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Efficient Sugar Release by the Cellulose Solvent-Based Lignocellulose Fractionation Technology and Enzymatic Cellulose Hydrolysis

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Efficient liberation of fermentable soluble sugars from lignocellulosic biomass waste not only decreases solid waste handling but also produces value-added biofuels and biobased products. Industrial hemp, a special economic crop, is cultivated for its high-quality fibers and high-value seed oil, but its hollow stalk cords (hurds) are a cellulosic waste. The cellulose-solvent-based lignocellulose fractionation (CSLF) technology has been developed to separate lignocellulose components under modest reaction conditions (Zhang, Y.-H. P.; Ding, S.-Y.; Mielenz, J. R.; Elander, R.; Laser, M.; Himmel, M.; McMillan, J. D.; Lynd, L. R. *Biotechnol. Bioeng.* 2007, *97* (2), 214−223). Three pretreatment conditions (acid concentration, reaction temperature, and reaction time) were investigated to treat industrial hemp hurds for a maximal sugar release: a combinatorial result of a maximal retention of solid cellulose and a maximal enzymatic cellulose hydrolysis. At the best treatment condition (84.0% H_3PO_4 at 50 °C for 60 min), the glucan digestibility was 96% at hour 24 at a cellulase loading of 15 filter paper units of cellulase per gram of glucan. The scanning electron microscopic images were presented for the CSLF-pretreated biomass for the first time, suggesting that CSLF can completely destruct the plant cell-wall structure, in a good agreement with the highest enzymatic cellulose digestibility and fastest hydrolysis rate. It was found that phosphoric acid only above a critical concentration (83%) with a sufficient reaction time can efficiently disrupt recalcitrant lignocellulose structures.

KEYWORDS: Biomass; biorefinery; cellulose solvent; cellulosic waste; enzymatic cellulose hydrolysis; lignocellulose fractionation

INTRODUCTION

Production of second-generation biofuels and biobased products from low-cost lignocellulosic biomass would promote rural economy, decrease greenhouse gas emissions, and enhance national energy securities (*1*−*4*). Biological conversion of biomass usually involves several sequential steps: lignocellulose pretreatment/fractionation, enzymatic cellulose hydrolysis, and fermentation. The largest technological and economic challenge for biomass biorefineries is cost-effective release of fermentable soluble sugars from lignocellulosic biomass (*5*−*7*). When costs of sugars isolated from lignocellulosic biomass are competitive with those made from corn kernels or sugar cane, sustainable biomass refineries will come true (*7, 8*). Currently, cellulosic ethanol production on a small scale is still too costly because of the relatively premature technologies for large-scale commercialization, high processing costs, and huge capital investment ($/gallon capacity) (*6, 8, 9*).

Hemp is one of the most environmentally friendly crops, because it requires little or no pesticides or fertilizers, replenishes soil with nutrients, and eliminates competing weeds. Industrial hemp, a number of varieties of *Cannabis sativa* L. species, is cultivated for agricultural and industrial purposes (*10, 11*). Industrial hemp contains low concentrations of tetrahydrocannabinols (THC), a key chemical in marijuana (*11*). Hemps containing less than 1% THC and 0.3% THC are legally cultivated in Canada and Europe, respectively. European farmers have grown industrial hemp for more than 20 years without any problem related to marijuana (*11*). The main product of industrial hemp is high-quality fibers around the hollow core of the hemp stalk. Hemp fiber excels in fiber length, strength, durability, and absorbency, as well as anti-mildew and antimicrobial properties, as compared to other bast fibers (flax, kenaf, jute, and ramie) (*12*). In addition to high-quality fibers, hemp seed oil contains a very high content of polyunsaturated essential fatty acids, including γ linoleic acid (GLA), an

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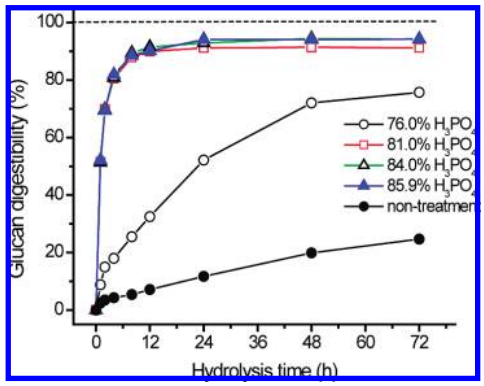

