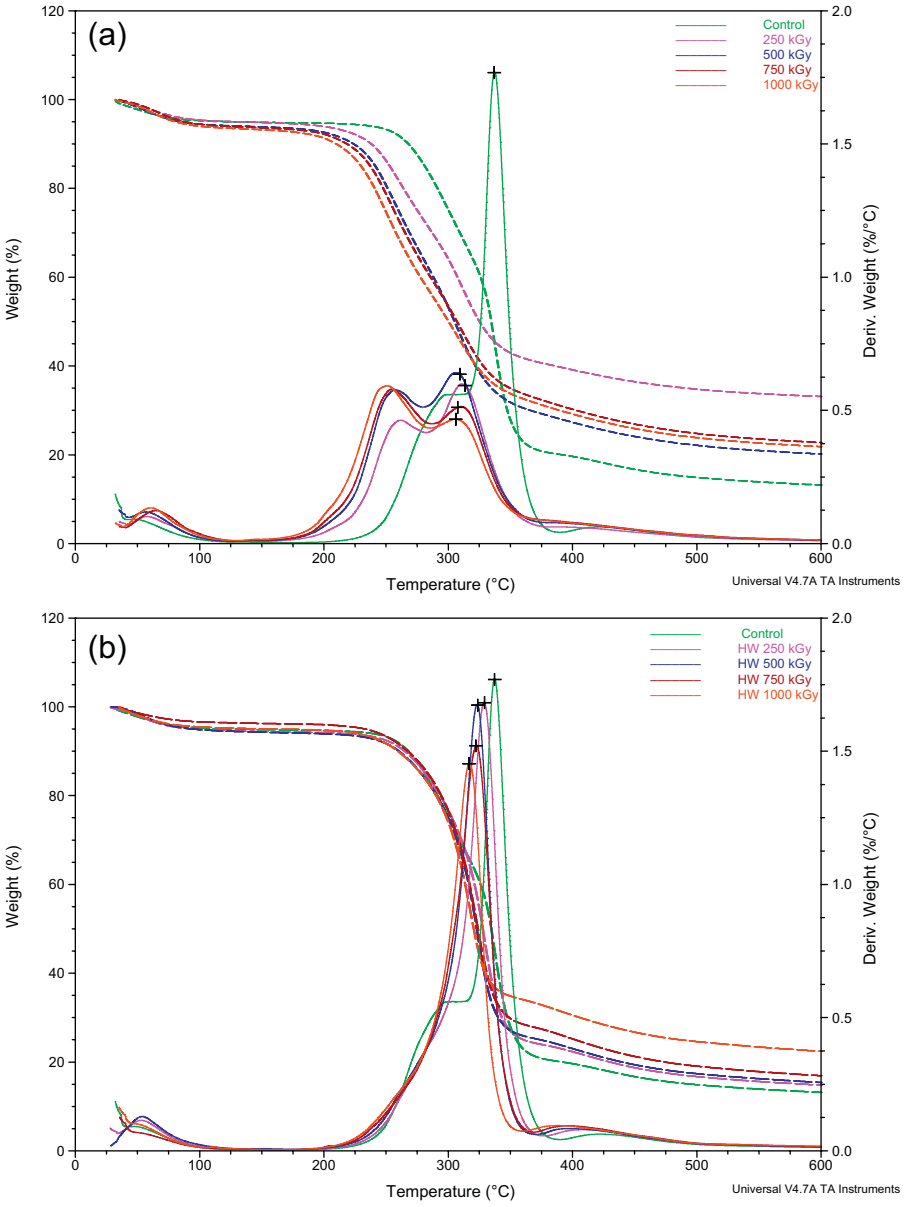

Fig. 1. TG (Y1 axis, dashed line) and DTG (Y2 axis, solid line) curves of EB pretreated switchgrass (a) before and (b) after HW extraction. One repetition per sample.

typical biomass DTG curve represents hemicellulose (Bridgeman et al., 2007; Carrier et al., 2011; Lee & Fasina, 2009; Serapiglia, Cameron, Stipanovic, & Smart, 2008). Therefore, when comparing Fig. 1a and b, the results show that the hemicellulose peak has been largely removed for the post-HW extracted samples that were EB pretreated.

