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Soaking in Aqueous Ammonia (SAA) Pretreatment of Whole Corn Kernels for Cellulosic Ethanol Production from the Fiber Fractions

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Abstract: Corn fiber is a co-product of commercial ethanol dry-grind plants, which is processed into distillers dried grains with solubles (DDGS) and used as animal feed, yet it holds high potential to be used as feedstock for additional ethanol production. Due to the tight structural make-up of corn fiber, a pretreatment step is necessary to make the cellulose and hemicellulose polymers in the solid fibrous matrix more accessible to the hydrolytic enzymes. A pretreatment process was developed in which whole corn kernels were soaked in aqueous solutions of 2.5, 5.0, 7.5 and 10.0 wt% ammonia at 105°C for 24 h. The pretreated corn then was subjected to a conventional mashing procedure and subsequently ethanol fermentation using a commercial strain of natural Saccharomyces cerevisiae with addition of a commercial cellulase. Pretreatment of the corn with 7.5 wt% ammonia solution plus cellulase addition gave highest ethanol production, which improved the yield in fermentation using 25 wt% solid from 334 g ethanol/kg corn obtained in the control (no pretreatment and no cellulase addition) to 379 g ethanol/kg corn (a 14% increase). The process developed can potentially be implemented in existing dry-grind ethanol facilities as a "bolt-on" process for additional ethanol production from corn fiber, and this additional ethanol can then qualify as "cellulosic ethanol" by the EPA's Renewable Fuels Standard and thereby receive RINS (Renewable Identification Numbers).

Keywords: Ethanol; corn; dry-grind process; bolt-on process; corn fiber; soaking in aqueous ammonia pretreatment; cellulase; cellulosic ethanol.

1. Introduction

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The growing demand for renewable fuels comes in response to the global crisis of relieving dependency on petroleum-based resources. Depletion of oil and other fossil fuels has shifted the focus to renewable resources to assist in the demand for fuel production [1]. Fossil fuels account for approximately 80% of the world's energy consumption; another 12% comes from renewable resources with biomass-derived fuels accounting for half of that [2, 3]. Corn starch is the primary feedstock used in the production of ethanol, a biomass-based fuel [4]. In 2016, ethanol produced from corn starch accounted for 95% of all biofuel production in the US. However, ethanol only accounts for about 10% of the total gasoline fuel consumption in the US [3]. Developing methods so as to increase ethanol yield as an alternative fuel are being studied to assist in the progression of the fuel industry from fossil fuels to renewable fuels.

Corn fiber makes up about 9% of the composition of the corn kernel on dry basis [5]. This fiber is comprised of lignocellulose material that acts as the structural make-up of the pericarp, or outer

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kernel covering, and the cell wall of the endosperm [6]. This aspect of the kernel consists of 18% cellulose, 35% hemicellulose, and 20% starch [7]. Corn fiber has potential to be utilized to produce additional starch-based ethanol as well as cellulosic ethanol, but the rigid, tightly packed structural composition poses a problem for accessibility to the non-starch polysaccharides for enzymatic hydrolysis into the fermentable sugars glucose and xylose [6]. With modification to the structure of corn fiber, this fraction of the corn kernel has potential to be utilized in a more economical sense. Thus, developing a process in which the corn fiber is converted to fermentable sugars is needed.

In 2005, the Environmental Protection Agency (EPA) introduced the Renewable Fuel Standard (RFS). Each year, the RFS sets a statutory requirement mandate for volume of renewable fuel produced in the US. The end of the 2016 marketing year saw production for biofuel from cellulosic feedstock of 0.16 billion gallons of cellulosic ethanol [8], which pales in comparison to the RFS set mandate of 4.5 billion gallons of cellulosic ethanol [9]. The potential for additional corn-starch ethanol and cellulosic ethanol production from corn fiber would not only help achieve the RFS standard, but would also give renewable fuel producers the opportunity to accumulate supplementary Renewable Identification Numbers (RIN) [10]. Renewable fuel producers must meet individual fuel volume requirements by acquiring Renewable Identification Numbers (RINs), which are unique values assigned to each gallon of renewable fuel generated [11]. Dry-grind ethanol plants that use the "bolt-on" technology proposed in this study to produce additional corn-starch ethanol and cellulosic ethanol would acquire RIN credits for conventional biofuel (D-code 6) as well as for cellulosic biofuel (D-code 3) [12].