Figure 1. Effect of the phosphoric acid concentration on enzymatic glucan digestibility. Pretreatment reaction conditions: different phosphoric acid concentrations at 50 °C for 1 h. Enzymatic hydrolysis condition: 10 g/L glucan, 15 FPU cellulase, and 60 IU β-glucosidase per gram of glucan in 50 mM citrate buffer (pH 4.8) at 50 °C.

omega-6 essential fatty acid (*13*). Hurds, core fiber, are a residue after removal of high-quality fiber from the sturdy hemp stalks. They have some minor applications, such as animal bedding, garden mulch, or as a component in lightweight concrete or plaster. Production of biofuels from the hurds of industrial hemp will be valuable because it not only would provide efficient removal of solid cellulosic wastes and generate more revenue but would also secure ultra-low-cost feedstock. Most times feedstock costs may account for a great fraction of biocommodity prices (e.g., ~30−50%) (*14*).

Recently, a cellulose solvent-based lignocellulose fractionation (CSLF) has been developed for separating lignocellulose under modest reaction conditions (e.g., ~50 °C and atmospheric pressure) by using a cellulose solvent, an organic solvent, and water (*15*). The key ideas of CSLF are (1) decrystallization of cellulose fibers (i.e., more cellulose accessibility, so that cellulase can work on the substrate more efficiently) (*16, 17*), (2) removal of partial lignin and hemicelluloses from cellulose (i.e., fewer substrate obstacles to enzyme, so that cellulase can access the substrate more efficiently) (*6, 8, 18, 19*), and (3) modest reaction conditions (i.e., a decrease in sugar degradation, less inhibitor formation, lower utility consumption, and less capital investment) (*20*). Our previous results suggested that CSLF with some modifications (e.g., acid concentration and reaction time) in pretreatment conditions efficiently broke the recalcitrance of several feedstocks: corn stover, switchgrass, and poplar (*15*).

In this paper, we investigated the effects of the pretreatment conditions (acid concentration, reaction time, and temperature) for industrial hemp hurds on enzymatic cellulose digestibility. The best pretreatment condition for hemp hurds were identified on the basis of a maximum sugar release from biomass: a combined result of a maximal retention of solid cellulose after the pretreatment and maximal enzymatic cellulose hydrolysis. Also, the scanning electron microscopic images for the intact and pretreated biomass were presented for the first time, suggesting that there was no fibril structure for the CSLF well-pretreated biomass, i.e., complete destruction of biomass recalcitrant structures. The overall sugar and lignin balance during the pretreatment process and enzymatic hydrolysis was presented for the first time.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals were reagent-grade and purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA), unless otherwise noted. *Trichoderma* cellulase (Spezyme CP) was a gift from Genencor International (Palo Alto, CA).

Industrial hemp stalks, provided by the Equator Group (Los Angeles, CA), were grown in Canada. The hurds were obtained after manual removal of the fiber of the hemp stems. The dry hurds were knife-milled (laboratory model 4, Arthur H. Thomas Company, Philadelphia, PA) and the lignocellulose particles (>60 mesh screen and <40 mesh screen) were used for all experiments. The hurds were dried in a convection oven at 105 ± 3 °C for 4 h or longer until a constant weight was achieved. The samples were then removed from the oven and allowed to cool to room temperature in a desiccator. The biomass samples before pretreatment were prepared in the same way as the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) (*21, 22*).

Carbohydrate and Lignin Assays. The structural carbohydrate composition of industrial hemp hurds was determined on the basis of a modified quantitative saccharification (QS) (*23*). In the modified QS, the secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid, rather than 4% sulfuric acid, at 121° for 1 h for more accurate determination of acid-labile carbohydrates (e.g., xylan and arabinan) (*23*). Monomeric sugars were measured by a Shimadzu HPLC (Kyoto, Japan) with a Bio-Rad Aminex HPX-87P column (Richmond, CA) at 80 °C with a mobile phase at a rate of 0.6 mL of distilled water per minute (*15*). Lignin and ashes were measured according to the standard National Renewable Energy Laboratory (NREL) biomass protocol (*24*). The carbohydrate and lignin composition of hurds were 42.37% glucan, 19.20% xylan, 2.63% galactan, 4.74% arabinan, 1.44% mannan, and 17.5% acid-insoluble lignin, respectively. The concentrations of glucose and xylose in the enzymatic hydrolysate were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H chromatography column using 0.1% (v/v) sulfuric acid as a mobile phase at a flow rate of 0.6 mL/min and a column temperature of 65 °C (*15, 25−27*).