Fig. 1a also illustrates the changes in the structure of the lignocellulose polymers that have been EB pretreated. The DTG curve of the untreated control sample appears very similar to the shape and profile that is expected from a typical biomass DTG curve under nitrogen, with one sharp cellulose peak at 337.0 °C and a hemicellulose shoulder peak around 300 °C. For the EB treated samples, however, the DTG curve changes considerably, with two very broad, short peaks with maxima around 245 and 300 °C observed. These peaks still represent the cell wall polysaccharides of hemicellulose and cellulose, respectively, but they have been shifted to lower temperatures and broadened. The maximum peak temperature decreases with increasing EB treatment intensity as shown in Table 2. The broadening and temperature reduction of DTG decomposition peaks are likely due to a decrystallization and increase in the breadth of the molecular weight distribution of the cell wall polymers (Cheng, Winter, & Stipanovic, 2012; Driscoll et al., 2009; Kim, Eom, & Wada, 2010).

Fig. 1b shows that after HW extraction the control sample remained essentially unchanged. However, the DTG curves of the EB-treated samples shift into single peaks. Because mass loss is observed in the entire region between 200 and 400 °C, these single peaks likely encompass both cellulose and the remaining hemicellulose. Possible hypotheses for the nondistinct hemicellulose peak include deacetylation and/or higher molecular weight of the remaining hemicellulose. EB pretreatment is known to partially deacetylate hemicellulose (Jeffries, 1994), and hemicellulose that is highly acetylated is known to be more water-soluble than nonacetylated hemicellulose (Sjöström, 1993). Hemicellulose is also more water-soluble at low molecular weight (Xu et al., 2007). Therefore, it can be argued that the shorter and more acetylated hemicellulose would be extracted by HW, leaving the longer and nonacetylated chains behind, which would degrade at a higher temperature. Thus, this hemicellulose degradation may be incorporated into the cellulose peak rather than appearing as a distinct

Table 2
Highest degradation peak temperature before and after HW extraction for EB-treated switchgrass, with one repetition per sample. Peak temperatures were determined using the Peak Maximum function of the TA Universal Analysis software.

	Control	250 kGy	500 kGy	750 kGy	1000 kGy
Non-extracted	337.0	313.7	309.3	307.6	306.0
HW extracted	337.3	329.2	323.8	322.2	316.7

shoulder peak. Table 2 shows that the peak temperatures are greater for the HW extracted EB-treated samples. This data suggests that the lower molecular weight polymers have been extracted.

3.3. Cellulase hydrolysis

In order to produce ethanol from cellulosic materials at an economically viable scale, the rate of production and ultimate glucose yield must be improved. In this study, switchgrass was treated with a mixture of endo- and exo-cellulases plus a β-glucosidase to determine the effect of EB treatment on glucose production rate and yield, as shown in Fig. 2a. The effect of HW extraction after EB treatment is given in Fig. 2b.

It can be seen in Fig. 2a that the amount of glucose liberated from the cellulose chain increases with increasing EB dosage, which is consistent with previously reported results (Shin & Sung, 2008,

