Efficiency of Catalytic Liquid Hot Water Pretreatment for Conversion of Corn Stover to Bioethanol

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ABSTRACT: Lignocellulose is a promising raw material for the production of second-generation biofuels. In this study, the effects of acid-catalyzed liquid hot water (LHW) on pretreatment of corn stover (CS) for subsequent hydrolysis and conversion to ethanol were studied. The effects of reaction temperature, acid concentration, and residence time on glucose yield were evaluated using a response surface methodology. The optimal condition was 162.4 °C for 29.5 min with 0.45% v/v of sulfuric acid, leading to the maximum glucose yield of 91.05% from enzymatic hydrolysis of the cellulose-enriched fraction. Conversion of the solid fraction to ethanol by simultaneous saccharification and fermentation resulted in a theoretical ethanol yield of 93.91% based on digestible glucose. Scanning electron microscopy revealed disruption on the microstructure of the pretreated CS. Increases of crystallinity index and surface area of the pretreated biomass were observed along with alteration in the functional group profiles, as demonstrated by Fourier transform infrared spectroscopy. This work provides an insight into the effects of LHW on the enzymatic susceptibility and modification of the physicochemical properties of CS for further application on bioethanol production in biorefinery.
Among these strategies, liquid hot water treatment requires no or less amount of chemicals and short residence time, and is considered an energy-efficient process. Under high pressure, water in liquid form becomes acidic and can cause autohdyalysis of the glycosidic bonds, which result in solubilization of the hemicellulose and partial delignification of the associated lignin, which leads to increased susceptibility of the cellulose fraction to enzymatic hydrolysis.\(^{13}\) The carbohydrate fractions of the lignocellulose are converted into sugars by enzymes, and the sugars are subsequently used in fermentation for biofuel production.\(^{14}\) Therefore, it is imperative that the pretreatment method be evaluated with respect to not only the cleavage of the hemicellulose and the removal of lignin but also the evaluation of the actual impact of the process on the next step, enzymatic hydrolysis.\(^{12}\)

In this study, the liquid hot water (LHW) pretreatment process with an added homogeneous catalyst of corn stover was developed for corn stover (CS) to maximize sugar yield with low generation of inhibitory byproduct. The developed pretreatment process was applied for subsequent conversion to ethanol by simultaneous saccharification and fermentation (SSF) process. The work provides a basis for further study on the conversion of corn stover to second-generation ethanol in biorefineries.

### RESULTS AND DISCUSSION

#### Composition of Corn Stover.

The composition of native CS used in this study was determined as 30.12 ± 0.72% cellulose, 18.27 ± 0.45% hemicellulose, 35.12 ± 0.53% Klason lignin, 2.38 ± 0.64% ash, and 14.10 ± 0.25% others on dry basis. The carbohydrates content of native CS accounted for 48.39% (as cellulose and hemicellulose). The other components that are chemically bound are water- and ethanol-soluble materials and proteins.

#### Effects of Different Acid Catalysts on Liquid Hot Water Pretreatment of Corn Stover.

The performance of noncatalytic LHW pretreatment in the presence of various acid catalysts on corn stover was first investigated. Several processes were investigated for the pretreatment efficiency based on the glucose yield, as shown in Figure 1. The standard condition was tested at 180 °C for 30 min with an acid concentration of 0.5% v/v. The presence of catalysts in LHW pretreatment could clearly enhance the glucose yield after enzymatic hydrolysis. The use of sulfuric acid significantly improved the glucose yield (91.34%) compared to the noncatalytic process (52.56%). In addition, alternative acid catalysts (formic acid and phosphoric acid) were also investigated to study the effect on LHW pretreatment, which corresponds to the efficiency of the enzymatic hydrolysis. The presence of formic acid and phosphoric acid gave glucose yields of 58.37 and 79.33%, respectively. Sulfuric acid led to a higher glucose yield than either of the alternative acid catalysts. The different final concentration of strong acid was compared with a weak acid (based on pK\(_a\)). Since the initial acid concentration was controlled at 0.5% v/v, the final concentration of hydronium ion (H\(_3\)O\(^+\)) of sulfuric acid was higher than the hydronium ion content of the alternative acid catalysts. According to the pretreatment method, various catalysts play different important roles in the physical structure of the CS material. The change in the microstructure is related to the efficiency of the digestibility of carbohydrate fractions by enzyme. Further explanation for the low glucose yields obtained from the reaction with alternative acid catalysts could be the inhibition of the byproducts on enzyme digestibility.\(^{19}\) The conversion of lignocellulosic biomass has been reported to lead to the formation of furfural (FF), 5-hydroxymethyl-2-furaldehyde (HMF) and other organic compounds from degradation of sugars and lignin. The concentrations of byproducts are dependent on the severity of the pretreatment process, which could reduce the glucose yields.\(^{20}\) Therefore, the optimization of the LHW process in the presence of sulfuric acid was further evaluated in the next section to study the effect of various parameters on pretreatment to maximize the glucose yield.