Lignocellulose Fractionation. One gram of dry industrial hemp sample was put into a 50 mL disposable plastic centrifuge tube. The samples were mixed with 8 mL of various concentrations of phosphoric acids by a glass rod for 30, 60, or 120 min. After incubation at various temperatures (40, 50, and 60 °C) in a water bath, approximately 30 mL of acetone was added to each tube to precipitate the lignocellulose slurry. Each sample was inverted vigorously or vortexed for even mixing. The mixtures were centrifuged in a swing bucket centrifuge at 3600 rpm at room temperature for 20 min. The pellets were resuspended by adding ~40 mL of acetone and centrifuged twice. The combined acetone-washing supernatant called "black liquor" contained mostly phosphoric acid, acetone, and dissolved lignin. After removal of acetone, the precipitated high-quality lignin was centrifuged, washed, dried, and weighed. After acetone washing, the pellets containing amorphous cellulose, hemicellulose, and some lignin were suspended in ~40 mL of distilled water and centrifuged at 3600 rpm for 20 min. After the supernatant was decanted, the pellets were resuspended in water and centrifuged 4 times. The combined water-washing supernatant called "white liquor" contained water-soluble hemicellulose and acetone. After acetone removal followed by posthydrolysis [1% (w/v) sulfuric acid, 121 °C, and 1 h] (*23*), the monomeric hemicellulose sugar concentrations were determined by high-performance liquid chromatography (HPLC). The residual (amorphous) cellulosic material after treatment of the cellulose solvent and the organic solvent, having a nearly neutral pH, was used for the sequential enzymatic hydrolysis.

Enzymatic Cellulose Hydrolysis. All enzymatic hydrolysis experiments were carried out in a rotary shaker at 50 ± 0.2 °C. Enzyme loadings were 15 filter paper units (FPU) of Genencor Spezyme CP cellulase and 60 international units (IU) of Novozymes 188 β-glucosidase per gram of glucan. Because the fractionation process removed some fraction of glucan from initial lignocellulosic biomass, another replicate of lignocellulosic biomass was freeze-dried and its remaining carbohydrate composition was measured by the modified QS (*23*). On the basis of the carbohydrate composition of the parallel pretreated samples, the pretreated biomass was diluted by 1 M citrate buffer (pH 4.5) and water to the initial glucan concentration of 1.0% (w/v) in a 50 mM citrate buffer (pH 4.8) plus 0.25% sodium azide. Eight hundred microliters of well-mixed hydrolysate samples were taken at various times and immediately centrifuged at 13 000 rpm for 5 min. After centrifugation, exactly 500 μL of the supernatant was transferred to a

new microcentrifuge tube and kept at room temperature for 1−2 h for complete conversion of cellobiose to glucose. A total of 500 μL of the supernatant was acidified by adding 30 μL of 10% (w/w) sulfuric acid and then was frozen overnight. The thawed liquid samples were mixed well and centrifuged at 13 000 rpm for 3 min to remove any solid precipitates. Glucose and xylose concentrations in the clear supernatants were measured by HPLC. After 72 h of hydrolysis, the remaining hydrolysate was transferred to a 50 mL centrifuge tube and centrifuged at 3600 rpm for 10 min. After removal of the supernatant, 20 mL of deionized water was added to resuspend the pellet. After centrifugation, the freeze-dried pellet was analyzed for residual glucan and xylan by QS.

