Low-Moisture Anhydrous Ammonia (LMAA) Pretreatment of Corn Stover

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Abstract. *Ethanol has been widely used as an alternative to fossil fuels. In recent years, much attention has been devoted to ethanol production from lignocellulosic biomass. In previous research, low-moisture anhydrous ammonia (LMAA) pretreatment was investigated due to its high efficiency and less washing compared to other pretreatment methods. The purpose of this study was to determine optimal conditions resulting in highest glucose yield and lowest ammonia input using the LMAA processing. In this experiment corn stover was used with different moisture contents (20, 50, 80 wb %) and particle sizes (<0.09, 0.09-2, >2 mm). Corn stover was ammoniated with a loading rate of 0.1g NH_3 /g biomass. Ammoniated corn stover then was subjected to different pretreatment times (24, 72,144 h). After that, compositional analysis, including ash content, moisture content, structural carbohydrates and lignin in biomass, and enzymatic digestibility were used to determine the glucose yield. This study focused on designing a pretreatment method which could improve the efficiency of pretreatment processing.*

Keywords. Ammonia, biomass, cellulosic ethanol, pretreatment.

Introduction

In recent years, there has been increasing interest in renewable, domestic sources of fuels to replace fossil fuel due to concerns about environmental, long-term economic, and national security (Mosier et al., 2005). Bioethanol, which is renewable and environmentally-friendly, can be used as an alternative to gasoline. Currently, bioethanol is mainly produced from sugar- or starch- based materials, such as corn, which is efficient, but problematic due to land use and competition with food crops (Sim et al., 2010). Bioethanol can be also produced from lignocellulosic biomass, which is known as second generation biofuel (Cheng and Timilsina, 2011). Generally, there are four major processes in converting lignocellulosic biomass to ethanol: pretreatment, hydrolysis, fermentation, and ethanol recovery (Naik et al., 2010). Among these steps, pretreatment is critical and expensive because of the need to break down cellulose, which is protected under a shield of hemicellulose and lignin, to monosaccharides, then the resulting glucose can be fermented into ethanol (Mosier et al., 2005).

Over the past few decades, numerous efforts have investigated various pretreatment methods to enhance enzymatic digestibility and improved ethanol yield from biomass (Alinia et al., 2010; Alizadeh et al., 2005; Bals et al., 2006; Gao et al., 2011; Garlock et al., 2009; Gupta and Lee, 2009; Hanchar et al., 2007; Kim and Hong, 2001; Kim and Lee, 2005; Lau et al., 2009; Lee et al., 2010; Narayanaswamy et al., 2011; Screenath et al., 1999; Srinivasan and Ju, 2010; Teymouri et al., 2004, 2005; Wan and Li, 2011; Wang et al., 2012; Yoo et al., 2011; Yourchisin and Walsum 2004;

Zhang et al., 2011). Additionally, various pretreatment reagents have been studied, each with its own advantages, disadvantages, and efficiencies.

For example, carbon dioxide has been used due to its many advantages: environmentally friendly, inexpensive, and easy to recover after use. The supercritical carbon dioxide (SC-CO_2) pretreatment method has been applied to a few lignocellulosic biomasses, such as aspen and south yellow pine (Kim and Hong, 2001), wheat straw (Alinia et al., 2010), guayule (Srinivasan and Ju, 2010), switchgrass, and corn stover (Narayanaswamy et al., 2011). The maximum glucose yield for corn stover under 3500 psi at 150°C was 30g/100g dry biomass (Narayanaswamy et al., 2011). However, low-efficiency and high capital cost for high-pressure equipment may be barriers to large-scale production using the SC-CO_2 pretreatment method (Kim and Hong, 2001).

Hot water has also been used as a reagent in pretreatment studies. Hot water has been used for aspen (Yourchisin and Walsum, 2004), soybean straw (Wan and Li, 2011), corn stover (Wan and Li, 2011; Yourchisin and Walsum, 2004), alfalfa (Screenath et al., 1999), and cattails (Zhang et al., 2011). Liquid hot water as a pretreatment method may be effective for soybean straw when combined with fungal degradation pretreatment, but it is not efficient for corn stover compared with fungal degradation pretreatment alone (Wan and Li, 2011).

