High Ethanol Concentration (77 g/L) of Industrial Hemp Biomass Achieved Through Optimizing the Relationship between Ethanol Yield/Concentration and Solid Loading

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ABSTRACT: In this study, the relationships between ethanol yield/concentration and solid loading (6−21%) were investigated to enhance ethanol titer and avoid a random choice of solid loading for simultaneous saccharification and fermentation (SSF). Alkali-pretreated hemp biomass was used for SSF in four scenarios including Case I: 30 filter paper unit (FPU)-cellulase and 140 fungal xylanase unit (FXU)-hemicellulase/g-solid; Case II: 40 FPU-cellulase and 140 FXU-hemicellulase/g-solid; Case III: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid with 1% Tween80; and Case IV: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid with particle size reduction (<0.2 mm). Results showed that bioethanol yield and concentration had a negative linear ($R^2 = 0.76−0.93$) and quadratic ($R^2 = 0.96−0.99$) correlation with solid loading (6−21%), respectively. As compared to Case I and previous studies, an enhancement in ethanol yield and concentration through increasing cellulase dose (Case II) and adding Tween 80 (Case III) was overestimated, whereas particle size reduction (Case IV) extended the “solid effect”, evidenced by the highest ethanol concentration (77 g/L) achieved from SSF at the focus point of a quadratic model. An interpretation of the relationship between ethanol yield/concentration and solid loading not only avoids a blind selection of solid loading for SSF but also reduces extra enzymes and water consumption.

INTRODUCTION

The inevitable depletion of fossil-based gasoline necessitates the exploration of sustainable and renewable biofuels. In particular, bioethanol obtained from lignocellulosic biomass has gained considerable attention as an alternative transportation biofuel. However, the efficient utilization of lignocellulosic biomass into bioethanol still confronts several technical barriers: robust recalcitrance limits enzymatic and microbial attack and lowers ethanol concentration which resulted in an increased cost for end product separation and purification. Moreover, raw biomass is a determinant factor in the final ethanol concentration. In comparison to the common biomasses (corn fiber, corn stover, and sorghum bagasse), industrial hemp biomass with high cellulose and less lignin contents is an excellent feedstock for bioethanol production.

Like other lignocellulosic biomass, thermochemical pretreatment is also an essential step prior to enzymatic hydrolysis and fermentation of hemp biomass. Steam explosion and dilute sulfuric acid pretreatments are commonly utilized to decompose and solubilize hemicellulose of hemp biomass. However, harsh pretreatment conditions often result in cellulose degradation and inhibitor formation. Besides, derivatives from the degradation of hemicellulose and cellulose have been reported to interact with the disrupted lignin units to form a pseudo-lignin complex, which can inhibit enzymatic absorption of cellulose significantly. Also, dilute alkali pretreatment can neutralize the released acids (such as acetic acid) and solubilize the disrupted lignin matrix. In our previous studies, dilute NaOH pretreatment achieved high lignin removal and sugar and ethanol yields.

Solid loading used for enzymatic saccharification and fermentation is a critical element that is associated with enzymatic doses and sugar and ethanol yields. Low solid loading could shorten enzymatic hydrolysis and fermentation duration and achieve high ethanol yield but result in undesirable ethanol titer, which was unable to meet the minimal (about 40 g/L) requirement for commercial ethanol distillation. Namely, at least 80 g/L of glucose should be
Theoretical available in hydrolysates for fermentation. Given 60% of potential glucose (54% glucan) in the pretreated solid biomass and 90% of glucan conversion efficiency, the lowest solid loading needed for fermentation should be higher than 15%. Therefore, maintaining high solid loading throughout the conversion process is preferred from cost-efficient and environmental standpoints.\textsuperscript{14}

Increasing solid loading within certain limits can generally improve the fermentable sugar and ethanol concentration, but conversion efficiencies from pretreated biomass to sugar and ethanol decrease inevitably as solid loading increases.\textsuperscript{6,15,16} For an orbital shaker or a conventional stirred tank, high solid loading also causes insufficient mixing because of the high viscosity of slurries.\textsuperscript{17} Several strategies, including Tween 80 addition,\textsuperscript{18} specific bioreactor,\textsuperscript{19} size reduction,\textsuperscript{20} and fed-batch fermentation,\textsuperscript{21} have been investigated to block the negative interaction between lignin with cellulase and enhance enzymatic saccharification and microbial digestion at high solid loading. However, a general phenomenon was observed that a random selection of high solid loading for SSF was performed to boost ethanol concentration without statistical reasons.\textsuperscript{6,15,22,23} Therefore, avoiding the selection of the solid loading for enzymatic hydrolysis and fermentation groundlessly is required.