Glucan digestibility (X_G) at the end of hydrolysis (hour 72) was calculated using the ratio of soluble glucose (G_{sol}) in the supernatant to the sum of G_{Sol} and the residual glucan−glucose equivalent (G_{res}) in the solid phase (eq 1). This calculation approach can obtain more reliable and accurate digestibility values than eq 2 (*28*).

$$X_G = \frac{G_{Sol}}{G_{sol} + G_{res}} \times 100\% \quad (1)$$

Glucan digestibility (X_G) during hydrolysis was calculated using the ratio of soluble glucose (G_{Sol}) to initial glucan content of the cellulosic pellets (G_{ini})

$$X_G = \frac{G_{Sol}}{G_{ini}} \times 100\% \quad (2)$$

Final xylan digestibility (X_X) during the enzymatic hydrolysis was calculated as below

$$X_X = \left(1 - \frac{X_{res}}{X_{ini}}\right) \times 100\% \quad (3)$$

where X_{ini} is the initial xylan content measured by QS and X_{res} is the residual xylan content of cellulosic samples after enzymatic hydrolysis. Because the hydrolysate volume decreased because of sampling, the calculation of soluble glucose and residual glucan had a volume adjustment.

Scanning electron microscopy (SEM) for the biomass materials was conducted by the Virginia Bioinformatics Institute. All samples were sputter-coated with gold and imaged by SEM, as described elsewhere (*17, 29−31*).

RESULTS

Overall sugar yield is among the most important factors for evaluating the overall performance of biomass saccharification because the economy of biorefineries relies on the tradeoff of several factors, such as product revenues (sugar yields, mainly), processing costs, capital investments, and so on. Lignocellulose pretreatment conditions impact substrate characteristics, resulting in different enzymatic hydrolysis performances (i.e., sugar yields and hydrolysis rates) at different enzyme loadings (*2, 8, 21, 22*). Three pretreatment conditions of CSLF (cellulose solvent concentration, reaction time, and temperatures) were investigated for a maximal sugar yield by using the recommended enzymatic hydrolysis conditions of CAFI (1% glucan, 15 FPU/g of glucan, 50 °C, and pH 4.8) (*15, 21, 22*).

Figure 1 shows the effects of phosphoric acid concentrations (76, 81, 84, and 85.9%) on glucan digestibility for pretreated lignocellulose at the enzyme loading of 15 FPU/g of glucan. When phosphoric acid concentrations exceeded 81%, the pretreated samples had very rapid hydrolysis rates and high digestibility. No significant difference was observed for 84 and 85.9% phosphoric acid treatment. The well-pretreated biomass was hydrolyzed by 93.3% at hour 12 and by 95.9% at hour 24, and then it leveled off. Glucan digestibility of the 76% H_3PO_4-treated biomass rose to 32.5% at hour 12, to 52.2% at hour 24, and to 75.7% at hour 72. When the phosphoric acid concentration (e.g., 76%) was below the critical point (about ∼83% for hemp hurds), the hydrolysis digestibility was low because a low-concentration phosphoric acid is a cellulose-swelling solvent but not a cellulose solvent (*17, 32*). The non-pretreated hurds show a very low digestibility (24%) at hour 72.

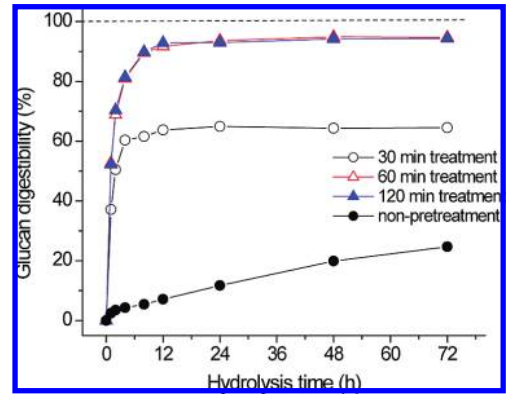

Figure 2. Effect of the pretreatment reaction time on enzymatic glucan digestibility. Pretreatment reaction conditions: 84.0% H_3PO_4 at 50 °C for 30, 60, and 120 min. The enzymatic hydrolysis condition was the same as that in **Figure 1**.

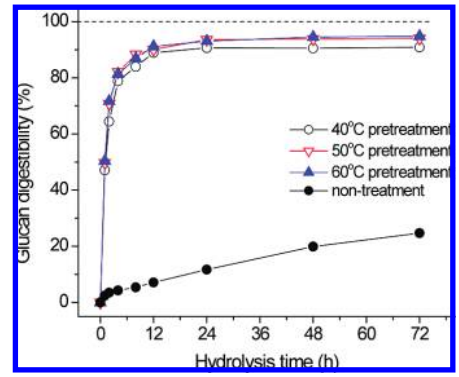

Figure 3. Effect of the pretreatment temperature on enzymatic glucan digestibility. Pretreatment reaction conditions: 84.0% H_3PO_4 at 40, 50, and 60 °C for 60 min. The enzymatic hydrolysis condition was the same as that in **Figure 1**.

