



Supercritical carbon dioxide pretreatment of corn stover and switchgrass for lignocellulosic ethanol production

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ABSTRACT

Supercritical CO₂ (SC-CO₂), a green solvent suitable for a mobile lignocellulosic biomass processor, was used to pretreat corn stover and switchgrass at various temperatures and pressures. The CO₂ pressure was released as quickly as possible by opening a quick release valve during the pretreatment. The biomass was hydrolyzed after pretreatment using cellulase combined with β-glucosidase. The hydrolysate was analyzed for the amount of glucose released. Glucose yields from corn stover samples pretreated with SC-CO₂ were higher than the untreated sample's 12% glucose yield (12 g/100 g dry biomass) and the highest glucose yield of 30% was achieved with SC-CO₂ pretreatment at 3500 psi and 150 °C for 60 min. The pretreatment method showed very limited improvement (14% vs. 12%) in glucose yield for switchgrass. X-ray diffraction results indicated no change in crystallinity of the SC-CO₂ treated corn stover when compared to the untreated, while SEM images showed an increase in surface area.

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1. Introduction

Bioethanol derived from bio-materials such as corn and sugarcane is the most popular alternate fuel today in energy diversification (Mabee, 2007). Increased demand in biofuels cannot be met by the use of corn and sugarcane. In the US, corn ethanol has increased food prices and its adverse impact on soil fertility is hotly debated (Rajagopal et al., 2007; Goldemberg and Guardabassi, 2009). Lignocellulosic biomasses such as wood, grass, agricultural wastes, sugarcane bagasse, corn stover, forest residues, and municipal wastes are abundant and widely available and could become a source of bioethanol that will unlikely adversely influence food prices (Cassman and Liska, 2007).

The production of lignocellulosic ethanol from biomass generally involves four major steps: pretreatment of biomass, hydrolysis of biomass, fermentation of released sugars, and ethanol separation. The barriers for hydrolysis of carbohydrates from biomass are the crystalline structure of cellulose, the protective sheath of lignin and the hemicellulose around cellulose. Lignin binds the cells together and reduces the surface area available for enzyme hydrolysis (Laureano-Perez et al., 2005; Mosier et al., 2005b). Without pretreatment, enzyme hydrolysis of biomass yields less than 20% of total sugars, whereas after pretreatment 90% can be achieved with some pretreatment methods (Alizadeh et al., 2005).

Numerous pretreatment methods are available, each with their advantages and disadvantages (Teymouri et al., 2004; Mosier et al., 2005a,b; Wyman et al., 2005; Alizadeh et al., 2005; Lloyd and Wyman, 2005; Kim and Lee, 2007; Hendriks and Zeeman, 2009; Zhu et al., 2010; Wu et al., 2011). The SC-CO₂ pretreatment method is an environment friendly method with its own niche in tactical biomass processing because the process does not discharge harmful chemicals, but research on its use has been limited to a few lignocellulosic materials such as Avicel cellulose, recycled paper and sugarcane bagasse (Zheng et al., 1998) or aspen and Southern yellow pine (Kim and Hong, 2001).

The present work investigated SC-CO₂ pretreatment of corn stover and switchgrass in a tubular reaction with various temperature, pressure and time settings. Two important lignocellulosic materials, corn stover and switchgrass were chosen to investigate the effectiveness of SC-CO₂ pretreatment. Glucose yields after enzyme hydrolysis using cellulase and β-glucosidase of the samples with and without SC-CO₂ pretreatment were used to measure the success of the pretreatment method.

2. Methods

2.1. Biomass

Corn stover (corn stalk) was collected from a local farm in Athens, Ohio. The switchgrass sample was supplied by Bob Hendershot (Ohio NRCS Grassland Management Fairfield Soil and Water District). The corn stover and switchgrass were cut and

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sieved using a US Standard Testing Sieve No. 16 (1.18 mm) and subsequently dried at 45 °C overnight.