#### Optimization of Catalytic Liquid Hot Water Pretreatment of Corn Stover.

In this study, the Box–Behnken design (BBD) based on the response surface methodology was used to evaluate the influence of parameters (e.g., H\(_2\)SO\(_4\) concentration, temperature, and residence time) to obtain maximum glucose yield in enzymatic hydrolysis. Temperature is considered as a major parameter to enhance the kinetic reaction, which is related to the cleavage of the chemical structure of corn stover. The effect of the acid concentration corresponds to the amount of addition of hydronium ion, which acts as the catalyst to disrupt the structure of corn stover. The residence time is also investigated to control the chemical reaction in the pretreatment process. The experimental design of the central composite is shown in Table 1. The experiments were designed with different levels of parameters within 20 runs. The remaining pulp was in the range of 50.09–80.72% w/w, which was dependent on the level of the severity condition. LHW pretreatment mainly eliminated the hemicellulose fraction, while the lignin fraction was partially removed, indicating that the increasing level of the severity could increase the disruption of the lignocellulose structure, which corresponded to more solubilized products being released into the hydrolysate.\(^{21}\) All pretreatment conditions of the corn stover were then investigated to enhance the glucose yield in enzymatic hydrolysis. The glucose yield was in the range of 60.96–90.34%. The effects of various parameters on glucose yield were further determined based on the RSM method.

According to the evaluation of the model, the predicted values were in good agreement with the experimental data (Figure S1). The analysis of variance (ANOVA) for response surface quadratic models, the significant parameters, and the effects of each variable on LHW pretreatment are presented in Table 2. The model was evaluated with the experimental results to estimate the significant effect of three parameters (acid concentration, temperature, and residence time) on glucose yield. The model in terms of actual value was fitted with a
Table 1. Responses in Solid Fraction from LHW Pretreatment Based on the Box–Behnken Design

| run no. | acid concentration (v/v) | temperature (°C) | time (min) | remaining solid composition (wt %) | glucose yield (%) |
|---------|--------------------------|------------------|------------|----------------------------------|-----------------|
|         |                          |                  |            | cellulose | hemicellulose | lignin | remaining solid pulp (%) |                  |
| 1       | 1                        | 150              | 45         | 24.35    | 0.09        | 23.08 | 50.09                     | 71.77            |
| 2       | 0.5                      | 180              | 15         | 27.42    | 0.74        | 31.61 | 63.15                     | 84.71            |
| 3       | 0                        | 120              | 45         | 28.69    | 9.57        | 34.22 | 75.93                     | 63.64            |
| 4       | 0.5                      | 180              | 30         | 27.08    | 0.11        | 31.04 | 61.56                     | 90.34            |
| 5       | 0                        | 150              | 15         | 28.18    | 5.26        | 33.94 | 70.85                     | 73.66            |
| 6       | 0                        | 120              | 15         | 29.36    | 13.68       | 34.24 | 80.72                     | 64.37            |
| 7       | 0.5                      | 180              | 45         | 26.76    | 0.11        | 30.72 | 60.74                     | 83.94            |
| 8       | 1                        | 180              | 30         | 25.32    | 0.07        | 22.42 | 50.37                     | 72.51            |
| 9       | 1                        | 120              | 15         | 28.43    | 1.16        | 27.87 | 60.35                     | 64.01            |
| 10      | 0.5                      | 150              | 45         | 27.23    | 1.32        | 32.14 | 63.96                     | 85.55            |
| 11      | 0.5                      | 180              | 30         | 27.08    | 0.11        | 31.04 | 61.56                     | 90.34            |
| 12      | 1                        | 150              | 30         | 25.59    | 0.10        | 23.39 | 51.71                     | 76.85            |
| 13      | 1                        | 120              | 15         | 26.63    | 0.22        | 24.16 | 53.57                     | 72.61            |
| 14      | 1                        | 150              | 45         | 26.61    | 0.28        | 25.38 | 54.78                     | 60.96            |
| 15      | 1                        | 120              | 45         | 27.63    | 2.31        | 32.57 | 65.97                     | 79.82            |
| 16      | 0                        | 180              | 30         | 27.68    | 3.32        | 33.32 | 67.79                     | 74.22            |
| 17      | 0                        | 150              | 45         | 28.65    | 7.98        | 34.54 | 74.57                     | 78.60            |
| 18      | 0.5                      | 120              | 30         | 28.65    | 7.98        | 34.54 | 74.57                     | 78.60            |
| 19      | 0.5                      | 120              | 45         | 27.95    | 7.35        | 34.22 | 72.88                     | 74.35            |
| 20      | 1                        | 180              | 30         | 25.32    | 0.06        | 22.42 | 50.37                     | 71.51            |

