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Abstract

As a promising alternative of fossil fuel, ethanol has been widely used. In recent years, much attention has been devoted to bioethanol production from lignocellulosic biomass. In previous research, it is found that the pretreatment method named low-moisture anhydrous ammonia (LMAA) has the advantage of high conversion efficiency and less washing requirements. The purpose of this study was to explore the optimal conditions by employing the LMAA pretreatment method. Corn stover was treated under three levels of moisture content: 20, 50, 80 w.b.% (wet basis), and three levels of particle size: <0.09, 0.09–2, >2 mm; it was also ammoniated with a loading rate of 0.1g NH₃/g biomass (dry matter). Ammoniated corn stover was then subjected to different pretreatment times (24, 96, 168 h) and temperatures (20, 75, 130 °C). After pretreatment, compositional analysis and enzymatic digestibility were conducted to determine the highest glucose yield. As a result, the highest glucose yield was obtained under the condition of 96 h and 75 °C with 50 w.b.% and 0.09–2 mm of corn stover. The main findings of this study could improve the efficiency of bioethanol production processing in the near future.

Keywords

Anhydrous ammonia, Corn stover, Cellulosic ethanol, Low-moisture anhydrous ammonia (LMAA), Pretreatment

Disciplines

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Comments

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Article

Anhydrous Ammonia Pretreatment of Corn Stover and Enzymatic Hydrolysis of Glucan from Pretreated Corn Stover

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Abstract: As a promising alternative of fossil fuel, ethanol has been widely used. In recent years, much attention has been devoted to bioethanol production from lignocellulosic biomass. In previous research, it is found that the pretreatment method named low-moisture anhydrous ammonia (LMAA) has the advantage of high conversion efficiency and less washing requirements. The purpose of this study was to explore the optimal conditions by employing the LMAA pretreatment method. Corn stover was treated under three levels of moisture content: 20, 50, 80 w.b.% (wet basis), and three levels of particle size: <0.09, 0.09–2, >2 mm; it was also ammoniated with a loading rate of 0.1g NH₃/g biomass (dry matter). Ammoniated corn stover was then subjected to different pretreatment times (24, 96, 168 h) and temperatures (20, 75, 130 °C). After pretreatment, compositional analysis and enzymatic digestibility were conducted to determine the highest glucose yield. As a result, the highest glucose yield was obtained under the condition of 96 h and 75 °C with 50 w.b.% and 0.09–2 mm of corn stover. The main findings of this study could improve the efficiency of bioethanol production processing in the near future.

Keywords: anhydrous ammonia; corn stover; cellulosic ethanol; low-moisture anhydrous ammonia (LMAA); pretreatment

1. Introduction

Due to concerns about environmental, long-term economic and national security, there has been increasing interest in renewable and domestic sources of fuels to replace fossil fuels in recent decades. [1]. Bioethanol, produced from renewable materials, is regarded as an alternative to gasoline. There are multiple raw materials to produce bioethanol; one of the most widely adopted is sugar- or starch- based material, such as corn. Bioethanol produced from corn is called first generation biofuel. It has been commercialized in several places and is considered quite efficient. However, a problem arose because of land use and competition with food crops, the so-called food versus fuel debate [2]. Bioethanol can also be produced from lignocellulosic biomass, which is known as second generation biofuel [3]. In general, four major processes are involved in converting lignocellulosic biomass to bioethanol: pretreatment, hydrolysis, fermentation, and ethanol recovery [4]. Among the four steps, pretreatment is critical because of the difficulties in removing the lignin-carbohydrate complex (LCC) structure in lignocellulosic biomass. With the assistance of pretreatment, the LCC structure could be removed, and the exposed cellulose could be broken down into monosaccharides, then the resulting glucose can be fermented into ethanol [1].

