Separate Hydrolysis and Fermentation of Untreated and Pretreated Alfalfa Cake to Produce Ethanol

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Abstract The use of lignocellulosic biomass to produce biofuel will add value to land and reduce emissions of greenhouse gases by replacing petroleum products. Valuable co-products derived from fractionation of alfalfa (Medicago sativa) give the resulting fibrous fraction an economic advantage as a feedstock for ethanol production. Freshly harvested alfalfa was dewatered using centrifugation and filtration, whereby alfalfa is separated into a fiber-rich cake and a nutrient-rich juice. Alfalfa solids was pretreated with alkaline soaking (1, 4, and 7 %) at room temperature to evaluate the effects on cellulose digestibility. The production of cellulosic ethanol from alfalfa fibers were investigated by this work using separate hydrolysis and fermentation (SHF). Results show the alkali pretreatment was able to effectively increase cellulosic digestibility of alfalfa solids. A maximal glucose yield of 61 % was obtained with filtered solids with 1 % NaOH pretreatment. The filtration process resulted in a solid fraction with a higher cellulose digestibility, which leads to a higher ethanol production.

Introduction

Declining fossil oil reserves, skyrocketing price, unsecured supplies, and environmental pollution are among the many energy problems we are facing today. These problems necessitate the development of alternative fuels such as biofuels (Xiu et al. 2010). One technology for doing so is the conversion of under-utilized lignocellulosic biomass sources, such as agricultural wastes, forest residues, and dedicated energy crops, into liquid fuel and chemicals that can partially replace petroleum and petrochemicals. However, the recalcitrance of lignocellulosic biomass to chemical and enzyme conversion hinders efficient production of cellulosic

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ethanol. Therefore, a suitable pretreatment process is important to reduce the recalcitrance and to make bioconversion processes more efficient, economic, and environmentally friendly.

Alkaline pretreatment is one of the current leading chemical pretreatment methods, particularly for dissolving lignin. In addition, acetyl groups and various uronic acid substitutes, which lower susceptibility of hemicelluloses and cellulose to hydrolytic enzymes, are also removed by alkaline pretreatment (Mosier et al. 2005). Agricultural residues and herbaceous crops have been shown to be more suitable to alkaline pretreatment than woody biomass (Galbe and Zacchi 2007; Wan et al. 2011). The most commonly used alkali base is NaOH (Li et al. 2004). Alkali pretreatment process has the advantages of utilizing lower temperatures and pressures compared to other lignin removal technologies (Zhang et al. 2010).

Alfalfa is a leguminous perennial crop that does not require either synthetic nitrogen fertilizer or yearly planting and tilling. Thus, the fossil energy inputs to produce a given amount of alfalfa are much less than for annual, non-leguminous species. Alfalfa is widely grown in the USA and has relatively high dry matter yields ranging from about 7 to 23×10^3 kg/ha-year. The technology for growing, harvesting, transporting, and storing alfalfa is already in place in US agriculture. This is an important advantage over any new energy crop.

The primary objective of this ongoing research is to evaluate the efficacy of separation methods and alkali pretreatment on alfalfa solids in terms of enzymatic digestibility. In this study, freshly harvested alfalfa from the North Carolina A&T State University farm was dewatered using centrifugation and filtration. The resulting solid cakes from the two processing methods were collected and processed with or without an alkali pretreatment process, followed by enzymatic hydrolysis. The bacteria Escherichia coli (*E. coli*) was then used to test the fermentability of the sugars enzymatically degraded from alfalfa cellulose.

Materials and Experimental Methods

Grass Harvest and Processing. Two maturities of alfalfa were harvested from existing fields on the NC A&T State University farm. Grass was hand-harvested to an average stubble height of 6 cm for immature and mature alfalfa and immediately transported to the laboratory. Subsamples were taken for assessment of dry matter content. Freshly harvested alfalfa stems were reduced in size using scissors. The biomass was then mixed with water and chopped in a commercial food processor, resulting in a mash with water: biomass ratio of 2:1. Subsequent juice separation was conducted with either a centrifuge (Centra-GP8R Centrifuge, ThermoIEC) or normal filtration (GE WhatmanTM, folded filters, diameter 240 mm). The centrifugation was carried out at a rotational speed of 3600 rpm for 10 min at 25 °C. The resulting juice from the two operations was collected and characterized for its potential in value-added processing and co-products

generation. The solid fraction was also dried at 105 °C for 24 h for chemical analysis. Subsamples of the green juice and solid cake were used fresh or kept in a freezer at −80 °C for downstream processing.

To determine how well each separation method would work, the performance of centrifugation and normal filtration was evaluated using the solid content of juices separated as well as the cellulose digestibility. A low solid content of the juice separated and a high cellulose digestibility from the solids fraction are desirable. All the experiments and analyses were performed in duplicate.