Table 2. Regression Model to Estimate a Significance of Variance by ANOVA Analysis

| source                        | sum of Squares | degree of freedom | mean square | f value | p-value |
|-------------------------------|----------------|-------------------|-------------|---------|---------|
| linear term                   |                |                   |             |         |         |
| acid conc.                    | 33.18          | 1                 | 33.18       | 20.54   | 0.0011  |
| temp.                         | 209.38         | 1                 | 209.38      | 129.60  | 0.0001  |
| time                          | 1.70           | 1                 | 1.70        | 1.05    | 0.3290  |
| quadratic term                |                |                   |             |         |         |
| acid conc. × temp.            | 15.98          | 1                 | 15.98       | 9.89    | 0.0104  |
| acid conc. × time             | 1.74           | 1                 | 1.74        | 1.07    | 0.3245  |
| temp. × time                  | 1.05           | 1                 | 1.05        | 0.65    | 0.4386  |
| interaction term              |                |                   |             |         |         |
| acid conc. × acid conc.       | 651.23         | 1                 | 651.23      | 403.09  | 0.0001  |
| temp. × temp.                 | 136.83         | 1                 | 136.83      | 84.69   | 0.0001  |
| time × time                   | 63.35          | 1                 | 63.35       | 39.21   | 0.0001  |
| residual                     | 16.16          | 10                | 1.62        |         |         |
| cor total                     | 1567.67        | 19                |             |         |         |

Typically, a p-value less than 0.05 indicates the significance of parameters. In this study, the quadratic model was evaluated as statistically significant at the confidence interval (95%). Table 2 demonstrates the coefficients of R-Square and R-Square (adj) to be 98.97 and 98.04%, respectively. All R-Square values were close to 100%, which suggests that the models are well fitted and of high accuracy for further prediction. According to the p-value, a linear term (acid concentration and temperature) was statistically significant for glucose yield. In addition, a quadratic term (acid concentration × temperature) and all interaction terms were also significant for glucose yield. According to the middle condition (150 °C, 0.5% v/v of acid concentration, and 30 min), a positive coefficient of the parameter suggested that increasing the level of the parameter would result in a positive effect on glucose yield. However, a negative coefficient would result in backward output (Figure S2). Acid concentration and residence time resulted in a negative coefficient, while temperature was presented as a positive coefficient based on the level of the middle condition. Moreover, temperature showed the most significant impact on glucose yield with the lowest p-value (0.0000), indicating that the increasing kinetic reaction of LHW pretreatment results in the removal of recalcitrant structures that further enhance the hydrolysis for glucose production. A response surface plot of glucose yield as a function of different levels of parameter is shown in Figure 2. The three-dimensional response surface plots demonstrate the effect of the level of each parameter on glucose yield and were evaluated to predict the optimum point of each factor for maximum response. As a result (Figure 2A–C), the glucose yield increases sharply with increasing acid concentration from 0 to 0.5% v/v, whereas the glucose yield decreased at high acid concentrations (>0.5% v/v) due to further degradation of the cellulose to organic acids and HMF via a dehydration reaction in the LHW pretreatment.22,23 Temperature was also observed to exercise the main effect on glucose yield. Increasing temperature was found to lead to substantial increases in the glucose yield. Further increases in temperature to above 170 °C led to decreases in the glucose yield, which corresponded to the