Numerous efforts have been invested in exploring various pretreatment methods on various biomass to enhance enzymatic digestibility. Additionally, various pretreatment reagents have been

studied, such as carbon dioxide, dilute acid, hot water, ammonia and alkaline. Based on the results of extensive research, each different reagent exhibited its unique characteristics. Several reagents are compared as following.

Carbon dioxide (CO₂) has many advantages, as it is environmentally friendly, inexpensive, and easy to recover after use. The pretreatment method based on CO₂ is supercritical carbon dioxide (SC-CO₂). It has been applied to a few lignocellulosic biomass, such as aspen and south yellow pine [5], wheat straw [6], guayule [7], and corn stover [8]. As for corn stover, the maximum glucose yield obtained under 3500 psi and 150 °C was 30 g/100 g dry corn stover [8]. However, the need for high-pressure equipment by using the SC-CO₂ pretreatment method may result in high capital cost; besides, the low efficiency of this treatment may be a barrier as well to large-scale production [5].

Hot water has also been used as a reagent in pretreatment studies. Hot water has been studied in materials like aspen [9], soybean straw [10], corn stover [9,10], alfalfa [11], and cattails [12]. As a convenient pretreatment method, liquid hot water is effective for soybean straw with the combination of fungal degradation pretreatment, but the combination of these two pretreatment methods is not efficient for corn stover, when compared with fungal degradation pretreatment alone [10].

Another reagent, ammonia, is also broadly explored in this field. Pretreatment methods of ammonia have attracted much attention due to its effectiveness in delignification. For example, ammonia fiber explosion [13–16], ammonia fiber expansion [17–20], and aqueous ammonia soaking [21–23] have been developed. In addition, the improvement in glucose yield is clearly observed. However, water consumption, environmental concerns, and high cost are problematic for ammonia-based pretreatment methods.

Yoo et al. [24] developed the low moisture anhydrous ammonia (LMAA) pretreatment method to eliminate the washing step and reduce capital costs in the ammonia-based pretreatment method. In their study, corn stover pretreated with 3% glucan loading at 80 °C for 84 h resulted in the highest ethanol yield, that is: 89% of theoretical ethanol yield. However, the reactor used in the research conducted by Yoo et al. [24] was a 2.9-inch (8.1 cm) internal diameter with a 6.5-inch (18.5 cm) length (690 mL internal volume). The small sealed reactor may not be capable of providing optimal conditions for bioethanol production at industrial scales. Yang and Rosentrater [25] and Cayetano and Kim [26] have expanded on this initial study. Yang and Rosentrater [25] investigated the effectiveness of LMAA as a method to both pretreat and preserve corn stover prior to fermentation, and found that LMAA is beneficial to preserving sugar yields during storage, with sealed containers being more effective at ammonia treatment.

The main objective of this study was to investigate the LMAA pretreatment process with a larger-scale reactor; four pretreatment conditions (moisture content, particle size, pretreatment temperature, and pretreatment time) were considered in this study. Furthermore, optimal conditions for higher ethanol yield were explored.

2. Materials and Methods

2.1. Biomass

In this study, freshly-harvested, air-dried corn stover was collected from central Iowa in 2012 and stored at ambient temperature. Prior to pretreatment, the corn stover was ground and sieved into three size fractions (<0.09, 0.09–2.0, and >2.0 mm). Then, the sieved corn stover was stored at room temperature (~21 °C) until use.

2.2. Equipment

The reactor (Figure 1), which was purchased from Pall Corporation, Ann Arbor, MI, USA, was used in the ammoniation process. Compared to Yoo's [24] study, this sealed reactor was about 4.35 times larger (the internal capacity is 3 L). It is anticipated that the potential errors caused by different ammonia loadings and reaction times could be eliminated by the use of a larger reactor.