The effects of different reaction times from 30 to 120 min on the glucan digestibility of the 84.0% H_3PO_4-treated materials were tested (**Figure 2**). There was no difference in glucan digestibility (∼96%) between the samples treated for 60 and 120 min, but a longer reaction time both led to lower volumetric productivity for biomass pretreatment and resulted in significant hydrolysis of cellulose and hemicellulose, accompanied by lower solid sugar retention efficiency. When the pretreatment time was as short as 30 min, the glucan digestibility was much lower, only 64%. This result suggests inefficient biomass dissolution by concentrated phosphoric acid within a short time.

We further investigated treatment temperatures on glucan digestibility by using 84.0% H_3PO_4. High reaction temperatures dissolve cellulose fibers quickly but can result in some sugar degradation and require more utility consumption, while low reaction temperatures can minimize sugar degradation and save utility costs. There was some improvement in enzymatic glucan digestibility when temperatures increased from 40 to 50 °C but no further improvement at higher temperature (60 °C). On the other hand, higher pretreatment temperatures (>50 °C) lead to weak hemicellulose degradation (data not shown). Therefore, the best treatment condition for industrial hemp hurds was 84.0% H_3PO_4, 50 °C, and 60 min. At this condition, there was no detectable degradation of hemicellulose and cellulose (<0.01%).

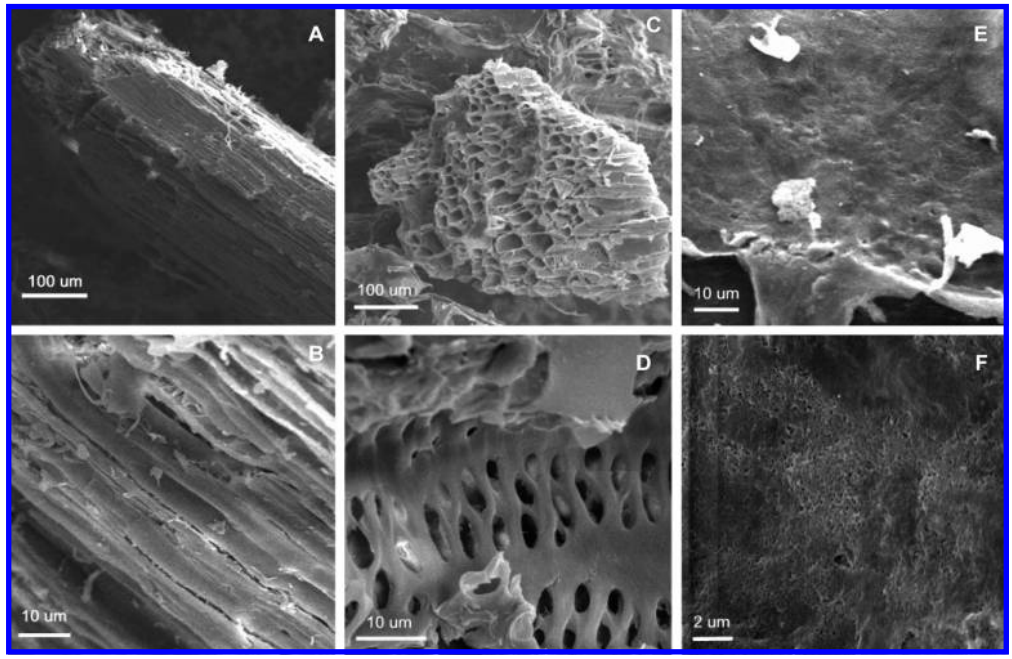

Figure 4. Scanning electron microscopic images of industrial hemp hurds at two magnitudes. A and B, the intact sample; C and D, the modestly pretreated sample at 84.0% H_3PO_4 at 50 °C for 30 min; and E and F, the well-pretreated sample at 84.0% H_3PO_4 at 50 °C for 60 min.

