

Video Article

# Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass

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## Abstract

Lignocellulosic materials are plant-derived feedstocks, such as crop residues (e.g., corn stover, rice straw, and sugar cane bagasse) and purpose-grown energy crops (e.g., miscanthus, and switchgrass) that are available in large quantities to produce biofuels, biochemicals, and animal feed. Plant polysaccharides (i.e., cellulose, hemicellulose, and pectin) embedded within cell walls are highly recalcitrant towards conversion into useful products. Ammonia fiber expansion (AFEX) is a thermochemical pretreatment that increases accessibility of polysaccharides to enzymes for hydrolysis into fermentable sugars. These released sugars can be converted into fuels and chemicals in a biorefinery. Here, we describe a laboratory-scale batch AFEX process to produce pretreated biomass on the gram-scale without any ammonia recycling. The laboratory-scale process can be used to identify optimal pretreatment conditions (e.g., ammonia loading, water loading, biomass loading, temperature, pressure, residence time, etc.) and generates sufficient quantities of pretreated samples for detailed physicochemical characterization and enzymatic/microbial analysis. The yield of fermentable sugars from enzymatic hydrolysis of corn stover pretreated using the laboratory-scale AFEX process is comparable to pilot-scale AFEX process under similar pretreatment conditions. This paper is intended to provide a detailed standard operating procedure for the safe and consistent operation of laboratory-scale reactors for performing AFEX pretreatment of lignocellulosic biomass.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57488/>

## Introduction

Ammonia fiber expansion (AFEX) is a thermochemical pretreatment that uses volatile ammonia as the main reactant for cellulosic biomass pretreatment. This process was originally invented by Bruce Dale to cost-effectively reduce the recalcitrance of lignocellulosic biomass and enhance biologically-catalyzed pretreated biomass deconstruction into fermentable sugars<sup>1,2</sup>. Unlike most other aqueous-based thermochemical pretreatments<sup>3</sup>, AFEX is a dry-to-dry process that causes no significant change in biomass composition and requires no washing step with its associated waste generation and expense. Recovery of excess volatile ammonia has been demonstrated at the pilot scale, resulting in reduced waste generation and processing costs. The pilot-scale packed bed AFEX reactor system developed by MBI (**Figure 1**) recovers residual ammonia using steam stripping and transfers the hot, concentrated ammonia to a new packed bed<sup>4,5</sup>. Following AFEX pretreatment, the minor amounts of nitrogen incorporated into the biomass are usable as non-protein nitrogen by ruminant animals and microorganisms. Additionally, by altering the biomass ultrastructure through various physicochemical mechanisms<sup>6,7,8</sup>, AFEX increases accessibility of the biomass to carbohydrate-active enzymes (CAZymes) and increases the rates of polysaccharides hydrolysis by several-fold<sup>8,9</sup>, which also increases its digestibility by ruminant animals via their cellulolytic microbiome<sup>4,10,11,12</sup>. Farmers have long employed a simpler version of this method to increase the digestibility of ruminant forages by incubating the biomass for days or weeks under plastic tarps in the presence of low anhydrous ammonia loadings (<4% w/w basis of dry biomass) and ambient pressures and temperatures<sup>10,11</sup>.

Anhydrous ammonia was first investigated for its potential to delignify wood in the 1950s and as a pulping chemical in the early 1970s<sup>13,14,15,16,17,18</sup>. In the early 1980s, pressurized, high-temperature, concentrated ammonia (>30% NH<sub>4</sub>OH) under sub-critical conditions was first used in the Dale laboratory to enhance the enzymatic digestibility and microbial fermentability of lignocellulosic biomass<sup>19</sup>. This process underwent several name changes over the years, starting as ammonia freeze explosion, and then ammonia fiber explosion, and finally, ammonia fiber expansion, or simply AFEX. Around this same time (mid-late 1980s), DuPont (now Dow-DuPont) also explored using supercritical and near-critical anhydrous ammonia based pretreatment processes to increase digestibility of biomass<sup>20,21,22</sup>. In recent decades, there has been