High Performance Liquid Chromatography (HPLC) with a Bio-Rad Aminex HPX-87P column (Aminex HPX-87P, Bio-Rad Laboratories, Hercules, CA, USA) and a refractive index detector (Varian 356-LC, Varian, Inc., Palo Alto, CA, USA) were used to measure sugar contents. Acid soluble lignin (ASL) content was determined by UV-Visible spectrophotometer (UV-2100 Spectrophotometer, Unico, United Products & Instruments, Inc., Dayton, NY, USA).



Figure 1. Ammoniation reactor with internal volume of 3 L.

2.3. Enzymes

In this study, GC 220 cellulase, purchased from Genencor International, Inc. (Rochester, NY, USA), was a mixture of endoglucanases and cellobiohydrolases. The cellulase activity was expressed in filter paper units (FPU); the average activity of GC 220 was determined to be 45 FPU/mL. The β -glucosidase enzyme (Novozyme 188), provided by Sigma-Aldrich, Inc. (St. Louis, MO, USA), was used to convert cellobiose to glucose. The activity of Novozyme 188 was 750 cellobiase units (CBU)/mL.

2.4. LMAA Pretreatment Process

The original moisture content was measured before ammoniation, then certain amounts of water were added to the corn stover in order to achieve the target moisture content (20, 50, and 80 w.b.%). Moisturized corn stover was equilibrated for 24 h afterwards.

The moisturized corn stover was placed in the sealed reactor, and ammonia gas was introduced. On top of the reactor, a pressure gauge and a temperature gauge were equipped to monitor the pressure and temperature change during the whole process. However, temperature change was not controlled during this study. The pressure of the anhydrous ammonia was maintained at 0.1 g NH₃/g DM biomass for 30 min in order to achieve a complete reaction. After the ammoniation process, the reactor was cooled down for 5 min and the lid was removed in the fume hood. Then the ammoniated corn stover was transferred into several glass bottles (250 mL) with screw caps. A pipe was connected between the top of the reactor and the fume hood to ventilate surplus ammonia.

The bottles packed with ammoniated corn stover were placed in various heating ovens at varying pretreatment temperatures (20, 75, and 130 °C) for 24, 96, and 168 h. As soon as the pretreatment

process was complete, the lid of the glass bottles was removed in the fume hood and surplus ammonia was evaporated for 12 h before compositional analysis.

2.5. Experimental Design

In this study, four independent variables that may have influence on the reaction severity were investigated. Biomass moisture contents were 20, 50 and 80 wet basis (w.b.) %; the pretreatment times were 24, 96, and 168 h; the pretreatment temperatures were 20, 75, and 130 °C; and the particle sizes were <0.9, 0.9–2.0 and >2.0 mm, respectively. By combining different levels of these four independent variables, 17 treatments were designed in this study, i.e., $2 \times 2 \times 2 \times 2 + 1$ center point. As dependent variables, moisture content, lignin, glucan, xylan, galactan, arabinan, mannan and ash content were measured and compared in the experiment. The experimental design for this study is shown in Table 1.

Table 1. Experimental design in this study. *

Treatment	Moisture Content (w.b. %)	Time (h)	Temperature (°C)	Particle Size (mm)
1	20	24	20	<0.9
2	20	24	20	>2.0
3	20	24	130	<0.9
4	20	24	130	>2.0
5	20	168	20	<0.9
6	20	168	20	>2.0
7	20	168	130	<0.9
8	20	168	130	>2.0
9	80	24	20	<0.9
10	80	24	20	>2.0
11	80	24	130	<0.9
12	80	24	130	>2.0
13	80	168	20	<0.9
14	80	168	20	>2.0
15	80	168	130	<0.9
16	80	168	130	>2.0
CP	50	96	75	0.9–2.0

* CP denotes center point of the design.

2.6. Compositional Analysis

Carbohydrates and lignin (both acid-soluble lignin and acid-insoluble lignin) contents were determined by NREL LAP [27]. Each sample was analyzed in duplicate. The glucan and xylan content in the corn stover were analyzed by HPLC, following the NREL standards. Acid soluble lignin was measured by UV-Visible Spectrophotometer. Moisture content was determined by an oven drying method [27].