2.2. Chemicals

Industrial grade liquid CO₂ (in a siphon-tube cylinder) was purchased from Airgas (Parkersburg, WV). Cellulase enzyme (EC No. 232-734-4) from *Trichoderma reesei* (ATCC 26921) was purchased from Sigma Aldrich (St. Louis, MO). According to the vendor, the activity of the cellulase was 700 U/g and density 1.2 g/ml. One unit of the cellulase enzyme, as defined by the manufacturer, corresponds to the amount of enzyme that liberates 1.0 μmol of glucose from cellulose in 1 h at pH 5.0 and 50 °C. β-glucosidase (EC No. 232-589-7) was purchased from Sigma Aldrich. The activity of the β-glucosidase was ≥6 U/mg (1 U of β-glucosidase enzyme defined by manufacture corresponds to the amount of enzyme that liberates 1 μmol of glucose per minute at pH 5.0 and 35 °C). The amount of glucose released by enzyme treatment was specifically determined using a glucose assay kit (Product No. GAHK20-1KT) from Sigma Aldrich.

2.3. Reactor and SC-CO₂ pretreatment

The reactor had an internal working volume of 94.7 ml. It was built using stainless steel components purchased from Scioto Valve & Fitting Co. (Westerville, Ohio). The reactor materials were rated 4000 psi. A 5 g biomass sample with known moisture content was placed in the reactor. The moisture content in the biomass sample was determined from the weight loss upon complete drying that was achieved in a 45 °C oven for 24 h. The reactor was then placed under vacuum to remove the air inside the reactor. The reactor was connected to the liquid CO₂ tank and liquid CO₂ flowed into the reactor, without a pump, due to a higher CO₂ cylinder pressure. When the pressure in the reactor equaled the pressure in the CO₂ tank, the reactor was placed in an ice bath to allow additional amount of CO₂ to flow in. The desired amount of CO₂ was initially estimated from thermodynamic equation of state calculations (without considering the presence of moisture and biomass) for a preset pressure and temperature and a fixed reactor volume (Narayanaswamy, 2010). The exact CO₂ amount needed to achieve the temperature and pressure was eventually determined from a test run. The ice bath temperature was maintained around 4 °C. When the desired amount of CO₂ (judged by mass increase measured on a balance) had entered the reactor, the reactor was sealed and disconnected from the CO₂ cylinder. This CO₂ loading method eliminated the need for an expensive CO₂ pump.

To reach the supercritical state, the reactor was heated to a desired temperature using a heating tape or a water bath depending on the temperature requirement. During the heating process, the reactor was rotated and shaken manually to allow better mixing of biomass with CO₂. After the biomass had been subjected to the preset pressure and temperature for a specific time period, the pressure was released instantaneously using a quick release ball valve. The temperatures studied in this work were 100, 120, and 150 °C and the pressures 2500, 3200 and 3500 psi, respectively. The pretreated biomass sample was taken out of the reactor and dried at 45 °C in an oven for 24 h before enzyme hydrolysis.

2.4. Enzyme hydrolysis of SC-CO₂ treated biomass

100 mg of a dried biomass sample, 50 U of cellulase and 20 U of β-glucosidase were mixed in 30 ml of citrate buffer solution (0.05 M, pH 4.8), in 50 ml conical tubes. The buffer solution and conical tubes were autoclaved before use to eliminate microbial contamination. The conical tubes were incubated in a shaking water bath maintained at 47 °C for 24 h. The hydrolysate was sam-

pled at fixed time intervals and placed in a water bath at 90 °C for 10 min to denature the enzymes. Then the samples were centrifuged at 4500g for 30 min in a Marathon Micro A Model AR centrifuge (Fisher Scientific, Pittsburgh, PA) and the supernatants were frozen at –18 °C for further analysis. The supernatants were analyzed for glucose using a glucose kit. The enzymatic hydrolysis of each pretreated and untreated cellulosic biomass samples were carried out in duplicates.

2.5. Quantification of glucose content

The glucose contents of samples were determined using a glucose kit (Product No. GAHK20-1KT) from Sigma Diagnostics. A 96 plate chamber UV-VIS spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) was used to measure absorption at 340 nm. The concentration of glucose present in a solution was determined from a calibration curve generated using glucose standard solutions. Glucose yield was measured based on the glucose released after enzyme hydrolysis vs. the dry biomass amount.

2.6. X-ray diffraction (XRD)

An X-ray diffractometer (Rigaku Geigerflex, 2000W, Rigaku USA Inc., Danvers, MA) was used to determine the crystallinity of untreated and the SC-CO₂ treated corn stover samples. The biomass samples less than 0.42 mm in length were used for X-ray diffraction analysis. A scan type of theta-2-theta with a step size of 0.05° was carried out at 0.05°/min.