increased emphasis on using dilute aqueous ammonia solutions as a pretreatment reagent including ammonia recycle/percolation<sup>23</sup> (ARP), soaking in aqueous ammonia (SAA), or the Dow-DuPont process without ammonia recycle<sup>24</sup>. A few additional methods have looked at use of anhydrous ammonia (low-moisture anhydrous ammonia (LMAA), and low-liquid ammonia pretreatment<sup>25</sup> (LAA). In the last few years, two new advanced organosolv-type pretreatment technologies utilizing liquid anhydrous ammonia<sup>26,27</sup> and ammonia-salt based solutions<sup>28</sup> at high liquid to solid loadings were recently developed that enable selective lignin fractionation and high efficiency enzymatic hydrolysis of pretreated cellulosic biomass at ultra-low enzyme loadings. A recent review article has highlighted the similarities and distinct differences between various forms of ammonia-based pretreatments<sup>29</sup>. However, until recently<sup>4</sup>, there were no pilot-scale demonstrations of ammonia-based pretreatment processes (like AFEX) that were efficiently coupled with closed-loop chemical recycle of concentrated ammonia used in the process.

In this paper, we describe in detail the most commonly used AFEX protocol for pretreating cellulosic biomass at the lab scale to produce gram scales of pretreated biomass (e.g., 1 to several 100 g). Typically, biomass is mixed with water (0.1–2.0 g H<sub>2</sub>O/g dry biomass) and loaded into a custom-built stainless-steel tubular or Parr type reactors. Anhydrous ammonia is then added (0.3–2.0 g NH<sub>3</sub>/g dry biomass) to the reactor and the mixture is heated to the desired reaction temperature (60–180 °C). Earlier publications on the AFEX process from the 1980s-1990s started the pretreatment residence time (e.g., 5-60 min) immediately after the temperature ramp. However, as the reactions occur as soon as the ammonia is added to the reactor, the current AFEX procedure is to start monitoring the residence time immediately after ammonia addition to the reactor. For temperatures of 90 °C or greater, it is often necessary to preheat the biomass before loading the ammonia in order to keep the initial temperature ramping to a minimum time period (i.e., <5 min). At the completion of the residence time, a valve is opened to rapidly release the pressure, and gas-phase contents into a suitable chemical fume hood. The rapid conversion of ammonia from liquid to gas phase also causes the reactor to cool down. Small reactors (<100 mL reactor volume) can often be unloaded in the fume hood immediately, while larger reactors (>100 mL reactor volume) may need additional time to cool. For user safety, at the larger scale (>100 g ammonia per reactor run), purging with nitrogen is recommended to remove as much residual ammonia as possible from the vessel and assist in cooling the reactor contents before unloading. Typically, no attempt is made at the lab-scale to recycle and/or recover the ammonia. One of the key design challenges for scaling-up the AFEX pretreatment process has been the recycling of ammonia with minimal capital and operating costs. Also, adding liquid ammonia to biomass generally drives partial flashing of the liquid that cools the biomass, requiring heating of the biomass-ammonia mixture before AFEX treatment can begin. Rather than adding ammonia as liquid, adding ammonia vapor to biomass offers two advantages: First, the high porosity of bulk biomass allows ammonia vapor to be transported rapidly, resulting in even ammonia distribution throughout the biomass. Second, ammonia vapor readily and exothermically dissolves into the water entrained in moist biomass, resulting in heat generation that rapidly and evenly heats the biomass. To exploit these advantages, both the MSU Dale lab and MBI have developed AFEX treatment methods using ammonia vapor. The Dale lab has developed the Gaseous Ammonia Pretreatment (GAP) process<sup>30</sup>, and MBI has developed the packed bed AFEX reactor process (Figure 1)<sup>4</sup>, which has been demonstrated at the pilot scale. The packed bed AFEX reactor system is capable of semi-batch mode operation with complete recycling of ammonia using a steam stripping method<sup>4,5</sup>. This novel MBI pilot-scale process exploits the chemical and physical characteristics of ammonia to efficiently pretreat biomass while efficiently recycling the ammonia.

