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Optimization of Low Moisture Anhydrous Ammonia (LMAA) Pretreatment for Corn Stover Enzymatic Digestibility during Hydrolysis Process

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Abstract.

Corn stover is one of the most common lignocellulosic biomass used in bioethanol production. In bioethanol production, pretreatment is the first step to break down recalcitrant structure of biomass which is also a critical process for further sugar release and fermentation. Among all chemical pretreatment processes, ammonia is one of the base reagent, and the low moisture anhydrous ammonia (LMAA) process could minimize water and ammonia input in bioethanol production. For obtaining the optimal fermentable sugar yields with the most efficient chemical loadings and pretreating time, several factors were examined for enzymatic digestibility optimization. In LMAA pretreatment process, the ammonia loading, ammoniation time and the particle size of corn stover are the main factors for enzyme digestibility in the hydrolysis process. As the particle size of corn stover was reduced from 1mm to 0.5 mm, the anhydrous ammonia loading was increased from 0.1g to 0.18g NH_3/g DM biomass under 75^{o}C and the ammoniation incubation was extended from 72 hr to 144 hr, the enzyme digestibility would increase from 71.6% to 83.69% with about 17% increments.

Keywords. Corn stover, LMAA, Hydrolysis, Enzyme digestibility, Optimization

Introduction

With the increasing of energy demands, the renewable resources have been regarded as a critical material for alternative fuels generation. Due to arguments resulted from first generation bioethanol produced from starch, the second generation bioethanol mainly converted from lignocellulosic materials has been getting lots of attractions (Cheng and Timilsina, 2011). Typically, pretreatment, hydrolysis (saccharification), fermentation and ethanol recovery are the four main steps of bioethanol production (Naik et al., 2010).

Corn stover is the most abundant agricultural crop residue in Midwest, and that includes stalks, leaves and cobs which has high contents cellulose and hemicellulose, over 50% (Sokhansanj et al., 2002, Tumbalam et al., 2016, Xu et al., 2016). In ethanol production, cellulose and hemicellulsoe are main materials, because they are composed of linear polymer of glucose and branched polymer of pentose respectively which can be released during hydrolysis and used for further fermentation. Pretreatment is used to break down the recalcitrant structure of biomass to increase the accessibility of fermentable sugars. Also, the pretreatment process makes up to one-third of the total production costs and can be regarded as one of the main barriers for commercial scale production (Saha, 2004, Saha et al., 2016).

There are several technologies for pretreatment have been developed which include physical, chemical and even cooperated with both effects generally (McMillan, 1994). Milling, hydrothermolysis and steam explosion are the common methods used in physical pretreatment in order to reduce particle size and increase the reaction surface (Mosier et al., 2005). For chemical methods, acids or bases could improve the efficiency of further hydrolysis and fermentation by open structure and removing lignin and hemicellulose. Sulfuric acid and sodium hydroxide are used in chemical methods the most commonly (Mosier et al., 2015). However, acid pretreatment is able to remove hemicellulose and also break down cellulose into glucose even degrade glucose to furfural (Harris and Begliner, 1946, Zeitsch, 2000). Though acid pretreatment provides higher efficiency for loosening structure and releasing glucose, that also results in decrease the amount of fermentable sugar. Base pretreatment is efficient to remove lignin with adding air/oxygen to the reaction mixture which is able to retain fermentable sugars, hexose and pentose, for ethanol production (Chang and Holtzapple, 2000).

Ammonia is one of base pretreatment, and ammonia fiber expansion (AFEX), common technology used in ammonia pretreatment, uses concentrated ammonia to break down the inner structure of lignocellulosic biomass for the enzymatic hydrolysis and fermentation process (Lau et al., 2010). Beside, soaking in aqueous ammonia (SAA) is also used in ammonia pretreatment which is able to retain the hemicellulose at low temperature and increasing the fermentation yield (Kim and Lee, 2005). For increasing the efficiency of pretreatment and ammonia usage, the low-moisture anhydrous ammonia (LMAA) process is developed to minimize the water and ammonia input for bioethanol production (Yoo et al., 2011). According to the research from Yoo et al., (2011), a small sealed batch reactor (690 mL internal volume) was used and achieved 89% of theoretical ethanol yield. However, the optimal conditions of small reactors may not be accurate when scaled up. Yang and Rosentrater (2014) applied LMAA in larger scale of corn stover pretreatment than Yoo's research (2011). The loading of ammonia, particle size and moisture content of biomass and the different time for ammoniation are the main variables for the pretreatment in Yang and Rosentrater's study which gave to the best enzymatic digestibility in hydrolysis process around 75%, and that was lower than Yoo's study.

For improving the enzymatic digestibility, this study mainly focused on trying harsher conditions and improving the ammonia dispersion based on Yang and Rosentrater's experiment in 2014. The optimization of LMAA used in corn stover pretreatment is another topic in the study as well.