Given that ethanol yield had a negative linear correlation with solid loading used for simultaneous saccharification and fermentation (SSF) (eq 1), corresponded ethanol concentration would form a quadratic equation (parabola, eq 2) with solid loading. Therefore, the decrease in ethanol yield can offset the advantages of conversion efficiency at high solid concentration. For example, given the focus point at 25% solid loading using the quadratic model, ethanol concentration obtained from 20% solid loading with 90% ethanol yield would be the same as 30% solid loading with 60% ethanol yield. Thus, an extra 10% solid loading coupled with enzymes and water addition was wasted. Furthermore, the vertex (highest ethanol concentration) of this simulated parabola can be obtained at the focus point (axis of symmetry, \(-b_2/2k_x\)). Based on the previous analysis, exploring the focus point (corresponded to solid loading) of ethanol fermentation might lower extra enzymes, solid biomass, and water input. It would also avoid a random choice of high solid loading for SSF.

\[ Y_E = k_1x + b_1 \]  
\[ C_E = k_2x^2 + b_2x + c \]

where \(Y_E\) and \(C_E\) are the ethanol yield and concentration, respectively; \(k_1\) and \(b_2\) are the linear coefficients; \(b_1\) and \(c\) are the constant coefficients; and \(k_2\) is an indeterminate variable.

To our knowledge, there are no studies conducted using industrial hemp biomass with high solid loading (more than 10%) to boost bioethanol concentration.\textsuperscript{5} In this study, industrial hemp (Tygra variety) biomass pretreated with dilute NaOH was used for SSF. The relationship between ethanol yield/concentration and solid loading (6–21%) in four scenarios, including Case I: 30 filter paper unit (FPU)-cellulase and 140 fungal xylanase unit (FXU)-hemicellulase/g-solid; Case II: 40 FPU-cellulase and 140 FXU-hemicellulase/g-solid; Case III: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid with 1% Tween 80; and Case IV: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid with particle size reduction (<0.2 mm), was evaluated and simulated. Then, the focus points (corresponded to solid loading) obtained from the simulated quadratic equations were used for SSF to achieve high ethanol concentration, thus avoiding a random choice of high solid loading for SSF.

## RESULTS AND DISCUSSION

### Physicochemical Properties of Hemp Biomass

Chemical compositions of raw and alkali-pretreated hemp biomass are listed in Table 1. Raw hemp biomass contains 40.66% glucan, 13.25% xylan, 15.74% lignin, and 14.40% extractives. After alkali pretreatment, glucan content increased to 77.54%, whereas xylan and lignin contents decimated to 8.72 and 11.05%, respectively (Table 1). During pretreatment, the efficient solubilization and elimination of hemicellulose, lignin, and extractives resulted in the increase in glucan content in pretreated biomass.\textsuperscript{5} High glucan in pretreated hemp biomass would be beneficial for subsequent ethanol concentration.\textsuperscript{5} These results also indicate that alkali pretreatment functioned to cleave chemical bonds (ester and ether bonds) between

### Table 1. Compositional Analysis of Raw and Pretreated Biomass\textsuperscript{a,d}

| sample               | glucan (%) | xylan (%) | lignin (%) | extractive (%) | CrI (%) |
|----------------------|------------|-----------|------------|----------------|---------|
| raw                  | 40.66 ± 0.09a | 13.25 ± 0.01b | 15.74 ± 0.05b | 14.40 ± 0.95 | 53.6 ± 2.5a |
| NaOH-pretreated\textsuperscript{b} | 77.54 ± 0.23b | 8.72 ± 0.01a | 11.05 ± 0.07a | ND\textsuperscript{d} | 76.7 ± 1.6b |