2.7. Enzymatic Digestibility Test

The enzymatic digestibility test was done in duplicate under conditions of pH 4.8 (0.1 M sodium citrate buffer) with 40 mg/L tetracycline and 30 mg/L cyclohexamide in 250 mL Erlenmeyer flasks according to NREL LAP [28]. The initial glucan concentration was 1% (*w/v*). Cellulase enzyme (GC 220) loading was 15 FPU/g of glucan, and β -glucosidase enzyme (Novozyme 188) loading was equal to 30 CBU/g of glucan. Flasks were incubated at 50 ± 1 °C and 150 rpm in an incubator shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA). Time for enzymatic digestibility test ranged from 0 to 168 h for sugar analysis.

Total glucose detected from HPLC was used to calculate the glucan digestibility following Equation (1) below. The conversion factor for glucose to equivalent glucan was 0.9 based on the calculation. The quantification of glucose in HPLC is based on the separation of the solvent into its constituent parts due to the different affinities of different molecules for the mobile phase and stationary phase. All the statistical results were analyzed by SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

$$\text{Glucan digestibility [\%]} = \frac{\text{Total released glucose} \times 0.9}{\text{Initial glucan loading}} \times 100\% \quad (1)$$

3. Results and Discussion

3.1. Effects of LMAA Pretreatment on Biomass Composition

In this study, the employment of low-moisture anhydrous ammonia (LMAA) pretreatment method didn't result in significant changes in lignin, glucan, xylan, arabinose, mannan or ash contents. Table 2 exhibits the main effect. As can be seen by the letter, temperature had an effect mainly on lignin and ash. With higher temperature, the ash content increased as well as the lignin content. Time also had effect on lignin and glucan as well; longer time resulted in higher glucan. The effect of size is primarily on ash content; larger size resulted in lower ash content. The fourth factor, moisture content, did not have much influence on the compositions. According to Table 3, the majority of the *p*-values of interactions among these four independent variables were higher than 0.05, which indicates that little evidence of significant interactions among independent variables was observed. Similar findings were found in treatment effect (Table 4).

The reason for insignificant compositional analyses results in this study was because the ammonia used in the LMAA pretreatment process was meant to break the LCC structure for later enzymatic saccharification and ethanol fermentation process, not to change composition per se. This has also been studied by Cayetano and Kim [26]. Their work showed that the LMAA pretreatment method did not result in significant changes to the chemical composition.