second-order polynomial. The final equation of glucose yield can measure the response at different levels of parameters using the following equation (eq 1)

\[
glucose \text{ yield (\%)} = -106.57934 + 64.2127A + 2.0708B + 0.9957C - 0.1038AB - 0.0621AC + 0.00091BC - 50.25538A^2 - 0.00630B^2 - 0.01882C^2
\]  

(1)

where A, B, and C are the actual values for H2SO4 concentration, temperature, and residence time, respectively.
formation of byproducts from sugar dehydration in the pretreatment process. However, the residence time from 15 to 45 min showed no substantial change in glucose yield.

In addition, the product profiles in the liquid fraction were also investigated to study the effect of various parameters (Table 3). Typically, the hydrolysis of the carbohydrate fraction in the LHW pretreatment is presented in the form of organic acids and products of further degradation (HMF and FF). Severe conditions were clearly seen to enhance the hemicellulose removal, which resulted in high concentrations of xylose and arabinose in the liquid fraction (e.g., run nos. 1, 8, and 13). The concentrations of xylose and arabinose were approximately 10 and 2 g/L, respectively. In addition, a high concentration of glucose (~3 g/L) was also observed under the same conditions. The side reaction of cellulose degradation appeared to occur under harsh conditions. Nevertheless, the concentrations of organic acids, HMF and FF, were found to be in the same range.

According to the final regression model, the model predicted the optimal condition at 162.4 °C for 29.5 min with an acid concentration of 0.45% v/v. This condition corresponds to the maximum glucose yield of 91.05%. Verification of the predicted optimal condition for the most efficient glucose yield was then investigated. According to the result in enzymatic hydrolysis (data not shown), the glucose concentration increased rapidly during 24 h. The final glucose concentration was 28.62 g/L after 72 h of hydrolysis, which is equivalent to the hydrolysis rate of glucose (~3 g/L).
0.39 g/(L h). The predicted values agreed well with the experimental result, which was 90.04% of glucose yield. In addition, the composition of the pretreated CS under optimal conditions was 28.60 ± 0.38% cellulose, 0.41 ± 0.22% hemicellulose, 29.73 ± 0.79% lignin, and 1.58 ± 0.39% ash. Under acid-catalyzed LHW pretreatment, the process particularly extracts the hemicellulose fraction due to the presence of hydronium ion (H₃O⁺), leading to the breakdown of the lignin—carbohydrate linkage of native CS, resulting in the solubilization of the hemicellulose fraction in the form of derived sugars with a minor depolymerization of lignin fraction. The hydrolyzed hemicellulose corresponds to their characteristics including low degree of polymerization (amorphous structure), highly branched structure, and short lateral chains. This leads to easy hydrolysis compared to cellulose structure.26 According to the proposed mechanism, LHW pretreatment results in the enrichment of cellulose and partial lignin fraction in the solid residue.27,28 The acid-catalyzed LHW pretreatment shows the disruption of lignin—carbohydrate linkage and enrichment of cellulose content, which results in an enhancement of enzyme accessibility to produce fermentable sugars and consequently improved bioethanol production.