Material and Methods

Biomass

Air-dried corn stover was supplied from central Iowa in 2012 and stored at ambient temperature. According to the result from objective 1, the biomass was then ground and sieved into two various sizes prior to pretreatment (<0.5 mm, 0.5-1.0mm). The sieved corn stover was kept at room temperature (~21°C) until use.

Equipment

The 3L reactor was also applied. However, for improving the ammonia dispersion and the interaction with

biomass, the inlet was extended to the bottom of the reactor (Figure 1). Due to the lighter density of ammonia which causes the ammonia remaining on the top in the reactor and the interaction between ammonia and biomass was limited. That might be the reason which led to lowering the digestibility of enzymatic hydrolysis in Yang's study (2014). This modification would improve the efficiency of ammoniation. In order to measure monosaccharides, HPLC installed 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., CA, USA) were used. Acid soluble lignin (ASL) content was determined by UV-Visible spectrophotometer (UV-2100 Spectrophotometer, Unico, United Products & Instruments, Inc., Dayton, NY, USA). And acid insoluble lignin (AIL) content was determined by oven and furnace.

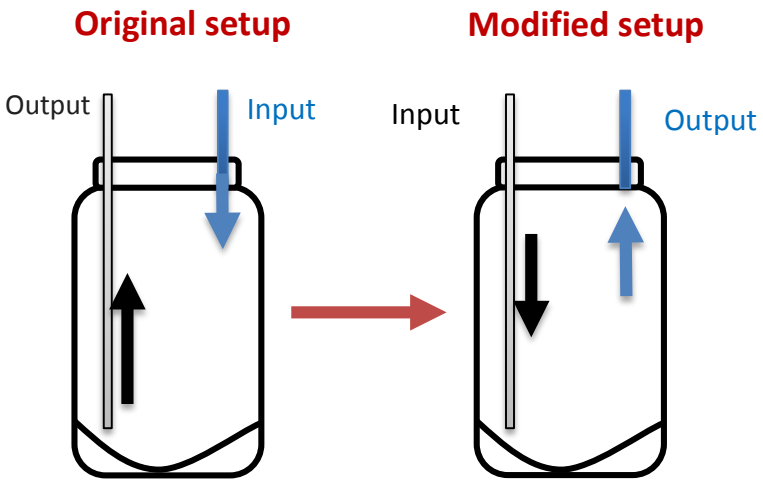


Fig. 1 Configuration change of ammoniation reactor

LMAA Pretreatment

According to the result of Yang and Rosentrater's study (2014), the moisture content of corn stover was 50 wb % and samples were equilibrated for over 24 hours has the best effect on LMAA treatment.

Moisturized corn stover was placed in the sealed reactor, and ammonia was introduced. A pipe was connected between the top of the reactor and the fume hood to ventilate surplus ammonia. A pressure gauge was equipped on the reactor to monitor the pressure change during the ammoniation process. Anhydrous ammonia was added up to the targeted pressure to achieve 0.18 g NH_3/ g dry matter biomass. The whole ammoniation process lasted up to 60 minutes in order to achieve a complete reaction. After the ammoniation process was finished, the reactor was cooled down for 5 minutes, the lid was removed in the fume hood, and then the ammoniated corn stover was transferred into several glass bottles (250 mL) with a screw cap.

The bottles packed with ammoniated corn stover were placed in heating ovens at various pretreatment temperatures 75°C for 72 h, and 144 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.

Enzymatic Digestibility Test

GC 220 cellulase was purchased from Genencor International, Inc. (Rochester, NY, USA). The cellulase activity was expressed in filter paper units (FPU). In this study, the average activity of GC 220 was determined to be 45 FPU / mL. The β-glucosidase enzyme (Novozymes 188) was obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA). The activity of Novozymes 188 was 750 cellobiase units (CBU) / mL.

Enzymatic digestibility was determined following NREL LAP (NREL, 2008). The test was done in duplicate under conditions of pH 4.8 (0.1M sodium citrate buffer) with 40 mg/L tetracycline and 30 mg/L cyclohexamide in 250 mL Erlenmeyer flasks. The initial glucan concentration was 1% (w/v). Cellulase enzyme (GC 220) loading was 15 FPU/g of glucan, and ß-glucosidase enzyme (Novozyme 188) loading was equal to 60 CBU/g of glucan. Flasks were incubated at 50°C ± 1°C and 150 rpm in an incubator shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA). Enzymatic digestibility time ranged from 0 h to 144h 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.

$$\text{Digestion } (\%) = \frac{grams\ cellulose\ digestied}{grams\ cellulose\ added} \times 100 \times 0.9 \qquad \text{Eq (1)}$$

Composition Analyses

Carbohydrates and lignin were determined followed by NREL LAP (NREL, 2011). Each sample was analyzed in triplicate. The content of holocellulose, which content cellulose (α-cellulose) and hemicellulose were analyzed according to Wise method (Wise et al., 1946), the content of α-cellulose was tested based on TAPPI standard (TAPPI T 203 cm-99). The content of hemicellulose was calculated by subtracting the content of α-cellulose from holocellulose. Acid soluble lignin (ASL) was measured by UV-Visible Spectrophotometer. And moisture content was determined by the oven drying method (NREL, 2011).