Table 2. Main effects of compositional analysis on corn stover. *

Factor	Levels	Lignin (%)	AIL (%)	ASL (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinose (%)	Mannan (%)	Ash (%)
Temperature (°C)	20	20.86a (0.73)	16.86a (0.74)	3.99a (0.44)	35.73a (2.97)	21.35a (2.96)	0.67a (0.34)	3.70a (0.47)	0.05a (0.05)	1.67a (0.69)
	75	21.20ab (0.26)	16.23a (0.48)	4.97b (0.74)	38.89a (2.75)	25.59b (3.07)	0.55a (0.06)	4.31a (0.64)	0.02b (0.01)	1.96ab (0.30)
	130	21.36b (0.80)	17.83b (0.87)	3.54c (0.57)	37.08a (2.86)	22.47ab (1.77)	0.83a (0.40)	3.88a (0.53)	0.04b (0.02)	2.20b (0.55)
Time (h)	24	20.89a (0.95)	17.27a (1.28)	3.62a (0.58)	35.38a (3.25)	21.89a (2.93)	0.75a (0.41)	3.75a (0.64)	0.05a (0.05)	1.94a (0.69)
	96	21.20ab (0.26)	16.23a (0.48)	4.97b (0.74)	38.89ab (2.75)	25.59b (3.07)	0.55a (0.06)	4.31a (0.64)	0.02b (0.01)	1.96a (0.30)
	168	21.33b (0.55)	17.42a (0.72)	3.91a (0.50)	37.43b (2.26)	21.92ab (2.00)	0.75a (0.34)	3.83a (0.32)	0.04b (0.02)	1.93a (0.66)
Moisture Content (w.b.%)	20	21.12a (0.95)	17.32a (1.08)	3.80a (0.45)	35.54a (2.76)	22.02ab (2.66)	0.82a (0.45)	3.88a (0.62)	0.06a (0.04)	1.79a (0.65)
	50	21.20a (0.26)	16.23a (0.48)	4.97b (0.74)	38.89a (2.75)	25.59a (3.07)	0.55a (0.06)	4.31a (0.64)	0.02b (0.01)	1.96a (0.30)
	80	21.10a (0.64)	17.36a (0.81)	3.73a (0.65)	37.27a (2.95)	21.79b (2.33)	0.69a (0.27)	3.70a (0.35)	0.03b (0.02)	2.08a (0.68)
Size	S	21.31a (0.92)	17.56a (1.12)	3.75a (0.60)	35.6a (2.77)	20.67a (2.38)	0.79a (0.36)	3.65a (0.46)	0.04a (0.04)	2.32a (0.59)
	M	21.2a (0.26)	16.23b (0.48)	4.97b (0.74)	38.89a (2.75)	25.59b (3.07)	0.55a (0.06)	4.31a (0.64)	0.02b (0.01)	1.96ab (0.30)
	L	20.91a (0.62)	17.12ab (0.67)	3.78a (0.51)	37.21a (2.98)	23.14b (1.91)	0.71a (0.39)	3.94a (0.52)	0.04a (0.03)	1.56b (0.51)

* Similar letters after means in each level of the main factor indicates insignificant difference for that dependent variable at $\alpha = 0.05$, LSD. Values in parentheses are standard deviation (S.D.). S denotes size less than 0.9 mm, M denotes size between 0.9 and 2.0 mm, and L denotes size larger than 2.0 mm. AIL stands for Acid-Insoluble Lignin; ASL stands for Acid-Soluble Lignin.

Table 3. Interaction effects of compositional analysis (*p*-values) on corn stover. *

Factor	Lignin (%)	AIL (%)	ASL (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinose (%)	Mannan (%)	Ash (%)
Temp	0.004	0.004	0.029	0.160	0.188	0.120	0.245	0.014	0.004
Time	0.978	0.612	0.150	0.038	0.967	0.955	0.580	0.003	0.978
MC	0.089	0.885	0.738	0.075	0.788	0.193	0.239	<0.0001	0.089
Size	<0.0001	0.150	0.865	0.097	0.008	0.418	0.072	0.807	<0.0001
Temp * Time	0.437	0.793	0.772	0.546	0.283	0.618	0.010	<0.0001	0.437
Temp * MC	0.285	0.110	0.065	0.426	0.466	0.672	0.036	<0.0001	0.285
Temp * Size	0.922	0.282	0.678	0.205	0.190	0.056	0.927	1.000	0.922
Time * MC	0.083	0.244	0.240	0.178	0.308	0.426	0.765	0.000	0.083
Time * Size	0.377	0.410	0.753	0.722	0.507	0.003	0.053	0.807	0.377
MC * Size	0.507	0.946	0.423	0.714	0.308	0.236	0.233	0.807	0.507
Temp * Time * MC	0.097	0.219	0.975	0.073	0.344	0.077	0.188	0.005	0.097
Temp * Time * Size	0.272	0.939	0.865	0.407	0.457	0.358	0.552	0.155	0.272
Temp * MC * Size	0.070	0.361	0.738	0.836	0.650	0.015	0.765	0.335	0.070
Time * MC * Size	0.512	0.852	0.701	0.315	0.635	0.654	0.510	0.100	0.512
Temp * Time * MC * Size	0.806	0.340	0.356	0.956	0.502	0.100	0.685	0.064	0.806

* Temp = Temperature; MC = Moisture Content; AIL = Acid-Insoluble Lignin; ASL = Acid-Soluble Lignin.