Table 3. Product Profiles in Liquid Fraction from LHW Pretreatment

| run no. | glucose (g/L) | xylose (g/L) | arabinose (g/L) | acetic acid (g/L) | formic acid (g/L) | lactic acid (g/L) | furfural (g/L) | HMF (g/L) |
|---------|---------------|--------------|-----------------|-------------------|-------------------|------------------|----------------|-----------|
| 1       | 3.58          | 10.83        | 2.71            | 0.01              | 0.31              | 0.29             | 0.03           | 0.07      |
| 2       | 2.00          | 10.40        | 2.60            | 0.01              | 0.31              | 0.29             | 0.03           | 0.03      |
| 3       | 1.06          | 5.16         | 1.29            | 0.01              | 0.31              | 0.29             | 0.02           | 0.05      |
| 4       | 2.28          | 10.77        | 2.69            | 0.01              | 0.31              | 0.29             | 0.02           | 0.02      |
| 5       | 1.45          | 7.72         | 1.93            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 6       | 0.56          | 2.71         | 0.68            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 7       | 2.51          | 10.77        | 2.69            | 0.04              | 0.31              | 0.29             | 0.01           | 0.01      |
| 8       | 3.58          | 10.82        | 2.70            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |
| 9       | 1.24          | 10.15        | 2.54            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |
| 10      | 2.15          | 10.06        | 2.52            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |
| 11      | 1.90          | 9.86         | 2.47            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |
| 12      | 2.28          | 10.77        | 2.69            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |
| 13      | 3.39          | 10.83        | 2.71            | 0.03              | 0.31              | 0.29             | 0.01           | 0.01      |
| 14      | 2.60          | 10.85        | 2.71            | 0.03              | 0.31              | 0.29             | 0.01           | 0.01      |
| 15      | 2.63          | 10.84        | 2.71            | 0.03              | 0.31              | 0.29             | 0.01           | 0.01      |
| 16      | 1.85          | 9.47         | 2.37            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 17      | 1.07          | 8.87         | 2.22            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 18      | 1.08          | 6.08         | 1.52            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 19      | 1.61          | 6.47         | 1.62            | 0.01              | 0.31              | 0.29             | 0.01           | 0.01      |
| 20      | 3.58          | 10.82        | 2.70            | 0.02              | 0.31              | 0.29             | 0.01           | 0.01      |

Figure 3. Scanning electron micrographs of (A) native CS and (B) pretreated CS under optimized conditions.
Physical Characterization of Native and Pretreated Corn Stover. Scanning electron microscopy (SEM) analysis revealed the morphological features and surface characteristics of the pretreated CS compared with the native CS. As shown in Figure 3A, the surface of native CS was highly ordered recalcitrant structures together with a smooth surface. After LHW pretreatment, the structure of the pretreated CS seems to lose the cover surface, resulting in the exposure of the internal structure and fibers (Figure 3B). The SEM image of the pretreated CS indicates that the pretreatment process proposes to remove the external recalcitrance, which increases the surface area of the cellulose to make the cellulose more accessible to the enzymes. Increasing the surface area in the pretreated solid residues was reported to improve glucose yield during the enzymatic hydrolysis of the solid residue.30

Crystallinity of the pretreated CS is an important property that has been shown to affect enzymatic hydrolysis.30 X-ray diffraction (XRD) is one method capable of determining relative changes in cellulose crystallinity.31 In this study, the features of pretreated CS under the optimal condition were compared with native CS, as shown in Figure 4. The crystallinity of native CS was determined to be 53.3%. After LHW pretreatment, the crystallinity of the pretreated CS increased significantly to 64.5%. In other words, increasing crystallinity was also related to the removal of hemicellulose and lignin under LHW pretreatment.32 In addition, the sharp increase in crystallinity indicated the high ratio of crystalline cellulose fraction in the remaining solid, resulting in an increase of cellulose surface accessibility.55

The Fourier transform infrared (FTIR) spectra of lignocellulosic materials were determined by the spectra of the functional group profiles.34 The FTIR spectra of native CS and pretreated CS are illustrated in Figure 5. The absorbance of the functional group in solid residues was assigned the peak from 3432 to 465 cm\(^{-1}\). The absorption at 3402 cm\(^{-1}\) is associated with O–H stretching. The peak at 2910 cm\(^{-1}\) is attributed to C–H stretching. The peak at 1631 cm\(^{-1}\) is probably related to the bending mode of absorbed water. A peak at 1428 cm\(^{-1}\) is related to CH\(_2\) symmetric bending, while that at 1392 cm\(^{-1}\) is related to CH bending. The absorbance at 1318 cm\(^{-1}\) is related to CH\(_2\) bending of carbohydrates. The peak at 1160 cm\(^{-1}\) is attributed to C–O antisymmetric stretching. A band at 1107 cm\(^{-1}\) is related to C–OH skeletal vibration. A band at 1061 cm\(^{-1}\) is due to the C–O–C pyranose ring skeletal vibration. The peak at 898 cm\(^{-1}\) corresponds to the glycosidic C\(_1\)–H deformation with a ring vibration contribution, which is a characteristic of \(\beta\)-glycosidic linkages in the glucose ring. In comparison, strong peaks at 1720 and 1247 cm\(^{-1}\) of native CS were attributed to the presence of the unconjugated carbonyl group (C\(\equiv\)O) and the C–O stretching signal, respectively. These peaks significantly decreased after acid-catalyzed LHW pretreatment process.