Results and Discussions

Effect of LMAA treatment with higher ammonia loading on biomass composition

Table 1 shows the main effects of LMAA pretreatment with higher ammonia loading on composition of corn stover**.** The higher ammonia loading and longer ammoniation incubation time resulted in the changes of cellulose, hemicellulose and AIL. The p-values of each factor are represented in Table 2.

Table 1 Main effects on biomass composition before and after LMAA pretreatment

Factor	Levels	Cellulose (%)	Hemicellulose (%)	AIL (%)	ASL (%)
Time (h)	72	36.55 (1.70)[b]	27.03 (1.04)[b]	13.31 (1.57)[c]	2.87 (0.15)[b]
	144	39.74 (1.46)[a]	24.82 (0.80)[c]	15.03 (0.87)[b]	2.71 (0.16)[b]
Particle Sizes (mm)	0.5	37.05 (2.33)[b]	25.95 (1.85)[b]	15.93 (0.95)[b]	2.93 (0.22)[a]
	1	39.25 (1.67)[a]	25.89 (1.09)[b]	14.88 (1.61)[c]	2.91 (0.12)[a]
Raw Materials		34.73 (1.20)[c]	38.8 (1.08)[a]	17.87 (1.09)[a]	3.19 (0.14)[a]

Values in parentheses are standard deviation, And each level of the main factor indicates insignificant differences at α=0.05

Table 2 p-values of each effect on LMAA pretreatment

Factor	Cellulose	Hemicellulose	AIL	ASL
Time	<0.0001	<0.0001	<0.0001	0.001
Particle Size	0.02	0.93	0.03	0.75
Time*Particle size	0.14	0.62	0.04	0.40

Each level of the main factor indicates insignificant differences at α=0.05

According to the results, LMAA pretreatment with higher ammonia led to increase the content of cellulose. This base treatment also decreased the contents of lignin and hemicellulose (Mosier et al., 2005). For ammoniation incubation time, it has the significant effect on the compositions changes for cellulose, hemicellulose and lignin. The longer incubation time results in higher cellulose, lower cellulose and lignin contents. That indicates the longer incubation time increases the accessibility for further hydrolysis and fermentation. As for different particle sizes, LMAA pretreatment increased the cellulose content and lowered the hemicellulose and lignin contents as well. However, that does not have the significant effects as incubation time especially on hemicellulose and ASL contents. That has the moderate effects on cellulose and AIL contents. In cellulose content, the smaller particle

size has lower cellulose content than larger particle size. That might be resulted from the cellulose which is easier to lose during the treatment because the smaller particle has the larger reactive surface and higher accessibility to the reagent.

Conclusively, the LMAA has the ability to open the recalcitrant chemical structure of biomass, and increase the amount of cellulose content. That indicates this pretreatment could increase the accessibility of further hydrolysis and fermentation to obtain the higher product yields.

Effect of LMAA pretreatment with higher ammonia loading on enzymatic digestibility during hydrolysis

According to the results from Yang and Rosentrater's study (2014), two different particle sizes biomass with 50% moisture content (wb) which pretreated with higher ammonia loading were applied in enzymatic hydrolysis. Otherwise, the dosage of β-glucosidase (Novozyme 188) was increased from 30 CBU to 60 CBU. The results of enzymatic digestibility are shown in Table 3.

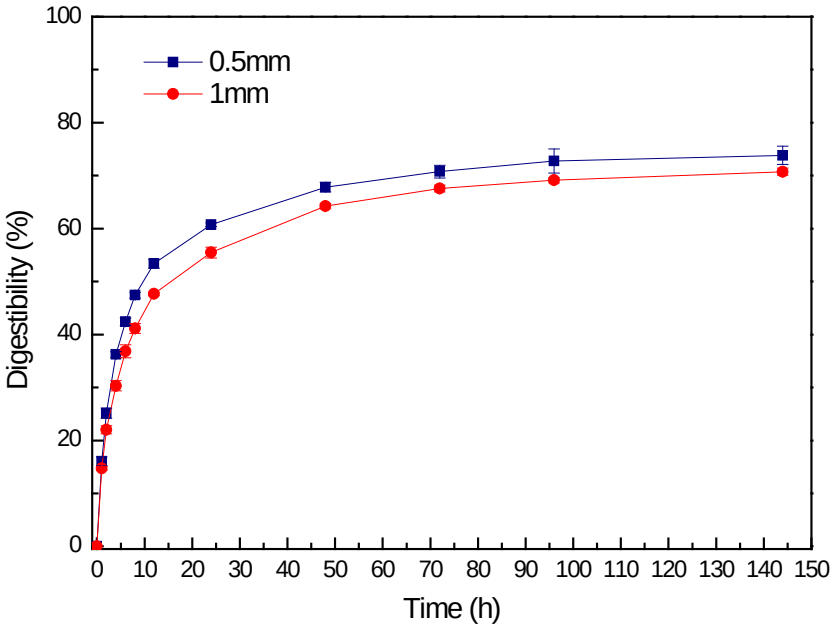
Table 3 Main effects of LMAA pretreatment on enzymatic digestibility