Pretreatment methods have been studied to determine the most effective way to disrupt the structure of the non-starch polysaccharides found in corn fiber [13]. Through alkali pretreatment, the cellulose and hemicellulose structures can be disrupted and degraded to allow access of enzymes for enzymatic hydrolysis into glucose and xylose for fermentation to ethanol [14]. Soaking in aqueous ammonia (SAA) is a relatively low cost, high conversion efficiency method for pretreatment of corn [15]. The SAA is a batch process that can be easily implemented in dry-grind ethanol facilities by "bolting-on" to the already existing facility structure.

Approximately 90% of total ethanol yield in the United States is produced from dry-grind ethanol facilities [9]. Dry-grind ethanol plants reduce capital cost by milling the entire kernel as opposed to fractionating the corn prior to processing, as is the case for wet milling [16]. Existing dry-grind plants only convert the starch fraction of corn to ethanol. The remaining non-starch fractions, the proteins and fibers, can be processed and dried as distiller's dried grains with solubles (DDGS). This coproduct is primarily sold as high-protein animal feed, and while there is a market for this product, there is a larger demand for increased ethanol yields [17]. Dry-grind ethanol plants can be modified to produce a higher yield of conventional ethanol as well as cellulosic ethanol by adding a "bolt-on" process that converts corn fiber to fermentable sugars. The "bolt-on" process will be a first-step process in dry-grind plants consisting of SAA pretreatment of the corn kernel.

2. Materials and Methods

2.1. Materials

Whole kernel corn was purchased from Tractor Supply Co. (Harleysville, PA, USA). The corn was hand-cleaned of debris and broken pieces and stored at 4°C prior to processing.

The enzymes were kindly provided by DuPont Industrial Biosciences (Palo Alto, CA, USA), which included Spezyme[®] Extra (reported activity of 14,000 α -amylase (AA) units per g), Fermenzyme[®] L-400 (reported activity of 350 glucoamylase (GA) units per g) and Accellerase[®] 1500 (reported endoglucanase activity of 2200-2800 carboxymethylcellulose (CMC) units per g and β -glucosidase activity of 525-775 para-nitrophenyl- β -D-glucopyranoside (pNPG) units per g).

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The microorganism used for ethanol fermentation was Red Star *Saccharomyces cerevisiae* yeast strain purchased from Lesaffre Yeast Corporation (Milwaukee, WI, USA). The dried yeast was stored at 4 °C.

The concentrated ammonium hydroxide solution (28-30 wt %) was purchased from J.T.Baker[®] (Center Valley, PA, USA). Other chemicals were purchased from various suppliers and were of reagent grade.

2.2. Methods

2.2.1. Pretreatment of corn kernels

Approximately 300 g (260g dry mass) of whole corn was soaked in 300 mL of ammonium hydroxide solutions having ammonia contents of 2.5, 5, 7.5, and 10 wt % in 1000-mL glass screw-cap bottles. The ammonium hydroxide solutions were prepared by dilution of the purchased concentrated solution with appropriate amounts of deionized (DI) water. The bottles containing the corn/ammonium hydroxide mixtures were placed in an oven set at 105 °C for 24 h. After the pretreatment period, the liquids were filtered off and the pretreated corn rinsed with DI water until the wash water was clear. The filtrate and wash water were collected, and the pretreated corn left in a fume hood at ambient temperature (about 25 °C) until no ammonia odor was detected. The filtrate and wash water were combined and 50 mL was used to determine the total solid content, which subsequently was used to calculate the loss of mass from the corn during the pretreatment.