The change in the crystal structure is often accompanied by variation in the surface area, which could further affect the cellulose accessibility.30 In this work, the surface area, pore volume, and pore size of native CS and pretreated CS were determined. According to the results (Table 4), the surface area of pretreated CS increased from 0.72 to 14.3 m\(^2\)/g, which was equivalent to 19.86 times the native CS. Additionally, the pore volume was obviously enhanced from 0.003 to 0.073 cm\(^3\)/g. The positive values indicated that the pretreatment using LHW in the presence of an acid catalyst leads to a significant modification of the surface morphology, thereby resulting in an enhancement of enzyme accessibility toward cellulose.55,36

Simultaneous Saccharification and Fermentation (SSF) for Ethanol Production. In this study, cellulose-enriched substrate was prepared under the optimal conditions of acid LHW pretreatment to apply as the starting material in the fermentation process for ethanol production (Figure 6). The substrate was predigested with CeliC Ctec2 and then inoculated with Saccharomyces cerevisiae for the fermentation process. In the prehydrolysis step, the pretreated CS was rapidly saccharified to glucose with a high accumulated glucose concentration of 12.22 g/L at 6 h, which is equivalent to the hydrolysis rate of 2.04 g/(L h). On the other hand, xylose was slowly released from the substrate. The glucose concentration profile was observed to decrease rapidly after 6 h in the fermentation process, which corresponds to the obvious increase in ethanol production. Only glucose was continually converted in the fermentation step, while xylose apparently showed no assimilation by the yeast. The highest ethanol concentration of 15.25 g/L was achieved at 72 h with the maximum ethanol yield of 0.47 g/g, which was equivalent to 93.91% of the theoretical yield based on the digestible glucose in the pretreated substrates with the production rate of 0.21 g/(L h). The residual glucose at the end of fermentation was 0.32 g/L, while xylose was not assimilated into the yeast cells, resulting in a constant residual concentration of 0.13 g/L. Variation in the ethanol concentration from the fermentation of lignocellulosic biomass depended on various factors including the nature of substrates, pretreatment methods, ethanologenic microorganisms, and the physical parameters of the fermentation processes. The ethanol yield obtained in this study was comparable to the ethanol yield previously reported for the fermentation of rice straw pretreated with acid and alkali, which was 84.6% of the ethanol yield.37 The hydrothermal pretreatment of corn stover was previously studied for ethanol production, which resulted in 68.7% of theoretical ethanol yield.38 Further fermentation of pretreated corn stover with steam pretreatment resulted in an ethanol yield of 72.4%.39

CONCLUSIONS

Acid-catalyzed LHW pretreatment of CS was optimized by BBD to maximize glucose yield in enzymatic hydrolysis, resulting in a marked improvement on sugar yield with low inhibitory byproducts contents. The pretreated solid could be used for ethanol fermentation with high efficiency. This work provided insights into the effect of acid LHW pretreatment on the modification of the physiochemical structure of CS, which can be applied for further utilization for biofuel production in integrated biorefinery.
EXPERIMENTAL SECTION

Materials. Corn stover was obtained from a local farm in So village, Phayao, Thailand. The raw material was dried in a hot-air oven at 60 °C for 24 h and ground to a particle size of 1−2 mm using a cutting mill (Retsch SM2000, Hann, Germany). The ground material was sieved using opening sieves of 1, 0.625, and 0.25 mm. The respective fractions were collected and stored in airtight containers at room temperature for further experiments. The composition of corn stover was determined according to the standard NREL method.16 All chemicals and reagents including sugar standards were purchased from major chemical suppliers, i.e., glucose, xylose, arabinose, 5-hydroxymethyl-2-furaldehyde (HMF), and furfural were purchased from Sigma-Aldrich (S.M. Chemical Supplies Co., Ltd, Bangkok, Thailand), sulfuric acid and phosphoric acid were purchased from Merck (Merck Ltd. Bangkok, Thailand), and formic acid was purchased from Fisher Scientific (Union Science Co., Ltd, Chiang Mai, Thailand).