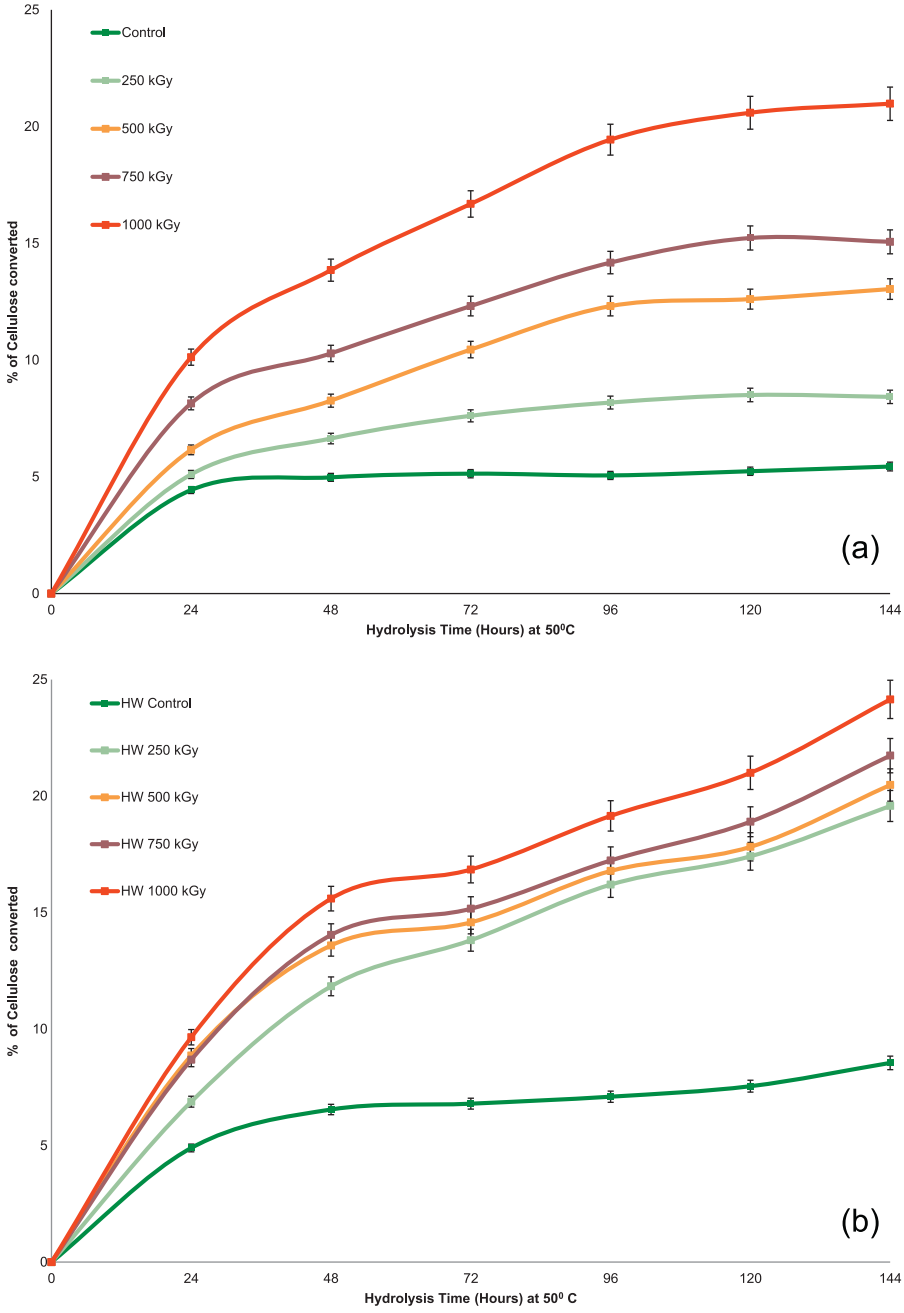

Fig. 2. EB-treated switchgrass hydrolyzed to glucose by enzymes (a) before and (b) after HW extraction (endo/exo glucanases and β-glucosidase at 35 FPU). Percent of cellulose converted to glucose calculated as shown in Eqs. (1)–(4). Error bars represent ±3.4% around the mean value of 2 repetitions, which is the stated within-run coefficient of variation (CV) provided from the GlucCell glucometer manufacturer's performance evaluations based on 20 replicates.

Table 3
Percentage of cellulose converted to glucose after (a) 48 and (b) 144 h of enzymatic hydrolysis. Percentages are based on the initial cellulose content and represent the average of two repetitions. The 48 h data is representative of initial rate, and the 144 h data is representative of ultimate yield. The CV provided by the GlucCell glucometer manufacturer is 3.4%.