Table 4. Treatment effects of compositional analysis on corn stover. *

Treatment	Lignin (%)	AIL (%)	ASL (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinose (%)	Mannan (%)	Ash (%)
1	20.01de	16.01d	4.00a–c	30.04c	16.37c	0.47c	2.90c	0.13a	2.23a–d
2	19.78e	16.22cd	3.56bc	34.44bc	23.34ab	1.20ab	3.69bc	0.12a	0.57e
3	21.46a–c	17.86a–c	3.60bc	36.69ab	20.61bc	0.55c	3.51bc	0.03cd	2.35a–c
4	21.02b–e	16.88b–d	4.14a–c	38.80ab	23.19ab	0.56c	3.70bc	0.03cd	1.81cd
5	20.99b–e	17.03b–d	3.96a–c	36.00ab	21.11a–c	0.79bc	3.95b	0.03cd	2.20a–d
6	20.94c–e	17.09a–d	3.85a–c	37.51ab	23.80ab	0.59c	3.98b	0.04b–d	1.48c–e
7	21.31a–c	16.76b–d	4.54ab	34.60a–c	20.12bc	0.65bc	3.92b	0.01d	1.47c–e
8	21.36a–c	16.99b–d	4.36ab	37.81ab	22.25ab	0.59c	4.00b	0.03cd	1.29de
9	22.47a	18.78a	3.69bc	35.15a–c	22.49ab	0.90bc	3.96b	0.04b–d	2.19a–d
10	21.12b–d	17.27a–d	3.85a–c	35.87ab	24.09ab	0.54c	4.92a	0.03cd	1.62cd
11	20.79c–e	17.78a–c	3.01c	37.10ab	22.04ab	0.56c	3.46bc	0.02d	2.80ab
12	20.47c–e	17.33a–d	3.14c	34.97a–c	23.01ab	1.22ab	3.89b	0.04b–d	1.96b–d
13	22.19ab	18.38ab	3.82a–c	37.09ab	21.45ab	1.52a	3.80b	0.03cd	2.30a–c
14	21.46a–c	17.78a–c	3.70bc	38.24ab	23.52ab	0.55c	3.89b	0.05bc	1.81cd
15	21.29a–c	17.89a–c	3.41bc	38.18ab	21.19a–c	0.91bc	3.72bc	0.07b	3.01a
16	21.11b–d	17.41a–d	3.70bc	40.05a	21.97ab	0.46c	3.43bc	0.04b–d	1.96b–d
CP	21.20b–d	16.23cd	4.97a	38.90ab	25.59a	0.55c	4.31ab	0.02d	1.96b–d

* Similar letter after means in each treatment indicates insignificant difference for the dependent variable at $\alpha = 0.05$, LSD. CP denotes center point. AIL stands for Acid-Insoluble Lignin; ASL stands for Acid-Soluble Lignin.

3.2. Effects of LMAA Pretreatment on Glucan Digestibility

Figure 2 shows the overall results of enzymatic digestibility for the 17 treatments mentioned in previous experimental design; moreover, the results of avicel (used as a reaction blank for the substrate) and untreated corn stover are indicated in Figure 2 as well. All the enzymatic digestibility results have been organized from the highest digestibility to the lowest in Figure 3. The Lineweaver-Burke linear regressions used to determine enzymatic digestibility kinetic constants are demonstrated in Table 5. As observed in Figure 2, the combinations of the four factors resulted in various digestibility. More clearly, in Figure 3, the highest glucose digestibility (57.23%) compared with the lowest one (29.02%) showed that LMAA pretreated corn stover was 1.97 times higher.