2.7. Scanning electron microscope (SEM)

The surface morphology of untreated and treated biomass samples was examined using SEM. The corn stover samples used for SEM analysis were first cut into small pieces using scissors. Care was taken to avoid outer surface damage. Samples were prepared by mounting them on sample holders using double-coated tape, and the sample was sputter coated with platinum to make the surface conductive for charge. The samples were sent to an independent lab for SEM analysis using a Hitachi S-4700 FEGSEM SEM machine and the SEM technician was unaware which samples were treated and untreated.

3. Results and discussion

3.1. SC-CO₂ pretreatment of corn stover

3.1.1. Effect of moisture

The presence of moisture did not prevent CO₂ from reaching the supercritical state. This was verified by placing CO₂ with moist biomass in a critical point drier (Model CPD-020, Balzers Union, Liechtenstein) which is equipped with a see-through glass window. When the CO₂ reached the desired temperature and pressure, the liquid and gas phases became indistinguishable, indicating supercritical state.

Two corn stover samples with different moisture contents were pretreated using SC-CO₂ at 120 °C and 3500 psi for one hour in two separate batch runs. The effect of moisture content on pretreatment of corn stover is shown in Fig. 1a. The glucose yields for samples with 75% moisture content, 0% moisture content and untreated biomass are 24%, 13% and 12% (w/w), respectively. This means SC-CO₂ pretreatment made very little improvement in glucose yield without moisture (13% vs. 12%) while with moisture, glucose yield doubled (24% vs. 12%). A similar increase in glucose yield for different moisture contents was observed for SC-CO₂

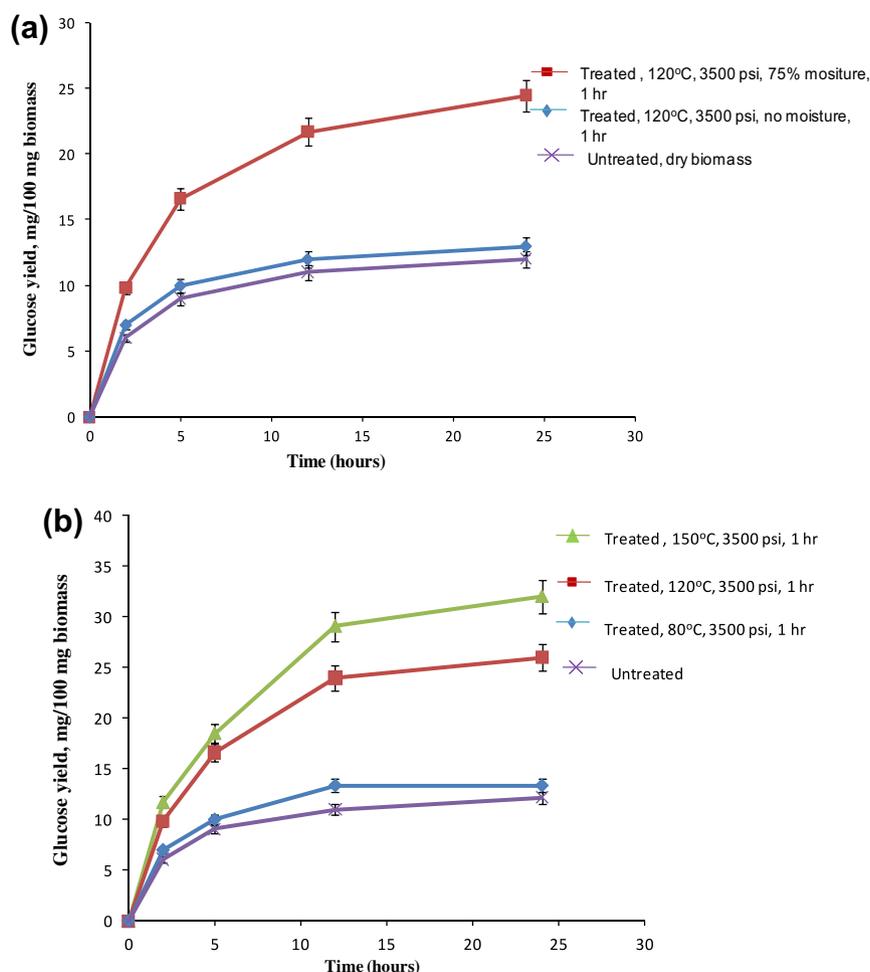


Fig. 1. Effect of moisture content (a), and effect of temperature (b) on SC-CO₂ pretreatment of corn stover. Glucose yield time courses during enzyme hydrolysis are shown.