Ammonia is another reagent widely used in pretreatment. Numerous pretreatment methods focused on ammonia have been investigated, such as ammonia fiber explosion (Alizadeh et al., 2004, 2005; Hanchar et al., 2007; Lee et al., 2010;

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Teymouri et al., 2005), ammonia fiber expansion (Bals et al., 2006; Garlock et al., 2009; Gao et al., 2011; Lau et al., 2010;), and aqueous ammonia soaking (Gupta and Lee, 2009; Kim and Lee, 2007). Previous studies have found effective delignification and glucose yield for a variety of lignocellulosic biomass materials using ammonia. However, economics, water and chemical consumption, and environmental concerns are problematic for ammonia use.

In order to eliminate the washing step and reduce capital costs in the pretreatment process, Yoo et al (2011) developed a low moisture anhydrous ammonia (LMAA) pretreatment method. In that research, corn stover treated at 80°C for 84 h with 3% glucan loading resulted in the highest yield (89% of theoretical ethanol yield). However, small sealed batch reactors (2.9 inch (8.1 cm) internal diameter × 6.5 inch (18.5 cm) length, 690 mL internal volume) in Yoo's (2011) research were very small scale, which may lead to inappropriate conditions for optimal ethanol production at larger scales.

In this study, a deeper investigation of the LMAA pretreatment process with a larger-scale reactor, under a range of pretreatment conditions (moisture content, particle size, pretreatment temperature, and pretreatment time), was studied. Optimal conditions for highest ethanol yield were sought.

Materials and Methods

Biomass

In this study, freshly-harvested, air-dried corn stover was supplied from central Iowa in 2012 and stored at ambient temperature. The biomass was then ground and

sieved into three size fractions prior to pretreatment (<0.09 mm, 0.09-2.0mm, >2.0

mm). The sieved corn stover was stored at room temperature (~21°C) until use.

Equipment

The reactor (Figure 1) used for the ammoniation process was purchased from Pall

Corporation, Ann Arbor, Michigan, USA. The capacity of the sealed reactor was 3 L,

which was about 4.35 times larger compared to Yoo's (2011) previous study. The use

of a larger reactor may reduce the potential errors that may be caused by different

ammonia loadings and reaction times. In order to measure sugars and ethanol content,

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., 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).

Enzymes

GC 220 cellulase was purchased from Genencor International, Inc. (Rochester,

NY, USA). The cellulose 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 (Novozyme 188) was provided from Sigma-Aldrich, Inc. (St.

Louis, Missouri, USA). The activity of Novozyme 188 was 750 cellobiase units (CBU)

/ mL.

LMAA pretreatment process

Before ammoniation, different amounts of water were added to the corn stover in order to meet the required moisture content (20, 50, 80 wb %); samples were equilibrated for 24 h.

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 and a temperature gauge were equipped on the reactor to monitor the pressure and temperature during the ammoniation process. Anhydrous ammonia was added up to the targeted pressure to achieve 0.1 g NH_3/ g biomass. The whole ammoniation process lasted up to 30 min in order to have a complete reaction. Temperature changes could be observed from the temperature gauge, but it was not controlled during this study. After the ammoniation process was finished, the reactor was cooled down for 5 min, 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 (20°C, 75°C, and 120°C) for 24 h, 72 h, and 144 h. As soon as the pretreatment process was complete, the lid of the glass bottles was removed in fume hood and surplus ammonia was evaporated for 12 h.

Experimental design

In this study, four independent variables that influenced the reaction severity were investigated. Biomass moisture contents were 20 wb %, 50 wb % and 80 wb %; the pretreatment times were 24 h, 72 h, 144 h; the pretreatment temperatures were

20°C, 75°C, 120°C, and the particle sizes were <0.9mm, 0.9-2.0mm and >2.0mm, respectively. By controlling these independent variables, there were 17 treatments in this study (i.e. 2 × 2 × 2 × 2 + 1 center point). Moisture content，lignin, glucan, xylan, galactan, arabinan, mannan and ash content were measured as dependent variables during the experiment. The experimental design for this study is shown in Table 1.