2.2.2. Ethanol fermentation

The pretreated corn was air-dried at ambient temperature overnight (about 16 h) before it was used in the ethanol fermentation experiments. The moisture content of the pretreated corn was determined (see the analytical section below) and used to calculate the quantity needed to prepare a 25 wt % corn mash (dry basis). The pretreated corn was ground using a Krups Type 203 coffee grinder (Parsippany, NJ, USA). The ground corn was transferred to a stainless steel beaker and brought to 25 wt % solids using DI water. The beaker was placed in an oil bath and its content was vigorously mixed with a mechanical agitator. The pH of the mash was adjusted to 5.6 with 2 N H₂SO₄ and Spezyme Extra (a thermostable starch hydrolyzing α -amylase) was added at 0.3 g/kg dry solids. The mash was maintained at 60 °C for 2 h, then the temperature increased to 90 °C and maintained for 1 h. DI water was added intermittently during the corn liquefaction to account for loss of water due to evaporation. The beaker was then removed from the oil bath and weighed. DI water was added as necessary to bring the mash back to 25 wt % solids. The beaker was placed in an ice bath and allowed to cool to approximately 55 °C. The pH of the mash was adjusted to 4.0 using 2 N H₂SO₄ before it was split into two approximately equal batches. Fermenzyme L-400 (a glucoamylase and protease mix) was added to both batches at 0.65 g/kg dry solids. Accellerase 1500 (cellulase) then was added to only one batch at 0.25 g/g dry solids. Each batch was thoroughly mixed and then distributed evenly into six 250-mL shaker flasks. A 5 wt % yeast slurry was prepared in DI water and stirred at ambient temperature for about 30 min. The rehydrated yeast slurry then was added to the flasks at 0.25 mL solution per 50 g mash. The flasks were corked with rubber stoppers punctured by small hypodermic needles for release of CO₂. The flasks were incubated at 32°C at 200 rpm for 72 h. Weight loss due to CO₂ production was determined by weighing the flasks at intervals. The weight loss was used to follow the progress of ethanol fermentation. At the end of the fermentation, samples were taken and centrifuged at 13,200 rpm for 3 min (Eppendorf model 5415D, Hauppauge, NY, USA.) The supernatant was filtered through 0.2 micron filters and used for HPLC analysis. In a separate experiment using corn treated with 7.5 wt % ammonia and with Accellerase 1500 addition, urea was also added to the mash at 0.4 g/kg mash. Experiments using untreated corn were performed in the same manner without and with addition of Accellerase 1500. Urea was added

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at 0.4 g/kg mash in both experiments. The results obtained in the experiment using untreated corn and without Accellerase 1500 addition were used as the baseline for a dry-grind ethanol process.

2.2.3. Analytical methods

The starch content of the untreated corn was determined by the modified Megazyme total starch assay procedure (amyloglucosidase/ α -amylase method) [18]. The ground corn samples were analyzed in triplicate. The average starch content of the untreated corn was determined to be 67.97 wt % on dry basis.

To determine the composition of the corn fiber the ground corn was first subjected to starch removal. Thus, a mash of 1000 g total weight at 5 wt % dry solids was prepared. Spezyme Extra and Fermenzyme L-400 were added at 5X the dosages described in the preparation of the ethanol fermentation corn mash previously (section 2.2.2). The mixture was placed in a 2-L flask, which was incubated at 55 °C and 250 rpm overnight (about 16 h). The solids were collected by centrifugation, washed three times with DI water, and dried in a 130 °C oven. The dried solids then were subjected to the NREL compositional analysis procedure [19]. The composition of the de-starched corn fiber is shown in Table 1.

Table 1.	Composition	of the	de-starched	corn fiber

Component	Content (wt %, dry basis)		
Glucan	17.51 ± 0.47		
Xylan	14.00 ± 0.46		
Arabinan	8.65 ± 0.32		
Lignin	20.09 ± 0.15		
Ash	0.16 ± 0.02		

The total solid contents of the combined ammonia solution filtrate and wash water obtained during the pretreatment of corn were determined by placing a pre-weighed 50 mL sample in an oven set at 105 °C for 24 h. The weights of the dried solids then were determined and the total solid contents calculated.

The moisture content of the untreated and treated corn was determined by drying about 3-5 g of material in a moisture balance (model MB45, Ohaus, Parsippany, NJ, USA).

Fermentation samples were analyzed for ethanol and other metabolites by HPLC. The HPLC was an Agilent 1200 series utilizing the Bio-Rad Aminex HPX-87H column (Hercules, CA, USA). Analysis conditions were as follows: 65 °C column temperature with 0.6 mL/min flow rate of 5 mM H₂SO₄ solvent.