pretreatment of Avicel cellulose by Zheng et al. (1998) and on aspen wood by Kim and Hong (2001). Moisture content above a particular level is not favorable for the SC-CO₂ pretreatment, as noted by Kim and Hong (2001).

The presence of water in the form of moisture has a positive influence on the glucose yield. It was reported that water and supercritical CO₂ could form weak carbonic acid at a high pressure (Puri, 1984; Meyssami et al., 1992). Carbonic acid can partially hydrolyze the hemicellulose fraction in biomass at low temperatures (Kim and Hong, 2001; van Walsum and Shi, 2004). This acidification of the wet biomass can also dissociate the hydrogen-hydrogen bonds that link cellulose microfibrils to hemicellulose polysaccharides, thus increasing the accessibility of the cellulase enzyme to its natural substrate (cellulose), and promoting cellulose hydrolysis (van Walsum and Shi, 2004). The other possible benefit of moisture is that water causes swelling of the biomass, which in turn can open the pores for CO₂ molecules to penetrate deeper into the biomass. Upon an explosive release of pressure, the CO₂ disrupts the biomass fibers exposing more surface area for enzyme action. In the aqueous ammonia pretreatment method, a lot of hemicellulose and lignin were removed from the solids (Kim and Lee, 2007). The dissolved hemicellulose had to be recovered from the liquid ammonia for higher yields of sugars. In the SC-CO₂ pretreatment method, hemicelluloses and lignin remain in the solids.

3.1.2. Effect of temperature

The SC-CO₂ pretreatment of corn stover was carried out at various temperatures, whereas the pressure (3500 psi), time (1 h) and

the moisture content (75%) were held constant. The glucose yields for 80, 120 and 150 °C were 13%, 24% and 30%, respectively for SC-CO₂ treated biomass compared with 12% for untreated biomass as shown in Fig. 1b. The glucose yield of 30% at 3500 psi and 150 and 80 °C is lower than the highest yields of 40% and 37% based on corn stover dry mass calculated by Wyman et al. (2005) from the experimental data reported by Teymouri et al. (2004) using the ammonia fiber explosion pretreatment of corn stover at 90 °C and the experimental data reported by Lloyd and Wyman (2005) for dilute sulfuric acid pretreatment, respectively. The SC-CO₂ pretreatment method has the advantage that no toxic chemical needs to be discharged. The SC-CO₂ pretreatment temperature of 80 °C appeared to have very little impact on glucose yield. This was likely due to the inferior diffusivity of the SC-CO₂ at low temperatures. Higher temperatures correlate with improved glucose yield, albeit, with the drawback of higher energy input. Similar trends of temperature on glucose yield were reported by Zheng et al. (1998) on Avicel and by Kim and Hong (2001) on wood using SC-CO₂. The SC-CO₂ pretreatment temperatures used in this work (150 °C and below) are considerably lower than 200 °C used in steam explosion pretreatment (Bonn et al., 1983).

3.1.3. Effect of pressure

Fig. 2a shows the effect of pressure on the SC-CO₂ pretreatment of corn stover at constant temperature (150 °C), time (1 h) and moisture content (75%). The glucose yields for 2500 and 3500 psi were 20% and 30%, respectively, and for the untreated biomass (control) 12%. At 2500 psi the glucose yield doubled compared