Chemical Analyses and Mass Flow Calculation. The alfalfa (parent material) and the solid cake fraction after extracting the juice were analyzed for elemental composition (e.g., C, H, O, N), ash content, solids content, volatile content, and carbohydrates (cellulose, hemicellulose, lignin). Two stages of acid hydrolysis were performed for determining the carbohydrate composition on the alfalfa samples according to NREL Ethanol Project Laboratory Analytical Procedure (Ruiz et al. 1996). The concentrations of ash and carbohydrates in the press juice can be calculated from the proportions of solid fraction and green juice in the alfalfa after separation. In addition to chemical analyses, the mass flow of dry matter from the alfalfa into the green juice and solid cake were calculated. The dry matter of all subsamples of the alfalfa, the solid cake and the green juice was determined by oven-drying at 105 °C for 24 h.

The elemental composition (C, H, O, N) of the alfalfa and the solid cake samples was determined using a PE 2400 II CHNS/O analyzer (Perkin Elmer Japan Co., Ltd.). The solids content analysis was determined using the APHA-AWWA-WPCF Standard Method 2540, which includes total solids (TS), volatile solids (VS), and fixed solids (FS).

Pretreatment of the feedstock. About 50 g of wet alfalfa solid was soaked in 0.25 L of NaOH at various concentrations (1, 4, 7 %) and left at room temperature for 24 h. The mixture was then centrifuged at 2600RCF for 20 min, the supernatant was decanted and the pellet was rinsed with water six times and twice with 0.05 M citric acid buffer (Ph 4.8). Samples were centrifuged and supernatants decanted between rinses.

Enzymatic Hydrolysis. Enzymatic hydrolysis tests were carried out under the same conditions for both the unpretreated and pretreated samples. A control was prepared with an identical amount of raw alfalfa parent material. The total amount of glucose released after 48 h of hydrolysis was measured to calculate the enzymatic digestibility. The conditions of the enzymatic hydrolysis were as follows: about 4.5 g of wet alfalfa was mixed with 0.05 M citrate buffer (pH 4.8) to a total volume of 50 mL. Screw-capped 250-mL Erlenmeyer flasks were used as reaction vessels and were agitated at 180 rpm in a constant temperature rotary shaker at 50 °C for 96 h. The samples were hydrolyzed using a cocktail of enzymes, which included cellulose loading (Novozyme, NS50013) of 25 FPU g/glucan, β-glucosidase (Novozyme, NS50010) at 4.5 CBU/g-glucan, and hemicellulose (Novozyme,

NS22002) at 2.5 FBG/g-glucan. After 96 h, the pretreated slurry was cooled and filtered for sugar determination using HPLC.

Fermentation. The bacterium *Escherichia coli* (*E. coli*) was used to ferment the enzymatically released sugars. For ethanol production, 1 mL of seed culture was used to inoculate 4 mL of Luria-Bertani Broth (LB) medium in a 250-mL Erlenmeyer flask. These were incubated in a shaker at 32 °C and 180 rpm and grown aerobically for 24 h. After 24 h, the cultures were transferred into 50 mL of the medium and the yeast was harvested by centrifugation at 2600 RCF for 15 min and washed with peptone solution three times. The supernatant was discarded, and the cells were transferred into 250-mL Erlenmeyer flasks containing 50 mL of the hydrolysate. The flasks were tightly closed to allow for the fermentation to occur under anaerobic conditions. The cultures were placed in a shaker and incubated at 30 °C for 72 h. Samples were taken at predetermined intervals (0, 3, 24, 72, and 96 h) and collected by filtering through 0.45-μm nylon membranes for ethanol and sugars analysis by HPLC. The ethanol yield was expressed as the percentage of the theoretical yield using the following formula:

$$\text{Yield}_{\text{ethanol}} = \left[\frac{C_{\text{ethanol},f} - C_{\text{ethanol},i}}{0.568f \cdot C_{\text{biomass}}}\right] \times 100\,\% \tag{1}$$

where $C_{\text{ethanol},\,f}$ is the ethanol concentration at the end of the fermentation (g/L), $C_{\text{ethanol},\,i}$ is the ethanol concentration at the beginning of the fermentation (g/L), C_{biomass} is the dry biomass concentration at the beginning of the fermentation (g/L), f is the cellulose fraction of the dry biomass (g/g), and 0.568 is the conversion factor from cellulose to ethanol.

Results and Discussion

Mass Flows into Juice and Solid Cake. Approximately 18–27 % of the dry matter contained in the raw alfalfa parent material (PM) was directed into the extracted juice during the centrifuge separation, while 73–82 % was left in the solid cake fraction (Fig. 1). For the filtration process, the mass flow of the dry matter into the juice was between 7 and 16 %, depending on the maturity of the alfalfa. Mature alfalfa results in better separation results with both separation methods than the immature alfalfa.