Liquid Hot Water Pretreatment. The pretreatment was performed in a 600 mL stainless steel high-pressure reactor equipped with a mixing system and a thermocouple for internal temperature measurement (Parr Reactor 4560, Parr Instrument Company, Moline, IL). The standard pretreatment was performed with the ratio of native CS to water of 1 g/15 mL. The biomass was pretreated under a set of conditions with varying temperatures (120−180 °C) and residence times (15−45 min). For catalytic LHW pretreatment, various acid catalysts (sulfuric acid, phosphoric acid, and formic acid) were added to the reaction by varying the concentration over 0, 0.5, 1.0% v/v. Nitrogen gas was flowed into the reactor for purging and adjusting the initial pressure to 20 bar with continuous stirring at 100 rpm. After the reaction, the vessel was extinguished in a water bath. Then, the pretreated corn stover was separated by filtering (Whatman No. 4). The pretreated substrate was dried at 80 °C for 12 h and kept at room temperature for further study. The liquid fraction was collected for analysis of the sugars and inhibitory products by high-performance liquid chromatography (HPLC).

Box–Behnken Design (BBD). In statistics, BBD is an experimental design for response surface methodology to estimate the effect of various parameters and their interaction with the process responses. Each variable was varied with three coded levels (lowest value coded as −1, middle value coded as 0, and highest value coded as +1). The BBD comprised 20 runs with different temperatures (120−180 °C), acid concentrations (0−1% v/v), and residence times (15−45 min). The target response was glucose yield with the investigation of the interaction effect and optimal conditions by response surface regression, fitted to the second-order polynomial multiple regression equation (eq 2).

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2
\]  

(2)

where \( Y \) is the predicted response; \( X_1, X_2, \) and \( X_3 \) are the independent variables; \( b_0 \) is the offset term; \( b_1, b_2, \) and \( b_3 \) are the first-order terms; \( b_{12}, b_{13}, \) and \( b_{23} \) are the interaction terms; and \( b_{11}, b_{22}, \) and \( b_{33} \) are the quadratic terms. The numbers 1, 2, and 3 are the index numbers of the parameters.

Enzymatic Hydrolysis. The pretreatment efficiencies were assessed based on biomass digestibility using a commercial cellulase (Cellic Ctec2, Novozymes A/S, Bagsvaerd, Denmark).

![Figure 5. FTIR spectra of (A) native CS and (B) pretreated CS under optimized conditions.](image)

![Figure 6. Fermentation of the cellulose-enriched substrate that was obtained under optimal conditions with a simultaneous saccharification and fermentation (SSF) process. The fermentation mixture contained 6.67% of the substrate at a controlled temperature of 40 °C and pH of 4.8 for 72 h.](image)

| responses                  | native CS | pretreated CS |
|----------------------------|-----------|---------------|
| BET surface area (m²/g)    | 0.72      | 14.3          |
| BJH pore volume (cm³/g)    | 0.003     | 0.073         |

Table 4. Specific Surface Area and Pore Volume of Native CS and Pretreated CS under Optimized Condition
The sugar yield from the enzymatic hydrolysis of the substrates was evaluated. Enzymatic hydrolysis reactions (total volume, 1 mL) contained 5% w/v pretreated substrate with 25 FPU/g of enzyme in 50 mM sodium citrate buffer (pH 4.8) and 1% w/v sodium azide. The reactions were incubated at 50 °C for 72 h with vertical rotation at 30 rpm. The hydrolysis experiments were performed in triplicate. Profiles of released sugars were analyzed on a high-performance liquid chromatograph (LDC Model 4100, Shimadzu, Kyoto, Japan) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Hercules, CA) operating at 65 °C with 5 mM H2SO4 as the mobile phase at a flow rate of 0.5 mL/min. Glucose yield was calculated as the percentage of glucose from enzymatic hydrolysis based on the amount of cellulose in the pretreated biomass according to eq 3.

\[
\text{Cellulose hydrolysis(%) = } \frac{\text{amount of glucose after hydrolysis (g)}}{\text{amount of glucose in pretreated material (g)}} \times 100
\]

Analysis of Products in Liquid Phase. The chemical components in the liquid phases were determined according to the standard protocols (NREL/TP-510-42623).