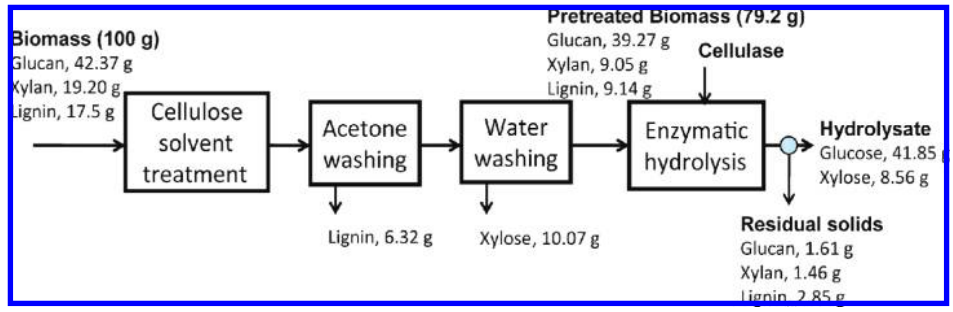

Figure 5. Mass balance of industrial hemp hurds after lignocellulose fractionation pretreatment and enzymatic hydrolysis. The pretreatment reaction condition was 84.0% H_3PO_4 at 50 °C for 1 h. The enzymatic hydrolysis condition was the same as that in **Figure 1**.

The degradation degrees of hemicellulose and cellulose were approximately estimated on the basis of the xylose and glucose degradation at the same experimental conditions, because degradation of polysaccharides involves hydrolysis of polysaccharide followed by degradation of monosaccharide (*23, 33*). The degradation of monosugars in the presence of acids have been measured elsewhere (*23, 34*). (**Figure 3**).

Cellulose solvents, including concentrated H_3PO_4, can completely dissolve cellulose fibers, disrupt orderly hydrogen bonds in crystalline cellulose (*17, 35, 36*), and increase cellulose accessibility to cellulase (*37, 38*). The intact biomass presents its plant cell vascular bundles and its fibril structure under SEM (parts A and B of **Figure 4**). Modestly pretreated conditions (e.g., 84.0% H_3PO_4 at 50 °C for 30 min) open larger holes on the surface of plant cell walls by removing the easily digested lignocellulose fraction (e.g., hemicellulose), but the supramolecular fibril structure remains with partial destruction (parts C and D of **Figure 4**). A well-treated lignocellulose sample (84.0% H_3PO_4, 50 °C, and 60 min) shows all fibrous structures of the lignocellulose completely disrupted (Parts E and F of **Figure 4**). The modestly pretreated biomass had a lower sugar yield and a slower hydrolysis rate than the well-pretreated samples (**Figure 2**), in agreement with the great differences in supramolecular structures (**Figure 4**).

Figure 5 presents the mass balance of industrial hemp hurds at the best lignocellulose pretreatment process (84.0% H_3PO_4 at 50 °C for 1 h) and enzymatic cellulose hydrolysis based on 100 g of dry hurds. The precipitated amorphous cellulose and hemicellulose were washed by acetone, removing 6.32 g of (acetone-soluble) lignin, followed by water washing, removing 10.07 g of water-soluble hemicellulose oligosaccharides. The remaining amorphous cellulose was hydrolyzed by 15 FPU of Spenzyme CP. The overall glucose yield was calculated to be 89.0% (95.9% glucan digestibility × 92.7% glucan remaining after the fractionation). The overall xylan recovery yield was 85.4%, the sum of 10.07 g of washed xylose equivalent in water plus 8.56 g of xylose equivalent in the enzymatic hydrolysate, where enzymatic xylan digestibility was 83.9%.

DISCUSSION

Developing a feedstock-independent lignocellulose pretreatment/fractionation is highly desired for profitable biorefineries because it uses diverse plant biomass feedstocks with relatively low-energy densities (GJ/kg feedstock). The strict pretreatment reaction condition required for a special lignocellulosic feedstock could result in higher transportation costs for the special biomass from much larger collection areas. Concentrated acid saccharification and mechanical milling are two main feedstock-independent treatment technologies (*20, 39, 40*). However, concentrated acid saccharification suffers from three technical barriers: solid acid/soluble sugar separation, acid recovery, and

acid reconcentration (*15, 41*); the milling technology requires extremely high energy input, so that it cannot be applied to any practical operation (*20*).