Here, we present a detailed outline for conducting AFEX pretreatment of corn stover at the lab-scale using custom-built 200 mL volume tubular reactors (Figure 2). The AFEX pretreated samples were digested to fermentable sugars using commercially available cellulolytic enzyme cocktails to demonstrate the efficacy of the pretreatment processes. The enzymatic hydrolysis results for the lab-scale AFEX reactor were compared to larger pilot-scale AFEX reactor generated samples. Our goal is to provide a standard operating procedure for the safe and consistent operation of lab-scale pressurized reactors for performing AFEX pretreatment on cellulosic biomass like corn stover. Additional supporting information regarding variations to this lab-scale AFEX pretreatment process (e.g., pilot-scale packed bed AFEX process) are further highlighted in the accompanying supplemental pdf file. A detailed report on the packed bed AFEX process operational steps will be highlighted in a separate publication and is available upon request from MBI-MSU.

## Protocol

### 1. Adjusting biomass moisture content

1. See the **Table of Materials** outlining all major equipment and materials necessary to perform bench or lab scale AFEX pretreatment using the custom-built tubular AFEX reactor (Figure 2).
2. Determine the total moisture content of biomass using a moisture analyzer, or an oven set at 105 °C for 8 h. For the oven method, transfer the samples to a heat-resistant desiccator to cool to prevent water adsorption prior to drying. Perform the process in duplicate or triplicate and calculate the average moisture content.
3. For a given dry biomass loading in the reactor (here, it holds 25 g), use the moisture content determined in step 1.2, to calculate how much wet biomass needs to be loaded.

$$m_{\text{wet}} = \frac{m_{\text{dry}}}{(1 - MC_{\text{TWB}})} \quad [1]$$

Where  $m_{\text{wet}}$  = total mass of biomass (wet weight basis);  $m_{\text{dry}}$  = mass of biomass on a dry weight basis;  $MC_{\text{TWB}}$  = biomass moisture content on a total weight basis

4. Weigh out this amount of biomass ( $m_{\text{wet}}$ ) in a plastic container.
5. Calculate how much water needs to be mixed with the wet biomass to achieve the desired moisture content. For corn stover, this is typically 0.6 g of H<sub>2</sub>O per g of dry biomass.

$$m_{\text{water}} = (x_{\text{water}} * m_{\text{dry}}) - (m_{\text{wet}} - m_{\text{dry}}) \quad [2]$$

Where  $m_{\text{water}}$  = mass of water added to the reactor (in addition to the water in the biomass);  $x_{\text{water}}$  = AFEX water loading (g/g dry biomass)

6. Using a spray bottle, slowly add this amount of water ( $m_{\text{water}}$ ) to the biomass that had been previously weighed out and mix well by hand.

### 2. Load and assemble the reactor

1. Assemble the reactor body by placing a cap and Teflon gasket on the bottom of the reactor tube. Bolt a clamp in place, tightening both nuts evenly using a ratchet.

2. Transfer the wet biomass to the assembled reactor base and place a plug of glass wool at the top of the biomass.
3. Place a Teflon gasket on the top of the reactor. Ensure the region is free of biomass and glass wool, which could prevent an effective seal, and place the reactor head on top, maneuvering the thermocouple through the glass wool and biomass.
4. Bolt the clamp to the top of the reactor using a ratchet evenly on both sides.
5. Weigh the reactor ( $m_{\text{reactor}}$ ) and record the weight.