Compositional analysis

Carbohydrates and lignin were determined by NREL LAP (NREL, 2008)**.** Each sample was analyzed in duplicate. The content of glucan and xylan in the corn stover were analyzed by HPLC, following the NREL standards. Acid soluble lignin was measured by UV-Visible Spectrophotometer. And moisture content was determined by an oven drying method (NREL, 2008).

Enzymatic digestibility test

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 approximately 15 FPU/g of glucan, and ß-glucosidase enzyme (Novozyme 188) loading was equal to 30 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 144 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.

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

(1)

Results and Discussion

Effects of LMAA pretreatment on biomass composition

In this study, the use of low-moisture anhydrous ammonia (LMAA) pretreatment didn't result in many significant changes in lignin, glucan, xylan, arabinose, mannan or ash contents, as shown in Table 2 (main effects) and Table 4 (treatment effects). What's more, as Table 3 shows, the majority of the p-values of interactions among these independent variables were higher than 0.05, which indicates little evidence of significant interactions among independent variables were obtained in this study. The reason for insignificant compositional analyses result was because the ammonia used in the pretreatment process was meant to separate lignin from cellulose and break down cellulose for enzymatic saccharification, not to change composition, per se.

Effects of LMAA pretreatment on enzymatic digestibility

Figure 2 shows the enzymatic digestibility results for the 17 treatments listed in Table 1, while Figure 3 compares digestibility results for avicel (used as a reaction blank for the substrate), untreated corn stover, and the best digestibility. From Figure 2, different combinations of the four factors resulted in various digestibility. As shown in Figure 3, the highest glucose digestibility (57.23%) of LMAA pretreated

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corn stover was 1.97 times compared to untreated (29.02%). Among the 17 treatments, the median treatment, which was 50% moisture content (wet base), 0.9-2.0 mm particle size, 72 h pretreatment time and 75˚C pretreatment temperature, achieved the highest glucose digestibility. In Yoo's research (2011), the optimal pretreatment condition was 80˚C for 84 h pretreatment time, which was also the median treatment in his study. Thus our results compare favorably with prior research on a smaller scale.

Four pretreatment factors were tested in this study: pretreatment temperature, pretreatment time, moisture content, and particle size. Among these factors, pretreatment temperature was selected as the most important variable, because it had the highest p-value (0.0013). Table 5 shows the differences of average glucose digestibility between high pretreatment temperatures and low temperatures, while other factors were kept constant (i.e., main effects). As can be seen from the table, the highest temperature resulted in decreased digestibility in this study.

In terms of pretreatment time, the difference between longer time and shorter time was also significant (Table 5). The average glucose digestibility at 168 h was considerably lower than the average for 24 h pretreatment time. Based on these results, there was an average of 92.7% increase from 6 h to 18 h, which was the maximum rate of increase during all the enzymatic digestibility tests. The reason for this may be longer pretreatment times cause the collapse of the structure of corn stover.

As for moisture content, it was observed that with higher moisture content, corn stover resulted in lower glucose digestibility. The reason for this may be the reduction

of retaining ammonia with higher moisture, which may result in lower delignification. As for the effect of particle size, as shown in Table 5, there were some differences between small size and large size corn stover, with larger stover pieces being somewhat more digestible.

Conclusions

In this study, the effect of LMAA pretreatment under various conditions was explored. As expected, LMAA pretreatment has the potential to achieve higher glucose yield. When corn stover (50 wb% moisture content) was pretreated at 75˚C and 96 h, the maximum glucose yield was obtained. What's more, LMAA pretreatment may eliminate water consumption because there was no washing step during this study.

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Table 1. Experimental design.*

Treatment	Moisture Content (wb %)	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	144	20	<0.9
6	20	144	20	>2.0
7	20	144	130	<0.9
8	20	144	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	144	20	<0.9
14	80	144	20	>2.0
15	80	144	130	<0.9
16	80	144	130	>2.0
CP	50	72	75	0.9-2.0