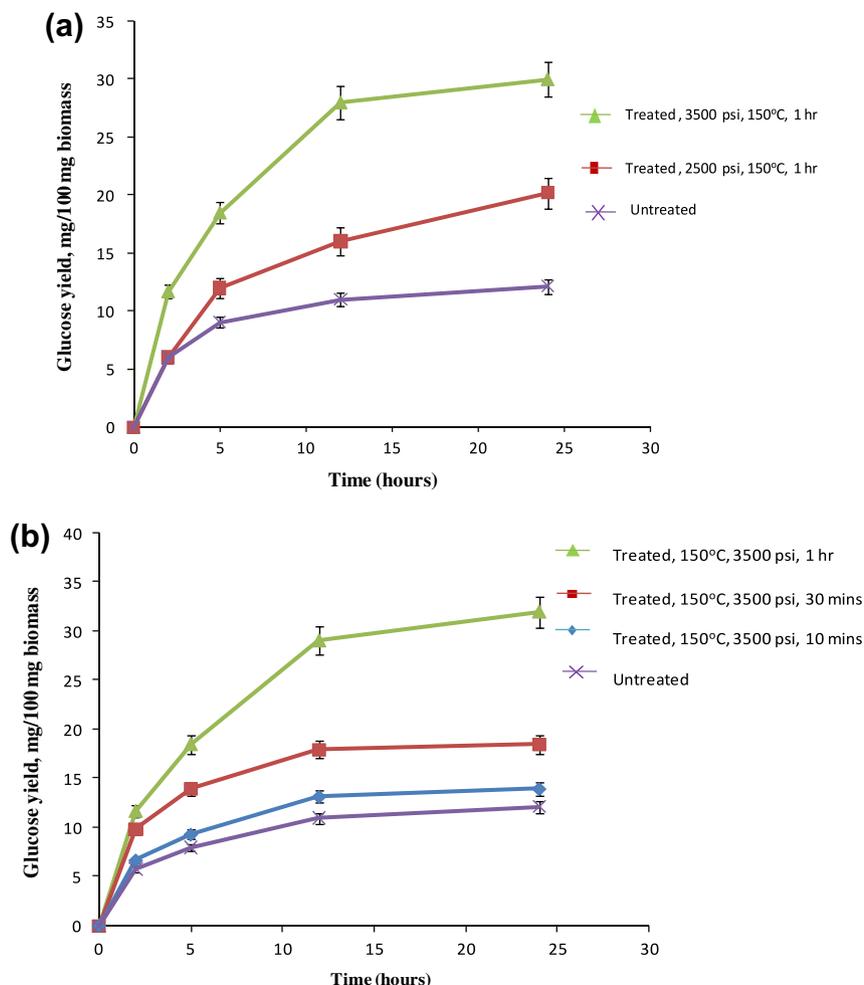


Fig. 2. Effect of pressure (a), and effect of pretreatment time (b) on SC-CO₂ pretreatment of corn stover.

with untreated biomass and at 3500 psi, the yield increased by 2.5 times. The results indicate that with an increase in pressure the glucose yield of biomass also increased. The higher pressure could result in a faster and deeper penetration of the SC-CO₂ into the biomass pores, and thus increasing the surface area for the enzyme action after a sudden release of pressure. A similar effect on glucose yield was observed by Zheng et al. (1998) on Avicel using SC-CO₂.

3.1.4. Effect of pretreatment time

The effect of time duration on the SC-CO₂ pretreatment of corn stover is shown in Fig. 2b. Pretreatment was carried out for various time durations at constant temperature (150 °C), pressure (3500 psi) and moisture content (75%). The glucose yields for 10, 30, and 60 min of pretreatment times were 14%, 18% and 30% of biomass, respectively compared with 12% for the untreated biomass. A pretreatment time of 10 min improved glucose yield by only 2% when compared with the untreated corn stover. 60 min resulted in a 30% yield that is considerably higher than the 18% glucose yield observed for 30 min. 30% is 77% of the theoretical maximum of 39.4 g glucose (from cellulose) per 100 g of the corn stover (Pauly and Keegstra, 2008). Further increasing the time will have rather limited improvement on the yield. Kim and Hong (2001) reported that the glucose yield for aspen wood pretreated with SC-CO₂ at 3100 psi and 165 °C for 30 min was 84.7% of theoretical maximum. In this work, the reactor was rotated and shaken manually during heating. It was likely that vigorous stirring or shaking might have reduced the reaction time.