\textsuperscript{a}Data are exhibited as means ± standard deviations. Biomass composition is on a dry basis. \textsuperscript{b}NaOH (1%, w/v) pretreatment condition was 10% solid loading at 170 °C for 30 min (48 reactors). \textsuperscript{c}CrI: crystallinity index. ND: nondetected. \textsuperscript{d}In each column, means with different letters are significantly different at \(p < 0.05\).
lignin and hemicellulose, thus reducing the recalcitrance of lignocellulosic biomass for scaling cellulose.\textsuperscript{10}

No notable variation in distribution ranges (1–1000 μm) of the particle size was exhibited among raw and alkali-pretreated hemp biomass (Figure 1a). However, it was noticed that alkali-pretreated biomass showed higher median and mean particle size as compared with raw biomass, which would be due to the solubilization of the disrupted lignin matrix and hemicellulose and swelling up of cellulose. Moreover, the maximum peak position between 2θ = 22° and 2θ = 23° (distance between hydrogen-bonded sheets in cellulose crystalline\textsuperscript{12}) for alkali-pretreated hemp biomass increased significantly compared with raw biomass (Figure 1b), accompanied by an increase in CrI (76.7 vs 53.6%) (Table 1). In other studies, alkali-pretreated biomass also presented higher CrI than raw biomass.\textsuperscript{25,26}

**Morphological Changes in Hemp Biomass.** Tracks left by alkali pretreatment were identified by Fourier transform infrared (FTIR) (Figure 2) and scanning electron microscopy (SEM) (Figure 3). In comparison with raw biomass, noticeable disappearance at 1730–1740 cm\textsuperscript{-1} (\textit{C}=\textit{O} stretching of an acetyl group from hemicellulose and aldehyde from lignin) in alkali-pretreated biomass was observed. The intensities of peaks at 1570–1590 cm\textsuperscript{-1} (\textit{C}=\textit{C} stretching due to aromatic skeletal vibration\textsuperscript{10}) weakened after alkali pretreatment. These changes were ascribed to the decreased xylan and lignin contents in alkali-pretreated hemp biomass (Table 1). The intensities of peaks at 1160–1170 cm\textsuperscript{-1} (asymmetric \textit{C}=\textit{O}–\textit{C} stretching from cellulose) and 1110–1120 cm\textsuperscript{-1} (\textit{C}–\textit{OH}

**Figure 2.** FTIR of raw and NaOH-pretreated hemp biomass.

| Wavenumbers (cm\textsuperscript{-1}) | 4000 | 3600 | 3200 | 2800 | 2400 | 2000 | 1600 | 1200 | 800 |
|-------------------------------------|------|------|------|------|------|------|------|------|-----|
| Transmittance (%)                  | 100  | 96   | 92   | 88   | 84   | 80   | 76   | 72   | 68  |

Figure 3. SEM of raw (a) and NaOH-pretreated (b) hemp biomass.
under low solid loading to elucidate and promote the function of pretreatment, whereas the low ethanol concentrations were neglected. Rapid reduction in sugar-to-ethanol conversion rates with the increase in solid loading (6–12% solids: 95.1–88.4; 15–21% solids: 86.9–67.6%) might be related to (1) the accumulated inhibitors (cellobiose and ethanol) limiting enzymatic and microbial activities; (2) lower water available slowing down mass transfer; and (3) hydrophobic interaction between residual lignin and cellulase reducing enzymatic absorption. Similarly, Lu et al. conducted enzymatic hydrolysis of steam-exploded corn stover with the solid loading ranging from 10 to 30% and found that sugar yields decreased from 75.6 to 72.1% but glucose concentration increased from 35.9 to 103.3 g/L. In addition, Cara et al. performed enzymatic hydrolysis of liquid hot water-pretreated olive tree biomass at solid loadings of 2–30% and observed that sugar yields reduced from 76.2 to 49.9%.