3. Results

3.1. Aqueous ammonia treatment of corn

The results of mass loss during aqueous ammonia treatment of the whole corn kernels are summarized in Table 2. The mass loss increased when the ammonia concentration was increased but subsequently leveled off after the ammonia concentration reached 7.5 wt %. Ammonia normally does not cause degradation of starch and has been used extensively in preservation of corn in silo storage. In fact, ammonia was found to reduce the rates of microbial starch degradation due to its anti-microbial activity [20]. In aqueous ammonia pretreatment of corn fiber and other lignocellulosic materials, lignin was extensively removed whereas high degrees of preservation of structural carbohydrates, in particular, glucan, were observed. The production rates and yields of fermentable sugars by subsequent enzymatic hydrolysis of the pretreated materials were significantly increased

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[21, 22]. In the present study, the highest mass loss, which was observed at 7.5 and 10 wt % ammonia, was only 5.4 wt % of the original mass.

Table 2. Mass loss during aqueous ammonia pretreatment of corn.

Concentration of NH4OH	2.5	5	7.5	10	
solution (wt %)					
Mass of starting corn	263.43	260.40	260.66	260.48	
(g dry basis)					
Mass of treated corn after	256.46	252.42	246.65	246.47	
washing (g dry basis)					
Mass loss	2.65	3.06	5.37	5.38	
(% of original mass)					

3.2. Ethanol fermentation

To illustrate the progress of ethanol fermentation, the weight loss due to CO₂ production obtained in the experiment using corn treated by 7.5 wt % ammonia solution and with addition of cellulase at the start of the fermentation is plotted against time in Figure 1. The rate of ethanol production, which was directly related to the rate of CO₂ production, was very high initially. Ethanol production gradually slowed down and eventually stopped due to exhaustion of substrate as well as ethanol inhibition. The progress of fermentation in other experiments followed similar trends. The curve shown in Figure 1 clearly indicates that the fermentation was completed at 72 h. Based on this observation, all fermentation experiments were terminated and final samples were taken at 72 h as mentioned previously in section 2.2.2.



Figure 1. Weight loss due to production of CO_2 in fermentation experiment using corn treated with 7.5 wt % ammonia solution.

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The final ethanol concentrations and the calculated yields are summarized in Table 3. To account for the effect of mass loss during pretreatment and to allow for a more realistic comparison from a process standpoint, the yields were re-calculated on the basis of g ethanol produced per kg raw (untreated) corn and showed in the last column.

Table 3. Final ethanol concentrations and calculated yields in control experiment and experiments using aqueous ammonia-treated corn.

Experiment	Final ethanol concentration (g/L)	Yield (g ethanol/kg treated corn)	Yield (g ethanol/kg raw corn)
Corn treated with 2.5 wt %	103.91 ± 1.05	349.9 ± 3.1	340.7 ± 3.0
ammonia without cellulase			
Corn treated with 2.5 wt %	106.31 ± 0.76	359.3 ± 2.2	349.7 ± 2.2
ammonia with cellulase			
Corn treated with 5 wt %	108.79 ± 0.24	369.0 ± 0.7	357.7 ± 0.7
ammonia without cellulase			
Corn treated with 5 wt %	110.83 ± 0.58	377.0 ± 1.7	365.5 ± 1.6
ammonia with cellulase			
Corn treated with 7.5 wt %	112.16 ± 1.40	382.3 ± 4.1	361.7 ± 3.9
ammonia without cellulase			
Corn treated with 7.5 wt %	116.84 ± 0.36	401.0 ± 1.1	379.5 ± 1.0
ammonia with cellulase			
Corn treated with 7.5 wt %	114.69 ± 1.0	392.4 ± 3.7	371.3 ± 3.5
ammonia with cellulase plus			
urea			
Corn treated with 10 wt %	104.86 ± 0.36	353.6 ± 2.9	334.6 ± 2.8
ammonia without cellulase			
Corn treated with 10 wt %	112.98 ± 0.54	385.6 ± 1.1	364.8 ± 1.0
ammonia with cellulase			
Untreated corn without	99.84 ± 1.67		334.2 ± 1.6
cellulase			
Untreated corn with	102.64 ± 1.25		345.0 ± 4.9
cellulase			

The SAA pretreatment combined with cellulase addition clearly gave improvement of ethanol yield over the baseline. The relationship between ammonia concentration in the SAA pretreatment and ethanol yield improvement is shown in Figure 2. Improvement of ethanol yield over the baseline increased with increasing ammonia concentration and reached the highest level of 13.6 % when 7.5 wt % ammonia was used before it declined when 10 wt % ammonia was used in the pretreatment. Thus, 7.5 wt % could be selected as the optimum ammonia concentration in the SAA pretreatment of whole corn kernels.