	Control	250 kGy	500 kGy	750 kGy	1000 kGy
(a) 48 h					
Non-extracted	5.0	6.7	8.3	10.4	13.9
HW extracted	6.5	11.8	13.2	13.7	15.7
(b) 144 h					
Non-extracted	5.4	8.5	13.5	15.0	21.0
HW extracted	8.5	19.6	20.5	21.7	22.8

2010). The control sample begins to plateau at around 5% conversion after only 24 h, indicating that only a very small portion of the cellulose within the switchgrass sample was accessible to the cellulase enzymes in the untreated biomass. With increasing EB treatment, however, the total amount of glucose released after 144 h increases approximately fourfold, and the plateau in enzyme activity is not seen until much further into the enzymatic hydrolysis experiment. These results suggest that the EB pretreatment greatly increases the number of sites at which the enzymes can hydrolyze the cellulose chain. This increase in accessible sites is in agreement with the previous studies showing reduction in cellulose crystallinity and molecular weight after EB treatment (Driscoll et al., 2009; Shin & Sung, 2008), which is also observed in the TGA results.

Fig. 2b shows the hydrolysis results after the switchgrass samples have been extracted with HW at 150 °C for 3 h. All samples show an increase in the amount of glucose that is liberated as well as an increase in the rate at which these higher glucose values are achieved compared to the non-HW extracted samples, with the EB-treated samples showing more marked enhancement than the control. Table 3 compares the glucose yield after 48 and 144 h of enzymatic hydrolysis, expressed as percentage of glucose liberated based on initial cellulose content, for the non-extracted and HW extracted switchgrass samples. The observed increase in glucose production at 48 and 144 h demonstrate the enhancement of the initial rate and ultimate yield, respectively, that is achieved with HW extraction. With a considerable portion of the hemicellulose removed during the HW extraction, there is less material hindering enzyme binding to the cellulose chain and beginning cleavage. The deacetylation of the remaining hemicellulose (as discussed in Section 3.2) could also contribute to the glucose yield enhancement, since increasing degrees of deacetylation have been shown to increase the yield of sugars obtained from enzymatic hydrolysis (Kong, Engler, & Soltes, 1992). Because lignin is known to be a possible inhibitor of cellulase activity (Zhang & Lynd, 2004), the HW extraction likely also has the positive benefit of removing the most easily extracted portions of the lignin prior to the enzymes coming into contact with the biomass. With much of the hemicellulose and water-extractable lignin removed, the rate and final yield of glucose liberated for all EB treatments after HW extraction are comparable or better than the 1000 kGy treatment for the non-extracted sample, and the observed glucose content has still not yet reached a plateau after 144 h.

4. Conclusion

In this study, EB pretreatment of switchgrass resulted in a greater than fourfold increase in sugar yield when the samples were hydrolyzed with cellulases. The increase in sugar yield was proportional to the dosage of EB treatment. HW extraction of the EB pretreated samples resulted in partial removal of hemicellulose, which in turn resulted in higher sugar yield. NIR spectroscopy was a helpful non-destructive tool in determining the changes in lignocellulosic component percentages before and after HW extraction, and TGA served to offer valuable insight into the impact that EB pretreatment and HW extraction had on the crystallinity and molecular weight of the cell wall polymers.

Rapid, high-yielding, and efficient production of glucose from cellulosic materials is paramount in the potential application of economically viable lignocellulose-based biofuels, and the results of this study demonstrate that EB pretreatment paired with HW extraction effectively increases the yield and rate of glucose production from the cellulose within switchgrass. Because both EB pretreatment and HW extraction are viable at a large scale, the enhanced sugar production demonstrated in this work is a potentially promising technology that could be implemented into lignocellulose-based biorefineries.

Future directions of this work include enhancing the ultimate glucose yield beyond the modest result of 22.8%. This study demonstrates the effect of EB pretreatment paired with HW extraction, but improvements can likely be achieved through the use of commercial-grade enzymes, industrial HW extraction procedures, and higher enzyme dosages, as well as coupling with other pretreatment methods such as biodelignification. Studies examining the sequence of pretreatment applications could also elucidate further possible synergies between various methods. Ultimately, partnerships with industrial collaborators will also help to determine the economics of these processes, i.e. cost per dose of EB.

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