*CP denotes center point of the design

Table 2. Main effects on resulting compositional analysis. *

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)	3.7a (0.47)	0.05a (0.05)	1.67a (0.69)
	75	21.2ab (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 (0.3)
	130	21.36b (0.8)	17.83b (0.87)	3.54c (0.57)	37.08a (2.86)	22.47ab (1.77)	0.83a (0.4)	3.88a (0.53)	0.04b (0.02)	2.2b (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	21.2ab (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 (0.3)
	168	21.33b (0.55)	17.42a (0.72)	3.91a (0.5)	37.43b (2.26)	21.92ab (2)	0.75a (0.34)	3.83a (0.32)	0.04b (0.02)	1.93a (0.66)
Moisture Content (wb%)	20	21.12a (0.95)	17.32a (1.08)	3.8a (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	21.2a (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 (0.3)
	80	21.1a (0.64)	17.36a (0.81)	3.73a (0.65)	37.27a (2.95)	21.79b (2.33)	0.69a (0.27)	3.7a (0.35)	0.03b (0.02)	2.08a (0.68)
Size	S	21.31a (0.92)	17.56a (1.12)	3.75a (0.6)	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 (0.3)
	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 at α=0.05, LSD, for that dependent variable. Values in parentheses are standard deviation. S denotes size less than 0.9 mm, M denotes size between 0.9-2.0 mm, while L denotes size larger than 2.0 mm. AIL = Acid Insoluble Lignin, ASL = Acid Soluble Lignin.

Table 3.   Interaction effects on resulting compositional analysis (p-values). *

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	<.0001	0.089
Size	<.0001	0.150	0.865	0.097	0.008	0.418	0.072	0.807	<.0001
Temp*Time	0.437	0.793	0.772	0.546	0.283	0.618	0.010	<.0001	0.437
Temp*MC	0.285	0.110	0.065	0.426	0.466	0.672	0.036	<.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 on resulting compositional analysis. *

Treatment	Lignin (%)	AIL (%)	ASL (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinose (%)	Mannan (%)	Ash (%)
1	20.01de	16.01d	4.00a-c	30.035c	16.365c	0.47c	2.895c	0.13a	2.225a-d
2	19.78e	16.22cd	3.56bc	34.435bc	23.34ab	1.195ab	3.685bc	0.12a	0.57e
3	21.46a-c	17.86a-c	3.6bc	36.685ab	20.61bc	0.55c	3.51bc	0.03cd	2.345a-c
4	21.02b-e	16.88b-d	4.135a-c	38.795ab	23.19ab	0.56c	3.7bc	0.025cd	1.805cd
5	20.99b-e	17.03b-d	3.955a-c	36ab	21.11a-c	0.785bc	3.945b	0.03cd	2.195a-d
6	20.94c-e	17.09a-d	3.845a-c	37.51ab	23.8ab	0.585c	3.975b	0.035b-d	1.475c-e
7	21.31a-c	16.76b-d	4.54ab	34.6a-c	20.12bc	0.645bc	3.92b	0.01d	1.47c-e
8	21.36a-c	16.99b-d	4.36ab	37.81ab	22.25ab	0.585c	3.995b	0.025cd	1.285de
9	22.465a	18.775a	3.685bc	35.145a-c	22.49ab	0.895bc	3.955b	0.035b-d	2.19a-d
10	21.12b-d	17.27a-d	3.845a-c	35.865ab	24.09ab	0.54c	4.92a	0.03cd	1.615cd
11	20.79c-e	17.78a-c	3.005c	37.1ab	22.04ab	0.56c	3.46	0.015d	2.795ab
12	20.47c-e	17.33a-d	3.135c	34.97a-c	23.01ab	1.22ab	3.885b	0.035b-d	1.955b-d
13	22.19ab	18.375ab	3.815a-c	37.09ab	21.45ab	1.52a	3.8b	0.03cd	2.295a-c
14	21.46a-c	17.78a-c	3.7bc	38.235ab	23.52ab	0.55c	3.89b	0.05bc	1.805cd
15	21.29a-c	17.89a-c	3.405bc	38.175ab	21.19a-c	0.91bc	3.72bc	0.065b	3.005a
16	21.11b-d	17.41a-d	3.695bc	40.045a	21.97ab	0.455c	3.43bc	0.035b-d	1.96b-d
CP	21.2b-d	16.23cd	4.97a	38.895ab	25.59a	0.55c	4.31ab	0.015d	1.955b-d

*Similar letter after means in each treatment indicates insignificant difference at α = 0.05, LSD, for the dependent variable.