CSLF has been demonstrated to treat herbaceous feedstock (corn stover and switchgrass) and hardwood (poplar) efficiently but not softwood (Douglas fir) (*15*). Here, we present its applicability to a new type of wood-like lignocellulosic biomass, industrial hemp hurds. This cellulose-solvent-based lignocellulose fractionation may be regarded as a new, nearly generic lignocellulose pretreatment method, because cellulose solvents (e.g., concentrated phosphoric acid, ionic liquid, and concentrated sulfuric acid) can disrupt the linkage among cellulose, hemicellulose, and lignin and further break the orderly hydrogen bonds of crystalline cellulose regardless of plant biomass types. Plant biomass pretreated by only cellulose solvents could still have relatively poor enzymatic digestibility (*42, 43*). The introduction of an organic solvent between the cellulose solvent and water brings some advantages, such as more removal of lignin, more efficient recycling of cellulose solvent, and separation of partially soluble hemicelluloses from solid cellulose (*8, 15*). Consequently, the highest enzymatic hydrolysis rates and very high sugar yields were obtained here and reported previously (*15*).

Concentrated phosphoric acid is a potential cellulose solvent applicable to biorefineries because of its low cost as compared to ionic liquids, tolerance to water presence, low-temperature cellulose dissolution capacity, nonvolatility, chemical stability, and easy recycling (*8*). Phosphoric acid can swell or dissolve cellulose, depending upon its concentration and biomass type (*17*). When the phosphoric acid concentration is lower than the critical concentration, it neither efficiently dissolves crystalline cellulose fibers nor greatly promotes cellulose digestibility and hydrolysis rate (*17, 44*). The biomass pretreated by 76% phosphoric acid had relatively low digestibility (**Figure 1**). The changes in supramolecular structure of lignocellulose before and after CSLF were drastic (**Figure 4**). The supramolecular structures of the intact plant cell walls were similar to the results presented elsewhere (*29, 31*). All fibrous structures of the well-pretreated lignocellulose were completely destructed (parts E and F of **Figure 4**), completely different from those pretreated by hot water (190 °C for 15 min) (*29*), diluted acid (*30*), or ammonia recycle percolation (170 °C, 2.3 MPa, 15 wt % ammonia, and 5 mL/min flow rate) (*31*). Decrystallized (amorphous) cellulosic materials have much higher cellulose accessibility to cellulase (~20-fold) than that of the non-pretreated samples (*37, 38*), resulting in much higher enzymatic hydrolysis rates and the highest cellulose digestibility (*15*−*17*).

Studies of pretreatment conditions clearly suggest that the acid concentration is the most important factor. The phosphoric acid concentration should be higher than a critical concentration to dissolve lignocellulose; reaction time must be as long as 1 h for hemp hurds, but a too long reaction time results in low overall sugar yields; and reaction temperature is the least important factor for pretreatment efficiency.

CSLF based on cellulose solvent and organic solvent is a new lignocellulose pretreatment technology. Although it shows some great promise for high overall sugar yields, short hydrolysis time, modest reaction conditions, and separation of lignocellulose components for co-use (*8, 15*), it has several shortcomings, such as large volume solvent recycling and possible high capital investment for a chemical recycling system. Therefore, it will be vital to further reduce solvent use and conduct an economic analysis based on the Aspen-plus process model.

In summary, biofuels and biobased product production based on cellulose-rich wastes from existing manufactures, such as hurds from industrial hemp fiber producers, corn fiber from corn ethanol biorefineries, wheat hull from flour processing facilities, and sawdust from wood processors, would not only solve solid waste disposal problems but would also produce value-added products, such as biofuels and biobased products. Much smaller biorefineries by using cellulosic waste from on-site manufacturers could be profitable because of the large saving in feedstock costs (~$30−90/ton of biomass, i.e., $0.35−1.00 per gallon of cellulosic ethanol) than those based on dedicated bioenergy plants and collected agricultural residues. The applicability of this nearly feedstock-independent CSLF technology to address local biomass residues from local manufacturers could provide great opportunities to build profitable small-size biorefineries (i.e., 100 tons of biomass per day) that can yearly produce 2.8 million gallons of cellulosic ethanol and ~1000 tons of acetic acid as a value-added co-product.

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