**Effect of Cellulase Loading on Ethanol Concentration and Yield.** Cellulase cost is the main bottleneck to restrain the industrial application of lignocellulosic biomass to ethanol. However, because of the "solid effect", more enzymes are generally needed to maintain the desired yield. In order to examine the effect of cellulase on ethanol concentration and yield, SSF of alkali-pretreated hemp biomass was carried out with increased cellulase loading from 30 to 40 FPU, as shown in Case II (40 FPU-cellulase and 140 FXU-hemicellulase/g-

Figure 4. Time courses of ethanol concentration of alkali-pretreated hemp biomass during SSF with solid loading from 6 to 21% under four scenarios [(a) Case I; (b) Case II; (c) Case III; and (d) Case IV].

Figure 5. Time courses of ethanol yield of alkali-pretreated hemp biomass during SSF with solid loading from 6 to 21% under four scenarios [(a) Case I; (b) Case II; (c) Case III; and (d) Case IV].
solid) with solid loadings of 6–21%. The time courses of ethanol concentration and yield during SSF are exhibited in Figures 4b and 5b.

For solid loading from 6 to 18%, the ethanol concentrations increased from 26.2 to 68.1 g/L during the fermentation period of 24–96 h; however, for extremely high solid loading (21%), the ethanol concentration decreased to 62.3 g/L (Figure 4b). The reduction in ethanol concentration (96 h vs 72 h) could be attributed to the volatilization of ethanol and sampling inhomogeneity as aforementioned. Similarly, Varga et al.32 reported that ethanol yield was markedly reduced when solid loading increased to 20% compared to those from 10 to 15% solid loading because of insufficient mass transfer caused by stirring hindrance. The decrease in ethanol yield (99.2–93.5%) with the solid loading increasing from 6 to 12% was significantly lower than that (90.2–67.4%) with the solid loading increased from 15 to 21% (Figure 5b), indicating that mass transfer efficiency and enzyme activities were notably affected by the “solid effect” as solid loading was greater than 12%. In comparison with Case I, ethanol concentration and yield after 96 h fermentation in Case II (increasing cellulose dose) increased 1.09–2.73 g/L and 2.72–5.17% as solid loading increased from 6 to 18%, respectively. However, no significant variation (p < 0.05) was observed when solid loading was up to 21% (Figure 5b), suggesting that other technical barriers such as insufficient mixing and inhibitory compounds from “solid effect” overwhelmed enzyme loading. This result also indicates that increasing enzyme loading results in enhanced sugar and ethanol yields and only applies to a certain extent of solid loading.

Effect of Tween 80 on Ethanol Concentration and Yield. Hydrophobic adsorption of cellulase onto residual lignin in SSF slurries has been reported to reduce enzymatic accessibility, thus resulting in lower sugar conversion efficiency.35,36 Tween 80 as a cost-efficient surfactant has been widely applied to enhance enzymatic hydrolysis of pretreated biomass at low solid loading through disassociating wall polymers and increasing enzymatic activities.18,35 Thus, the impact of Tween 80 addition on the SSF under Case III condition (30 FPU-cellulase and 140 FXU-hemicellulase/g-solid assisted with 1% Tween 80) was investigated. The ethanol concentration and yield are shown in Figures 4c and 5c.

As solid loading increased from 6 to 18%, ethanol concentration increased from 26.0 to 69.6 g/L, while it decreased to 61.8 g/L as solid loading increased up to 21% at 96 h compared with 18% solid loading (Figure 4c). Correspondingly, ethanol yields decreased from 98.3 to 66.8% after 96 h SSF, with solid loading increasing from 6 to 21% (Figure 5c). In addition, it was noticed that hydrolysates at a solid loading of 21% within 72 h fermentation were unavailable (Figure 4c). It was accredited to fibrous cellulose containing considerable negatively charged groups that attracted water molecules locally, causing enzymatic inaccessibility. Moreover, as compared to Case I, Tween 80 addition increased ethanol concentrations of 0.84–3.81 g/L and yields of 3.17–9.61% at the solid loading of 6–18%. However, no significant improvement was observed at 21% solid loading (p < 0.05). This result indicates that the improvement of Tween 80 on sugar and ethanol yields also applies to a certain extent of solid loading.

Effect of Size Reduction on Ethanol Concentration and Yield. Several studies have reported that mechanical size reduction of thermochemically pretreated biomass can increase the specific surface area of carbohydrates to enzymes by reducing its degree of crystallinity and polymerization, thus directly influencing sugar and ethanol yields.20,36 In our work, SSF was conducted under Case IV condition [30 FPU-
cellulase and 140 FXU-hemicellulase/g-solid combined with size reduction (<0.2 mm)].