The results in Table 3 also indicated the beneficial effect of cellulase addition on ethanol yield. For example, in the case of 7.5 wt % ammonia pretreatment, the improvement of ethanol yield without cellulase addition over the baseline was 8.2 %, which was only about 60 % of the improvement when cellulase was added (13.6 %). In the case of the untreated corn, addition of cellulase also resulted in higher ethanol yield. The improvement in this case, however, was relatively modest at 3.2 %. The cellulase formulation used in the present study, in addition to its main function of glucose production via cellulose hydrolysis, also has been oberved for its high viscosity reduction capability (qualitative observation in the corresponding author's laboratory; unpublished results.) In

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the case of the untreated corn, very low glucose production from the corn fiber fraction was expected. The increase in ethanol yield, therefore, probably was due mainly to viscosity reduction, which assisted in better distribution of enzymes, nutrients and yeast during the fermentation. Another special characteristic of the cellulase used in the present study is it is a whole broth product that is high in residual nutrients carried over from the manufacturing process [23]. It can be seen in Table 3 that no improvement of ethanol yield was observed when an urea dosage of 0.4 g/kg, which was the level normally used in a dry-grind corn ethanol process, was added to the mash prepared with 7.5 wt % ammonia-treated corn with cellulase addition. This observation indicated that the nutrients in Accelerase 1500 were sufficient to allow ethanol fermentation to proceed at high efficiencies.



Figure 2. The relationship between ammonia concentrations used the SAA pretreatment and improvement of ethanol yield over the baseline value.

4. Discussion

A process has been developed for production of ethanol from corn fiber in addition to ethanol produced from corn starch in a conventional dry-grind process. In the newly developed process, whole corn kernels were pretreated in an aqueous ammonia solution. Following removal of residual ammonia in a washing step, the pretreated corn then was subjected to mashing and ethanol fermentation where a commercial cellulase was added to generate glucose from the glucan fraction of the corn fiber. The fiber-derived glucose was fermented to produce additional ethanol. The optimum ammonia concentration was determined to be 7.5 wt %. Ammonia is easy to recover and recycle due to its high volatility. Processes for ammonia recovery and re-use in lignocellulosic biomass pretreatment have been suggested [24]. The relatively low concentration of ammonia required in the pretreatment solution will facilitate easy regeneration of such solution. Since the newly developed process does not require complex and expensive equipment, it has strong potential to be added to an existing dry-grind corn ethanol plant as a "bolt-on" process. The improvement of

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ethanol yield over the baseline value obtained in the new ammonia-based process compared favorably to other "bolt-on" processes that has been practiced commercially. Under optimum conditions, the ethanol yield improvement over the baseline value obtained in the ammonia-based process was 13.6 %, which is higher than the values obtained in the D3Max process (11 %) [25] and the Cellerate process (6 %) [26].

Since the yeast that was used in the present study was a natural strain, the additional ethanol produced only came from the glucose derived from hydrolysis of the glucan fraction of the corn fiber. Although Accellerase 1500 contained both cellulase and xylanase activities, the highly complex structure of the hemicellulose in corn fiber severely restricted release of xylose by enzymatic hydrolysis [16]. It has been demonstrated that to obtain high yields of both glucose and xylose, sequential enzymatic and dilute sulfuric hydrolysis of corn fiber was needed [21]. To improve ethanol yield in the ammonia-based process demonstrated in the present study, future investigation should include combined enzymatic and dilute acid hydrolysis of the SAA-treated corn and the use of strains capable of fermenting both glucose and xylose at high efficiencies.

5. Conclusions

A simple pretreatment process employing soaking in aqueous ammonia of whole corn kernels has been demonstrated. When the pretreated corn was used in a typical dry-grind process with addition of a commercial cellulase, significant improvements of ethanol yield over the baseline value were observed. Although the newly developed process has strong potential for implementation as a "bolt-on" process in an existing dry-grind corn ethanol plant, further development is still needed. It is highly recommended that future efforts should include not only yield improvement but also experimentation at pilot or semi-commercial scales.

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