According to the research of Yoo et al. [24], the optimal pretreatment temperature was 80 °C and the pretreatment time was 84 h. In our study, among the 17 treatments, treatment CP, which contained 50 w.b.% moisture content with 0.9–2.0 mm particle size, achieved the highest glucose digestibility with the conditions of 96 h pretreatment time and 75 °C pretreatment temperature. The results are similar to those of Yoo et al. [24], thus indicating that the consistency remained in small and large scale reactors.

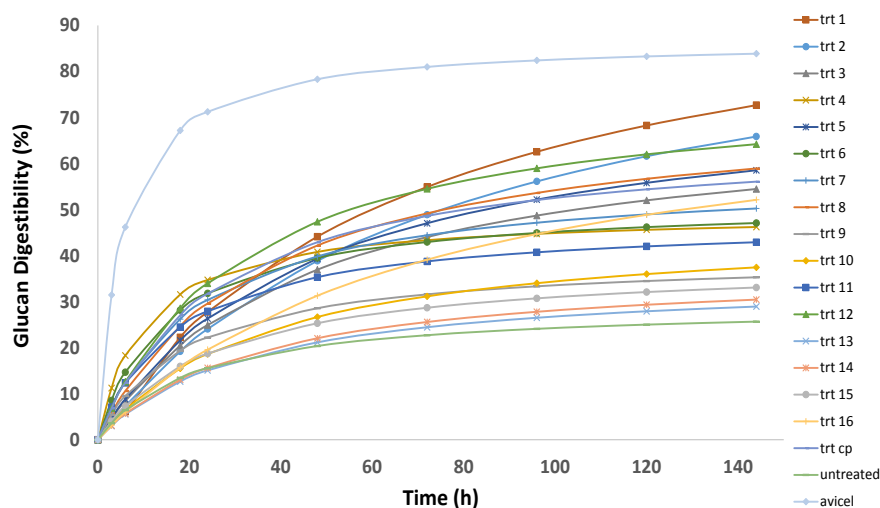


Figure 2. Glucan digestibility results for all treatments. Trt denotes treatment; CP denotes center point.

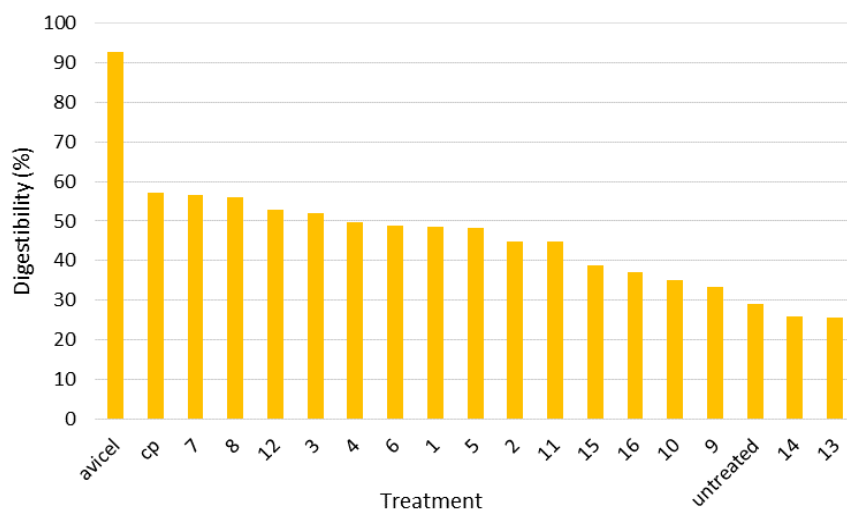


Figure 3. Enzymatic digestibility results of glucan (from highest to lowest) for all the treatments. CP denotes center point.