CP denotes center point in this study. AIL = Acid Insoluble Lignin, ASL = Acid Soluble Lignin.

**Table 5.** **Main effects on enzymatic 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)

Figure 1.   Ammoniation reactor.
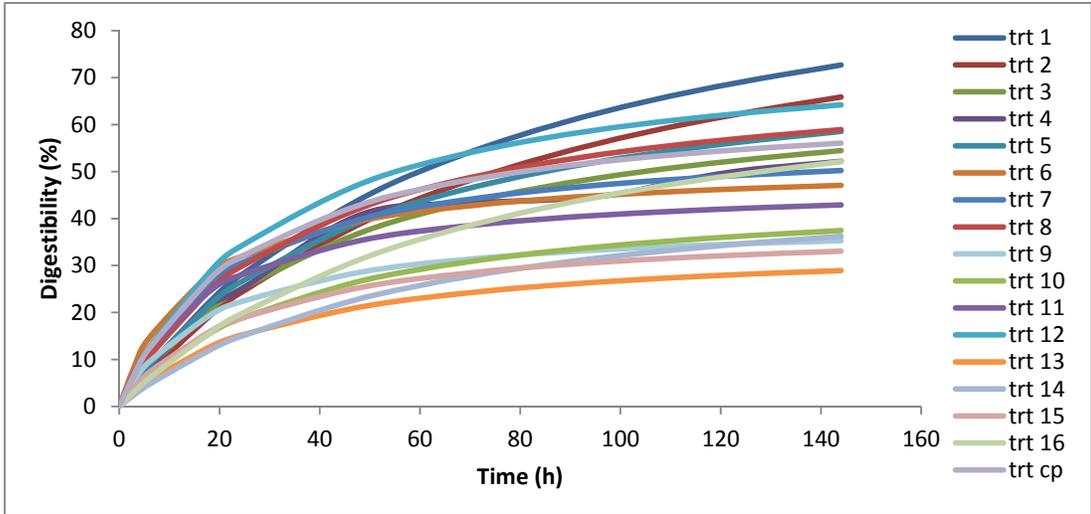
Figure 2. Enzymatic digestibility results for all treatments. Trt denotes treatment; CP denotes center point.
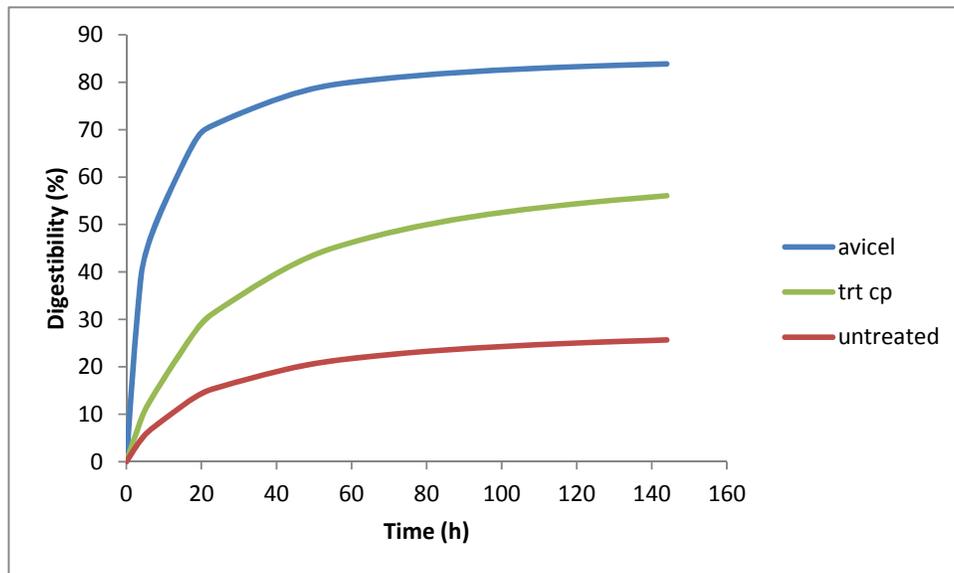
Figure 3. Enzymatic digestibility results for avicel, untreated corn stover, and maximum LMAA-treated corn stover (treatment cp). Trt denotes treatment; CP denotes center point.