3.1.5. Effect of enzyme loading

Tests were conducted to determine whether the SC-CO₂ pretreatment could reduce the amount of enzyme needed in hydrolysis. The effects of enzyme loading on both the untreated and the SC-CO₂ treated (150 °C, 3500 psi, 1 h and 75% moisture content) corn stover samples are shown in Fig. 3a. Two untreated samples were loaded with 50 and 84 U of cellulase enzyme, respectively, and were compared with the SC-CO₂ pretreated samples loaded with 50 U of cellulase enzyme. Glucose yield was measured at fixed time intervals during hydrolysis. The glucose yield from pretreated corn stover was 30% with 50 U of cellulase compared with 12% from untreated corn stover with 50 U, and 14% from untreated corn stover with 84 U. The data in Fig. 3a indicate that SC-CO₂ pretreatment was much more effective than increasing enzyme loading, and pretreatment was necessary because without it, increasing enzyme loading from 50 to 84 U improved glucose yield by only 2%. This also means that the pretreatment method is an effective way to save in the enzyme costs.

3.2. SC-CO₂ pretreatment of switchgrass

To investigate the effect of the SC-CO₂ pretreatment on glucose yield released from switchgrass, switchgrass samples were pretreated at 3200 psi for 1 h at 100 and 150 °C, respectively. Fig. 3b shows that glucose yields for 100 and 150 °C were 13% and 14%, respectively compared with 12% for the untreated switchgrass. Unlike SC-CO₂ pretreatment of corn stover, the pretreatment achieved

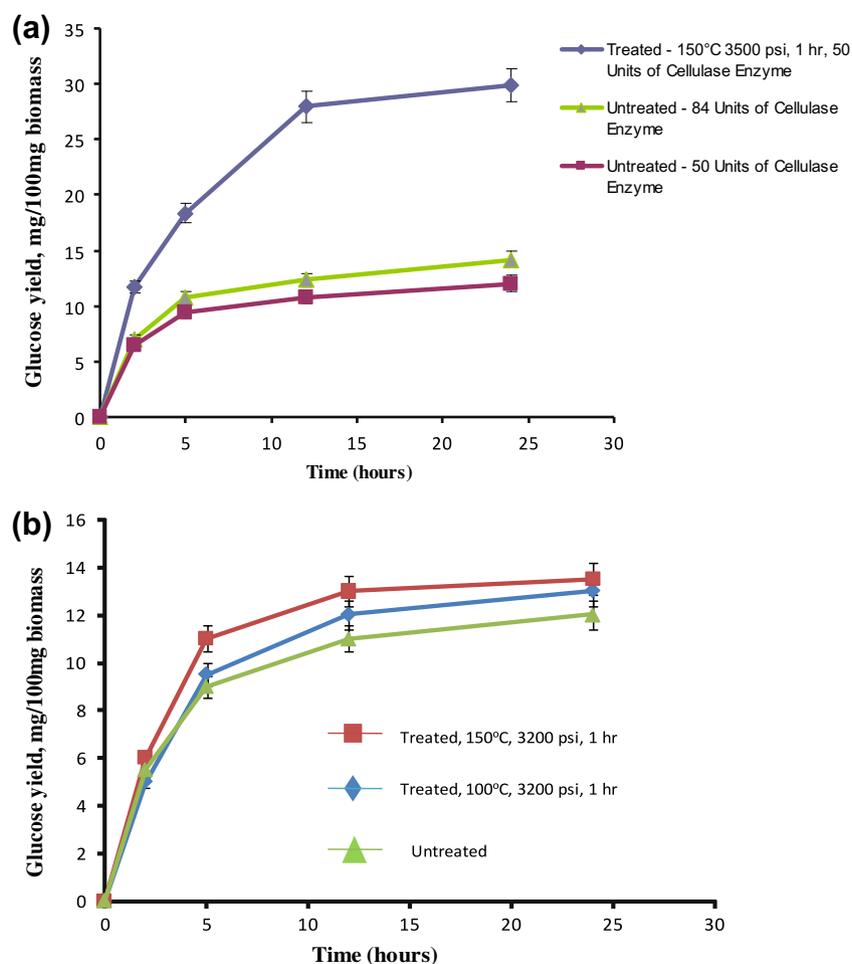


Fig. 3. Glucose yields of pretreated and untreated corn stover during enzyme hydrolysis with different enzyme loadings (a), and glucose yields from SC-CO₂ treated and untreated switchgrass during enzyme hydrolysis (b).

very limited, if any, improvement on glucose yield for switchgrass. Kim and Hong (2001) reported that SC-CO₂ pretreatment was highly effective for aspen (hard wood) while it was ineffective for southern yellow pine (soft wood). The reason for the very different results for corn stover and switchgrass could be due to the structural differences between their cell walls. Although both corn and switchgrass are grasses, their cell wall structures and compositions have some differences. Switchgrass typically has about 10% less glucan and slightly more xylan and lignin than corn stover on the dry mass basis less (Wyman, 1996). It is known that xylan backbones are substituted with glucuronic and ferulic acid residues that cross the polymer to itself or to lignin, which contribute to the recalcitrance of the cell walls in these grasses. Also, the switchgrass used for the study was from a long-standing and more mature crop.