Fermentable sugar profiles and inhibitory byproducts (5-hydroxymethylfurfural and furfural) were analyzed on a high-performance liquid chromatograph (SPD-M10A DAD, Shimadzu, Japan) equipped with a refractive index detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) operating at 65 °C with 5 mM H2SO4 as the mobile phase at a flow rate of 0.5 mL/min.

Physical Analysis of Solid Residues. The microstructures of the native CS and the pretreated CS obtained from the pretreatment process were analyzed by scanning electron microscopy (SEM) using a JSM-6301F scanning electron microscope (JSM-6301F, JEOL, Japan). The samples were dried and coated with gold for analysis. An electron beam energy of 20 kV was used for analysis. The crystallinity of the native and pretreated fractions was determined by X-ray diffraction (XRD) using a XPERT PRO diffractometer (PANalytical, Almelo, The Netherlands). The samples were scanned in the 2θ range of 10–30° with a step size of 0.02° at 500 kV and 30 mA. The crystallinity was calculated according to eq 4.

\[
\text{CrI (%) = } \left( \frac{I_{002} - I_{\text{amorphous}}}{I_{002}} \right) \times 100
\]

where \(I_{002}\) is the scattered intensity at the main peak of cellulose, which typically lies at the 002 plane, and \(I_{\text{amorphous}}\) is the scattered intensity of the amorphous portion evaluated at the 101 planes.

Chemical characteristics of the pretreated materials were determined by Fourier transformed infrared (FTIR) spectroscopy on a PerkinElmer System 2000 (PerkinElmer, Waltham, MA). The samples were prepared using the KBr pellet method. The measurement resolution was set at 4 cm\(^{-1}\) with a mirror velocity of 0.6329 cm/s. Infrared spectra were collected in the range 4000–400 cm\(^{-1}\) with 64 co-added scans. The peaks of cellulose were compared with the standards of functional groups. The total surface area of the raw material and solid residues from pretreatment was determined using the Brunauer–Emmett–Teller (BET) method. The surface area and pore volume of the untreated and treated corn stover were determined using nitrogen adsorption/desorption isotherms in a surface area analyzer (TriStar II 3020, Micromeritics Co.).

Ethanol Production by Simultaneous Saccharification and Fermentation (SSF). The SSF process was performed in a 2.0 L reactor (Biosart B 2, B. Braun (Thailand), Bangkok, Thailand) with a total operating volume of 1.2 L. The fermentation medium contained 5 g/L (NH4)2SO4, 0.025 g/L MgSO4·7H2O, 1.0 g/L yeast extract, pH 4.8 with 6.25% (w/v) of pretreated CS under the optimized acid LHW pretreatment conditions. The medium was sterilized at 121 °C for 15 min. The pretreated substrate was pregested with 25 FPU/g Cellic Ctec2 at 50 °C with mixing at 300 rpm for 6 h. The S. cerevisiae TISTR 5339 (www.tistr.or.th) yeast culture inoculum was grown at 30 °C for 24 h in YPD medium. Subsequently, the fermentation mixture was incubated at 40 °C with pH controlled at 4.8 by H3PO4 and NH4OH. Fermentation was operated for 72 h with continuous mixing at 300 rpm. The samples were collected periodically to analyze the concentration of ethanol, glucose, and xylose on a high-performance liquid chromatograph equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) as described above. The yield of ethanol was calculated in percent of theoretical ethanol yield of consumed glucose, 0.511 g-ethanol/g-sugar.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04054.

Linear plot of predicted value and experimental value of glucose yield based on RSM model and relative coefficient impact of acid concentration, temperature, and residence time on glucose yield in enzymatic hydrolysis (PDF)

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Notes

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