As shown in Figure 4d, after 96 h fermentation, ethanol concentrations increased significantly from 25.6 to 66.8 g/L, whereas ethanol yields decreased gradually from 96.8 to 72.3% as solid loading increased from 6 to 21% (Figure 5d). This result indicates that size reduction efficiently mitigated the “solid effect”. Compared to Case I (Figure 4a), after 96 h SSF, mechanical size reduction decreased ethanol concentrations (0.09–2.72 g/L) and yields (0.23–3.43%) at the solid loading of 9–18%, but it increased ethanol concentration (0.43 and 4.36 g/L) and yield (1.64 and 4.71%) for 6 and 21% solid loading, respectively. The former decrease would be due to an increased free lignin unit that limited enzymatic attack, and the latter increase is attributed to the sealed cellulose that was released gradually and then hydrolyzed by cellulase. Kapoor et al.20 reported that 10 mm of dilute acid pretreated rice straw exhibited higher sugar yield (93.4%) than those of S (79.5%) and 20 mm (72.8%) using 10 FPU/g-solid at 10% solid loading. However, an increasing trend in total sugar yield was observed with decreasing mean particle size from a 0.08 mm sieve screen (56 μm) followed by 2.0 mm (301 μm) and 6.0 mm (695 μm) screens.36

**Relationships between Ethanol Yield/Concentration and Solid Loading.** The responses of ethanol yield and concentration to solid loading under different SSF conditions are shown in Figure 5. Ethanol yield exhibited strong negative correlation with solid loading, and significant variations among cases were observed: \( Y_{E-I} = -1.503X + 105.4 \) for Case I with \( R^2 = 0.79 \) (Figure 6a); \( Y_{E-II} = -1.771X + 112.2 \) for Case II with \( R^2 = 0.79 \) (Figure 6b); \( Y_{E-III} = -1.839X + 113.9 \) for Case III with \( R^2 = 0.76 \) (Figure 6c); and \( Y_{E-IV} = -1.436X + 104.1 \) for Case IV with \( R^2 = 0.93 \) (Figure 6d). These results demonstrate that the decreased trend of cellulose-to-ethanol conversion with the increase in solid loading is similar for all four cases. Therefore, using sugar and ethanol yields obtained from low solid loading SSF (<10%) to promote a pretreatment method proposed could be controversial and cause misleading, especially when compared to other studies in which SSF was performed with higher solid loading.

As expected, ethanol concentration showed notable quadratic correlation with solid loading at various SSF cases: \( C_{E-I} = -12.99 + 7.025X + -0.1582X^2 \) for Case I with \( R^2 = 0.98 \) (Figure 6a); \( C_{E-II} = -16.45 + 7.971X + -0.11952X^2 \) for Case II with \( R^2 = 0.97 \) (Figure 6b); \( C_{E-III} = -17.41 + 8.235X + -0.2059X^2 \) for Case III with \( R^2 = 0.96 \) (Figure 6c); and \( C_{E-IV} = -2.819 + 5.081X + -0.08195X^2 \) for Case IV with \( R^2 = 0.99 \) (Figure 6d). Thus, the focus points (solid loading as an axis of symmetry) of 22.2% for Case I, 20.4% for Case II, 20.0% for Case III, and 31.0% for Case IV were obtained. The parabolic trend of ethanol concentration with solid loading provides biofuel technologists with an available clue to fabricate and optimize cellulose bioethanol production while avoiding a random choice of high solid loading for SSF.