3.3. X-ray diffraction analysis

The X-ray diffraction patterns of the SC-CO₂ pretreated and untreated corn stover are shown in Supplementary Fig. 1. Zheng et al. (1998) observed a reduction of 50% in crystallinity in SC-CO₂ pretreated Avicel cellulose when compared with untreated Avicel. However, in this work the SC-CO₂ pretreated corn stover showed no change in crystallinity in the cellulose fraction when compared with untreated corn stover. The reason could be that Avicel is pure cellulose and not associated with other cell wall polymers, whereas in corn stover, cellulose microfibrils are embedded in hemicellu-

loses, lignin and glycoproteins (Buchanan et al., 2000). Kim and Lee (2005) found that their ammonia recycle percolation pretreatment of corn stover improved glucose yield greatly, but detected no change in its crystalline structure. The presence of lignin and hemicelluloses in corn stover makes this lignocellulosic biomass rather resistant to change in crystalline structure. It should be noted that a change in crystallinity is not the only factor that influences the enzymatic hydrolysis of biomass.

3.4. SEM analysis

The surface morphology of untreated and SC-CO₂ treated (150 °C, 3500 psi, 1 h, 75% moisture) corn stover was examined under SEM. Supplementary Fig. 2 shows the SEM images of untreated (Panel A) and SC-CO₂ treated (Panel B) corn stover samples. These images demonstrate that the SC-CO₂ pretreatment exposed some internal areas in the biomass compared with the images for untreated samples. The biomass for SC-CO₂ pretreatment should be pre-wetted and swollen with moisture. Supplementary Fig. 3 shows a comparison between untreated corn stover and SC-CO₂ treated corn stover without moisture. The SEM images in Supplementary Fig. 3 indicate that the SC-CO₂ treatment did not open pores for unwetted corn stover unlike wetted corn stover in Supplementary Fig. 2. It was possible that after the biomass was swollen with moisture, the water inside the pores allowed CO₂ molecules to penetrate the pores under a high pressure and subsequently ruptured them during a sudden pressure release in

the SC-CO₂ treatment. Water in the form of moisture also dissolved CO₂ to form carbonic acid in the pores resulting in partial acid hydrolysis.

SC-CO₂ is a competitive pretreatment method especially for a tactical biorefinery because CO₂ is a green solvent and no expensive equipment is needed. The method does not generate any harmful chemical wastes. To eliminate the expensive transportation of bulky lignocellulosic biomass such as corn stover, a tactical mobile biomass preprocessor can be deployed on a truck to obtain concentrated hydrolysates for a centralized fermentation facility. Liquid CO₂ and dry ice that are by-products from ethanol fermentation can be used. By using liquid CO₂ or dry ice, there is no need for an expensive CO₂ pump to achieve supercritical CO₂ state in a reactor. Even if the CO₂ is not reused, the release of the CO₂ will not increase the carbon footprint because the CO₂ was obtained by the biomass used in ethanol fermentation from the atmosphere in the first place.

4. Conclusions

The SC-CO₂ pretreatment on corn stover enhanced glucose yield considerably. The glucose yield was increased when pressure and temperature increased. The highest glucose yield of 30% was achieved at 3500 psi at 150 °C when corn stover with 75% moisture content was treated for 1 h compared with 12% from untreated corn stover. SEM images showed considerably more pores in the SC-CO₂ treated corn stover with prior wetting and swelling compared with untreated corn stover, while XRD indicated no effect on crystallinity. The SC-CO₂ pretreatment was found ineffective for switchgrass, likely due to its more rigid cell walls.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biortech.2011.04.052](https://doi.org/10.1016/j.biortech.2011.04.052).

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