Table 5. Lineweaver-Burke linear regressions used to determine enzymatic digestibility kinetic constants. Dynamic changes in enzymatic digestibility over time are provided in Figure 2. *

Treatment	Equation
1	$y = 0.6417 \times x + 0.0093 (R^2 = 0.955)$
2	$y = 0.7603 \times x + 0.0099 (R^2 = 0.983)$
3	$y = 0.6274 \times x + 0.0140 (R^2 = 0.996)$
4	$y = 0.2067 \times x + 0.0202 (R^2 = 0.982)$
5	$y = 0.6021 \times x + 0.0129 (R^2 = 0.985)$
6	$y = 0.2939 \times x + 0.0192 (R^2 = 0.990)$
7	$y = 0.3751 \times x + 0.0173 (R^2 = 0.965)$
8	$y = 0.4849 \times x + 0.0136 (R^2 = 0.984)$
9	$y = 0.4805 \times x + 0.0250 (R^2 = 0.992)$
10	$y = 0.7780 \times x + 0.0213 (R^2 = 0.986)$
11	$y = 0.3611 \times x + 0.0208 (R^2 = 0.987)$
12	$y = 0.3995 \times x + 0.0128 (R^2 = 0.943)$
13	$y = 0.9155 \times x + 0.0282 (R^2 = 0.981)$
14	$y = 0.9004 \times x + 0.0266 (R^2 = 0.968)$
15	$y = 0.6683 \times x + 0.0256 (R^2 = 0.945)$
16	$y = 0.9196 \times x + 0.0128 (R^2 = 0.983)$
CP	$y = 0.3939 \times x + 0.0151 (R^2 = 0.957)$
Untreated	$y = 0.7294 \times x + 0.0339 (R^2 = 0.913)$
Avicel	$y = 0.0609 \times x + 0.0115 (R^2 = 0.986)$

* *y* stands for the reverse of digestibility; *x* stands for the reverse of time.

In this study, four independent variables were tested: moisture content, particle size, pretreatment temperature, and pretreatment time. Among the four variables, pretreatment temperature was regarded as the most critical due to the smallest *p*-value (0.0013). Table 6 shows the difference in average glucan digestibility between high and low levels of pretreatment temperature when other factors were kept constant, in particular, the other main effects. From Table 6, it is clear that higher pretreatment temperature led to decreased glucan digestibility in this study.

Table 6. Main effects on glucan digestibility results (at *t* = 144 h).

Factor	Levels	Digestibility (%)
Temperature (°C)	20	47.76 (16.11)
	75	56.07 (–)
	130	51.02 (9.56)
Time (h)	24	53.14 (13.83)
	96	56.07 (–)
	168	45.65 (11.55)
Moisture Content (%)	20	57.51 (8.47)
	50	56.07 (–)
	80	41.28 (11.60)
Size	S	47.02 (14.80)
	M	56.07 (–)
	L	51.77 (11.17)

As for pretreatment time, the difference between the longest time and the shortest one when other factors were kept constant was also significant as shown in Table 6. The average glucose digestibility at 168 h (47.76%) was relatively lower than the average for 24 h of pretreatment time (51.02%). This could be explained by the longer pretreatment times causing the collapse of the LCC structure of corn stover. It is observed from Figure 2 that from 6 to 18 h, there was an average of 92.7% increase in glucan digestibility, which was the maximum increase rate during all the enzymatic digestibility tests.

In terms of moisture content, higher moisture content resulted in lower glucan digestibility. The reason for this may be that the reduction of retaining ammonia with higher moisture content could result in lower delignification within its structure. As for the effect of particle size, there were some differences between the smallest size and the largest size of corn stover, as observed in Table 6. Larger corn stover particles tend to be more digestible than smaller ones.

4. Conclusions

In this study, the effect of the LMAA pretreatment method with four independent factors was investigated. As a result, LMAA pretreatment showed the potential to achieve higher glucose yield due to higher glucan digestibility. When corn stover (50 w.b. % moisture content) was pretreated at 75 °C for 96 h, the maximum enzymatic digestibility for glucan was obtained. What's more, because there was no washing step involved during the study, the LMAA pretreatment method has the potential to eliminate water consumption compared to other ammonia-based pretreatment methods.

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