**Ethanol Yield and Concentration after Verification.** To examine the accuracy of the simulated quadratic equation, we conducted the validation experiments at the focus points for all four cases, as shown in Figure 7. Marginal increases in ethanol concentrations and yields from validation were noticed as compared to those from the simulation: 1.95 g/L and 2.00% for Case I, 2.94 g/L and 3.27% for Case II, 1.15 g/L and 1.31% for Case III, and 0.98 g/L and 0.72% for Case IV (Figure 7). The maximum ethanol concentration of 76.92 g/L from validation was achieved under Case IV condition with 31.0% solid loading, which was significantly higher than that from Case I (66.95 g/L with 22.2% solid loading), Case II (67.86 g/L with 20.4% solid loading), and Case III (66.08 g/L with 20.0% solid loading). In contrast, Lan et al.37 reached 47.4 g/L ethanol concentration through cofermentation of fed-batched solid and pretreated filtrate with final solid loading up to 20%. Raj and Krishnan15 conducted low-temperature aqueous ammonia pretreatment of sugarcane bagasse and obtained 72.4 g/L ethanol concentration using a laccase-mediator system coupled with the fed-batch process at 40% solid loading. Gomes et al.38 optimized fed-batch saccharification of pilot-scale mild-acid- and alkali-pretreated sugarcane bagasse using enzymes and surfactants to achieve 62 g/L ethanol. These reports demonstrate that SSF at the focus point (solid loading) of the simulated quadratic model from alkali pretreatment assisted with size reduction of hemp biomass could be a promising method to not only achieve high bioethanol concentration without concentration and fed-batch processes but also avoid a random selection of solid loading with the saving of enzymes and water.

### CONCLUSIONS

Ethanol yield and concentration of alkali-pretreated hemp biomass showed a negative linear and quadratic correlation with solid loading, respectively. Increasing cellulose dose with a Tween 80 addition slightly improved ethanol concentration and yield as solid loading increased from 6 to 18% but showed an apparent enhancement at 21% solid loading. The maximum ethanol concentration (77 g/L) was obtained from alkali pretreatment assisted with size reduction of hemp biomass after pretreatment when SSF was conducted at the focus point from the simulated quadratic equation. This result indicates that the quadratic equation simulation applied for SSF of lignocellulosic biomass is meaningful for bioethanol production, which can avoid a random choice of solid loading for SSF.

### EXPERIMENTAL SECTION

**Materials.** Industrial hemp (Tygra) was cultivated and harvested on Kansas State University John C. Pair Horticul-
Biomass Pretreatment. The pretreatment conditions were adapted from our previous study. Five grams of biomass and 50 mL of 1% NaOH (w/v) solution were loaded into 75 mL stainless-steel reactors (Swagelok, Kansas City, KS) at a solid/liquid ratio of 1/10. In total, 48 reactors were rapidly submerged into a sand bath (Techno Inc., Princeton, NJ), which was supplemented with a continuous air blast at a target temperature of 170 °C for 30 min. Once it reached the designed time, these reactors were immediately taken into cold water (below 10 °C) to quench the further reactions (~20 min). After that, the resultant slurries were filtered into solid and liquid fractions using vacuum filtration. The resultant solid fraction was washed with distilled water until neutral pH was reached and then dried at 49 °C overnight in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) for further analysis.

Particle Size Distribution. After pretreatment, the particle size distribution of hemp biomass was determined using a laser scattering analyzer (Horiba LA-910, HORIBA, Ltd., Kyoto, Japan). In brief, samples were loaded into a distilled water cell and 40 mA. Diffraction intensities ranged from 10 to 30° (2θ) with a step rate of 0.05°/second. The crystallinity index (CrI) was calculated based on eq 3.

\[ \text{CrI} = \frac{I_{002} - I_{am}}{I_{002}} \]  

where \( I_{002} \) is the maximum peak intensity of the lattice diffraction angle (002) at 2θ ≈ 23° for cellulose crystalline I; the second peak, ranging from 2θ = 15° to 2θ = 16°, was associated with the allomorph of cellulose crystalline I. \( I_{002} \) positioned between the abovementioned two diffraction peaks at 2θ ≈ 18°, was ascribed to amorphous contributions.

FTIR Analysis. Chemical linkages of raw and pretreated industrial hemp biomass were examined using a 400 FTIR spectrophotometer (PerkinElmer Corp., Shelton, CT), which was equipped with an RT-DLaTGS detector. The determination details were followed with our previous study.

Scanning Electron Microscopy. Microstructural changes of raw and alkali-pretreated hemp biomass were identified using the S-3500 SEM (Hitachinaka, Ibaraki, Japan). Hemp samples were mounted on conductive adhesive tapes and then coated with a 4 nm-thick metal film (40% palladium and 60% gold). SEM captured microstructures of hemp biomass.