Compositions of the separated alfalfa solids. The compositions of the separated alfalfa solids are listed in Table 1. The composition of the fresh harvest alfalfa is also reported in Table 1 for comparative purposes. One of the most notable differences between the mature alfalfa and immature alfalfa is the significantly higher total solids content and lignin content of the mature alfalfa. Differences also

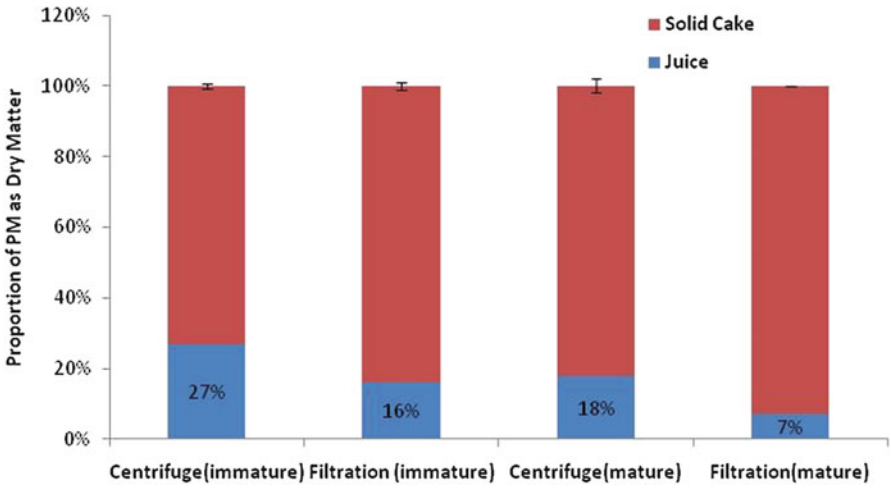

Fig. 1 Mean values of proportions of parent material (PM) that contribute to the mass flow of dry matter (DM) in the juice and solid cake for mature and immature alfalfa with different separation methods

Table 1 Characteristics of raw alfalfa and solid cakes

Group/specific	Mature alfalfa	Immature alfalfa	Immature alfalfa	Immature alfalfa
Separation method	None	None	Centrifuge	Filtration
Solids content				
Total solids, %wt	21.74	16.28	7.29	8.96
Volatile solids, % dry matter	93.01	89.19	91.89	94.28
Ash, % dry matter	6.99	10.81	8.11	5.72
H_2O, %wt	78.26	83.72	82.08	82.30
Composition of dry matter %wt				
Hemicelluloses	17.87	15.59	17.49	6.8
Cellulose	36	35.29	29.3	20.26
Lignin	21.59	14.35	17.78	15.62
Element group (%)				
C	45.06	45.41	45	44.3
H	6.26	6.09	6.39	6.18
O	41.53	42.48	39.9	43.53
N	6.18	5.29	7.76	5.11
S	0.97	0.73	0.95	0.88

existed in the carbohydrates group among these samples. For example, the hemicelluloses content was much lower in immature alfalfa solids with filtration, compared to the other alfalfa, with a value of 6.8 %. The elemental composition is very similar for all of the alfalfa samples.

Enzymatic Hydrolysis

Enzymatic hydrolysis of alfalfa cakes without NaOH pretreatment. Enzymatic hydrolysis was performed to evaluate the cellulose and xylan digestibility of centrifuge solids (CS) and filtered solids (FS) without pretreatment. Raw mature alfalfa was employed as a control. As shown in Fig. 2, the glucose yield increased with enzymatic hydrolysis time and began to level off after 72 h. More glucose was released from separated alfalfa solids than from the control experiment using raw mature alfalfa as a feedstock. The highest glucose yield of 50 % was obtained at 96 h from the FS, 80 % higher than the glucose yield of raw alfalfa. These results suggest that the separation process has a significant impact on the cellulose digestibility of raw alfalfa. The filtration process resulted in a solid fraction with a higher cellulose digestibility.

Figure 3 shows the xylose yield from enzymatic hydrolysis of CS and FS. As seen in Fig. 3, the xylose yield was improved using both separation methods. The FS produced the highest xylose yield, equivalent to 23 % of theoretical yield. Overall, compared to centrifuge separation of alfalfa, the filtration process resulted in higher cellulose and xylose digestibility in the separated solids.