Factor	Levels	Digestibility (%)	p-value
Time (h)	72	72.29 (2.05)^{b}	<0.0001
	144	80.50 (3.71)^{a}	
Particle Sizes (mm)	0.5	78.76 (5.61)^{a}	0.0003
	1	74.03 (3.67)^{b}	
Time*Particle Size	144*0.5	83.69 (1.72)^{a}	0.0662
	144*1	77.31 (0.94)^{b}	
	72*0.5	73.83 (1.72)^{bc}	
	72*1	70.74 (0.65)^{c}	

Values in parentheses are standard deviation, And each level of the main factor indicates insignificant differences at α=0.05

From the results, the digestibility of all conditions are increased above 70%, and the highest digestibility could reach 85% which was increase around 22% from the average digestibility from objective 1. The different ammoniation incubation time and particle sizes were taken as different factors for optimization test. The enzymatic digestibility during hydrolysis are shown in Figure 2. Also, according to the results, ammoniation incubation times plays a critical role in final enzymatic digestibility. The longer treating time leads to higher digestibility. For particle size, that has the significant effect on enzymatic digestibility as well, the smaller particle size has higher digestibility. These two factors have p-values smaller than 0.001.

(a)



(b)

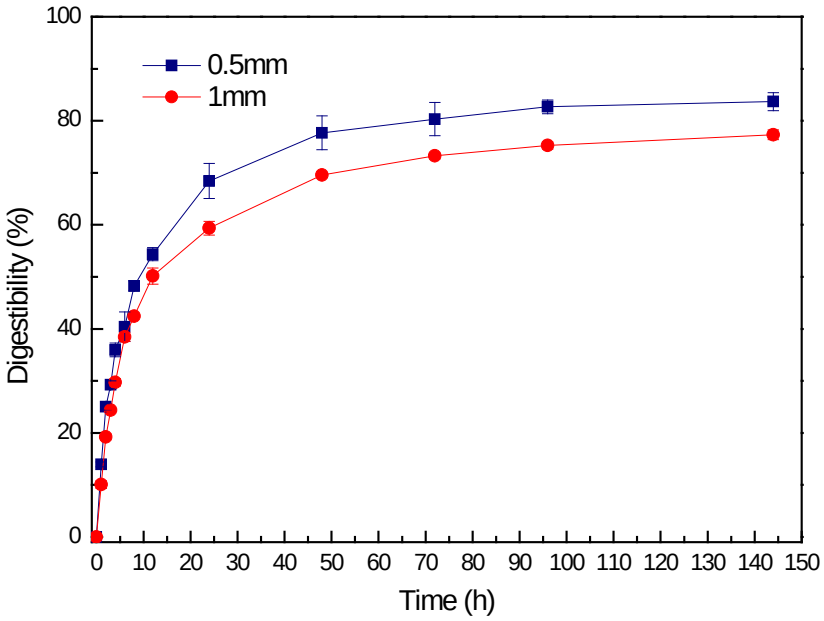


Fig. 2 Enzymatic digestibility during corn stover hydrolysis. (a) 72h ammoniation incubation; (b) 144 ammoniation incubation

As these two factors were considered together, there is the weak effect on the interaction between these two factors. However, the biomass with smaller particle size (<0.5 mm) was treated for 144 hours could has the highest average enzymatic digestibility of 83.69%. These results also indicates the smaller particle size and longer treatment gave the better accessibility for ammoniation. The higher digestibility could lead to the higher yield of further fermentation.

Conclusions

The LMAA with higher ammonia loading (0.18 g NH_3/ d biomass dm) could decompose parts of lignin and hemicellulose, and increase cellulose content of biomass. This base pretreatment is able to open the recalcitrant structure of biomass as well. Additionally, this treatment also increased the ammonia diffusion which increased the enzymatic digestibility. However, the time of treatment and particle sizes are the critical factors not only for increasing accessibility of biomass but also for obtaining higher digestibility. Hence, the biomass with smaller particle size treated with longer ammoniation incubation could give the highest digestibility.

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