Simultaneous Saccharification and Fermentation. Traditional industrial Saccharomyces yeast (ethanol red, Lesaffre, Milwaukee, WI, USA) was used to ferment NaOH-pretreated industrial hemp biomass. First, 10 g of glucose, 2.5 g of peptone, 1.5 g of yeast extracts, 0.5 g of KH₂PO₄, and 0.25 g of MgSO₄·7H₂O were mixed with 500 mL of distilled water to form culture broth. Then, 19 mL of culture broth was autoclaved for sterilization at 121 °C for 20 min. After it cooled to room temperature, 1.0 g of dry yeast was poured into the broth and incubated at 38 °C at 160 rpm for 25 min. The activated yeast culture had a cell concentration of about 10⁶ cells/mL. The buffer containing the designed amounts of cellulase, hemicellulase, and Tween 80 was prepared, including Case I: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid; Case II: 40 FPU-cellulase and 140 FXU-hemicellulase/g-solid; Case III: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid with 1% Tween 80; and Case IV: 30 FPU-cellulase and 140 FXU-hemicellulase/g-solid. For Case IV, alkali-pretreated hemp biomass was subjected to an SM 2000 cutting mill (RestSH Inc. Newton, PA) to obtain the particle size less than 0.2 mm (Scheme 1). The designed amount of NaOH-pretreated hemp biomass was loaded into an Erlenmeyer flask and the designed volume of sodium acetate buffer (50 mM, pH 4.8) was added with different solid–liquid ratios (6–21%, w/v). Saccharification and fermentation were initiated by inoculating 1.0 mL of the activated yeast culture and performed in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) at an incubation temperature of 37 °C with 140 rpm. During SSF, 80 μL of the supernatant of slurries was pipetted periodically (24, 48, 72, and 96 h) from each flask for measuring ethanol concentration. Ethanol yield was...
calculated based on comparing the ethanol concentration in slurries with theoretical ethanol amount obtained from glucan, as shown in eq 4.

\[ Y_e = \frac{C_e}{A_g \times 0.511} \times 100\% \]

(4)

where \( Y_e \) is the ethanol yield (%); \( A_g \) and \( C_e \) are the theoretical concentration of glucose and ethanol concentration in hydrolysates (g/L), respectively; and 0.511 is the conversion factor of glucose-to-ethanol.

**Compositional Analysis.** Extractives in industrial hemp biomass were measured through distilled water and ethanol extraction following the National Renewable Energy Laboratory (NREL). Extracted biomass (~0.3 g, accurate to 0.1 mg) was first hydrolyzed with 3 mL of 72% sulfuric acid in a water bath at 30 °C for 1 h. The slurry was diluted to a 4% acid concentration by pouring 84 mL of deionized water and then autoclaved at 121 °C for 1 h. When it cooled to room temperature, the mixture was filtered using a preweighed filter crucible, and 10 mL of the filtrate was neutralized with 0.40 g of potassium carbonate for 45 min. Monosaccharides from the compositional analysis were determined quantitatively using a 1200 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA). The separation column was an HPX-87H organic acid column (7.8 × 300 mm) and set at 60 °C. The mobile phase was 0.005 M H_{2}SO_{4} buffer with a flow rate of 0.6 mL/min. The refractive index detector temperature was 45 °C. Residual monosaccharides and ethanol from SSF were measured using an RCM-monosaccharide Ca^{2+} (7.8 × 300 mm, Rezex) column under the following conditions: the flow rate of the mobile phase (HPLC-grade water) was 0.6 mL/min; the temperatures of the column and refractive index detector were 80 and 45 °C, respectively.

**Statistics.** The relationships between ethanol yield (linear regression)/concentration (nonlinear unitary quadratic) and solid loading as well as variances and comparisons of collected data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). The experiment procedures are shown in Scheme 1.

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**Author Contributions**

J.Z. is responsible for experimental implementation, data interpretation, and manuscript writing. Y.X., W.W., and D.W. revised the manuscript. J.G. provided the raw biomass. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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