Comparison of alkali pretreatments. The yields of glucose and xylose from enzymatic hydrolysis of alkali pretreated alfalfa solids are shown in Table 2. In comparison with the alfalfa solids without NaOH pretreatment, the glucose yield obtained from both CS and FS with alkaline pretreatment was increased significantly due to a synergistic effect of degradation of hemicelluloses and lignin. However, a slight decrease in glucose yield at higher alkali loading was observed,

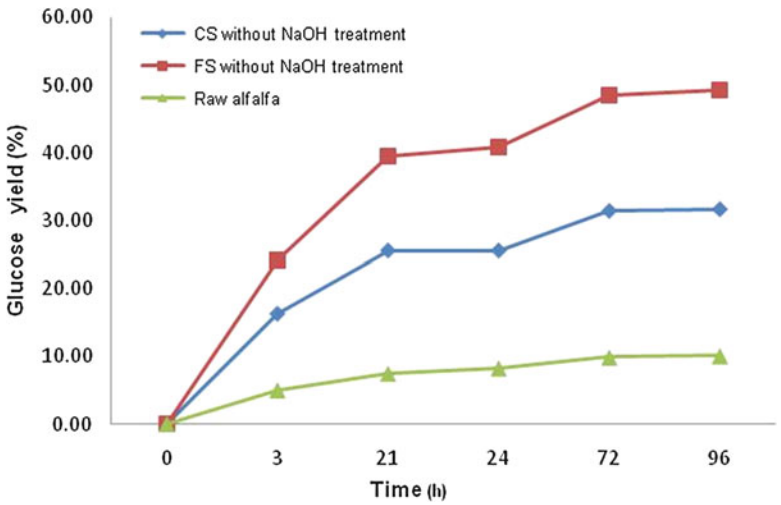

Fig. 2 Glucose yield after enzymatic hydrolysis of centrifuged solids and filtered solids without NaOH treatments (*FS* filtered solid, *CS* centrifuged solid)

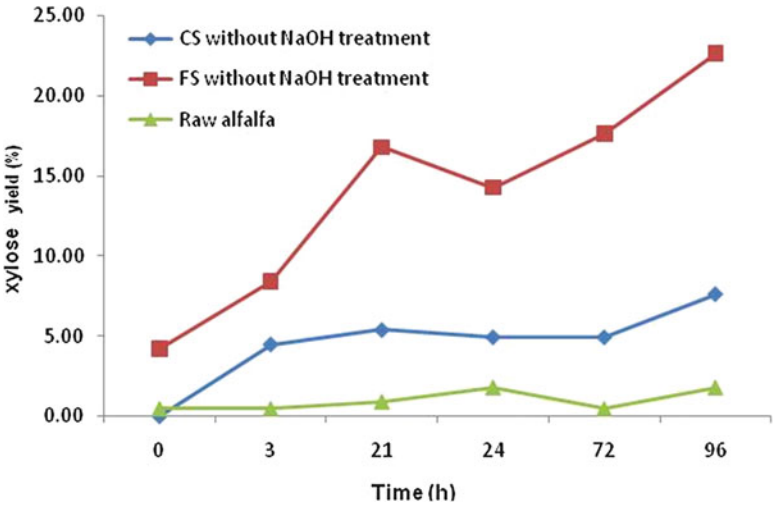

Fig. 3 Xylose yield after enzymatic hydrolysis of centrifuged solids and filtered solids (*FS* filtered solid, *CS* centrifuged solid)

Table 2 Effect of NaOH concentration on the glucose and xylose yields of pretreated alfalfa solids

Biomass type	Sugar yield (%)	0 % NaOH	1 % NaOH	4 % NaOH	7 % NaOH
CS	Glucose	31.71	48.8	49.63	48.05
	Xylose	7.62	21.43	22.75	23.26
FS	Glucose	49.3	60.56	58.59	50.25
	Xylose	22.66	42.88	45.51	46.53

probably due to the high severity pretreatment conditions may lead to undesired sugar loss through dissolution and degradation of hemicelluloses (Chen et al. 2013). The highest glucose of 60.25 % was obtained at 1 % alkali loading for the FS. The xylose yield was increased by alkaline pretreatment as the NaOH concentration was increased. The pretreated FS has higher cellulose digestibility than the pretreated CS samples, which consists with the untreated samples.

Fermentation of Alfalfa Solids for Ethanol Production. The ethanol yield was calculated according to (1). The final ethanol yields for FS and CS without pretreatment were 75 % and 51 %, respectively. These results suggest that the glucose and xylose produced from alfalfa separated solids can be efficiently fermented to ethanol.

Conclusions

1. In comparison with the centrifugal process, normal filtration proved to be more efficient at reducing the solids mass transfer to the juice.
2. The separation process has a significant impact on the cellulose digestibility of raw alfalfa. The filtration process resulted in a solid fraction with a higher cellulose digestibility.
3. Alkali pretreatment improved the enzymatic digestibility of alfalfa solids. Filtered alfalfa solid pretreated with 1 % NaOH produced the highest glucose yield of 61 %.
4. Glucose and xylose released from filtered alfalfa solids can be efficiently fermented to ethanol using *E. coli*, resulting in approximately 75 % of the theoretical ethanol yield.

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