### 3. Set up the reactor system and fill the ammonia transfer cylinder

1. Confirm that all equipment is plugged in and operable (temperature controller, temperature monitor, syringe pump, timers).
2. Set the timers to desired residence time for each reactor and sample to be run.
3. Turn on and, if using a programmable syringe pump, set up the ammonia delivery method on the syringe pump.
  - Step 1: Withdrawal.
  - Step 2: Wait for 15 seconds (to allow time to open and close valves).
  - Step 3: Infuse (to transfer the ammonia into the reactor).
    1. Save as the **AFEX** method to allow for easy reuse.
4. Verify that all valves into and out of the small ammonia cylinder are closed.
5. If the cylinder has been used previously and contains residual ammonia/nitrogen, slowly open valve A on the top of the small ammonia cylinder to bleed off any nitrogen and close the valve once liquid ammonia begins to sputter out.
6. To fill the small ammonia cylinder, open the large anhydrous ammonia cylinder and all valves on the ammonia line. Slowly open valve (B) near the top of the small ammonia cylinder until the pressure stabilizes. Wait for 5 min before continuing to the next step. Approximately 120 mL of ammonia gets charged from the main cylinder to the transfer cylinder during this time.
7. Close all valves between the ammonia tank and the small ammonia cylinder, working from the left to right, beginning from the small cylinder (valve B) and finishing at the main valve on top of the tank.
8. Set the nitrogen regulator to 350 psi. Open the valve on the nitrogen cylinder and the valve on the attached regulator. Open valve C on the small ammonia cylinder to slowly add nitrogen, overpressuring the system. Adjust the pressure of the small cylinder to 350 psi, as needed, by adjusting the set point on the regulator. Keep nitrogen lines open while dispensing ammonia.

### 4. Preheat the reactor (for reaction temperatures of >100 °C)

1. Plug in the temperature monitor to the thermocouple and the heating tape to the temperature controller.
2. Manually adjust the temperature controller to bring the reactor up to 60 °C.

### 5. Load the reactor with ammonia

1. Turn on the syringe pump if not already on.
2. Calculate the volume of ammonia required based on the desired ammonia loading (g:g dry biomass) and a previously determined ammonia calibration.
 
$$m_{\text{NH}_3} = (x_{\text{NH}_3} * m_{\text{dry}}) \quad [3]$$

NOTE: Because the ammonia pump loads on a volume basis, when first using it, calibrate to convert from the required mass to volume. Follow the same procedure used for AFEX, but end the run (vent the reactor) immediately after loading the ammonia and weighing the reactor. Follow the same procedure for unloading the reactor.
3. Set up the method to load the correct amount of ammonia:
  1. Select the **AFEX** method from section 3.3.
  2. Press **Step Definition | Step: 1 | Set Target Volume or Time**.
  3. Key in the volume required in mL using the number pad and press the green checkmark.
  4. If more than 85 mL is required, enter the target volume as half of the amount specified in the spreadsheet and fill the reactor twice using the same syringe volume.
  5. Repeat steps 5.3.2 through 5.3.4 for "Step: 3".
  6. Press the back button.
4. Open valve (D) on the bottom of the small ammonia cylinder towards the exhaust, and then close it once any residual ammonia has exited.
5. Open valve (E) on the end of the syringe pump towards the front of the fume hood, and then open valve (F) to release any residual ammonia. Close valves (E) and (F).
6. Disconnect the reactor from the temperature monitor and the temperature controller. Attach the reactor to the quick connect.
7. Open valve (D) towards the small ammonia cylinder and open valve (E) towards the small ammonia cylinder.
8. Press the green arrow on the pump to start the sequence and draw ammonia into the syringe.
9. When the syringe stops automatically for the wait period, turn the syringe valve (E) towards the reactor, and the reactor inlet valve so it is pointing towards the quick connect stem.
 

After the delay, the syringe will begin infusing, stopping automatically at the set point.
10. If more than 85 mL of ammonia is required, repeat steps 5.7 through 5.9.
11. Close the reactor valve and valve (D). Open valve (F) to release residual ammonia from the syringe, and then close valve (F) and close valve (E).
12. Open valve (D) towards the exhaust, and then close it once the residual ammonia has left.
13. Wearing cryogenic gloves, remove the reactor from the quick connect. Be careful of potential ammonia spray. Use the elephant trunk vent line to vent the released ammonia, if necessary.
14. Start the timer for the appropriate reactor.

15. Weigh the reactor unit to verify that the appropriate weight of ammonia was added based on the spreadsheet calculations.

## 6. Begin heating and monitor the reaction

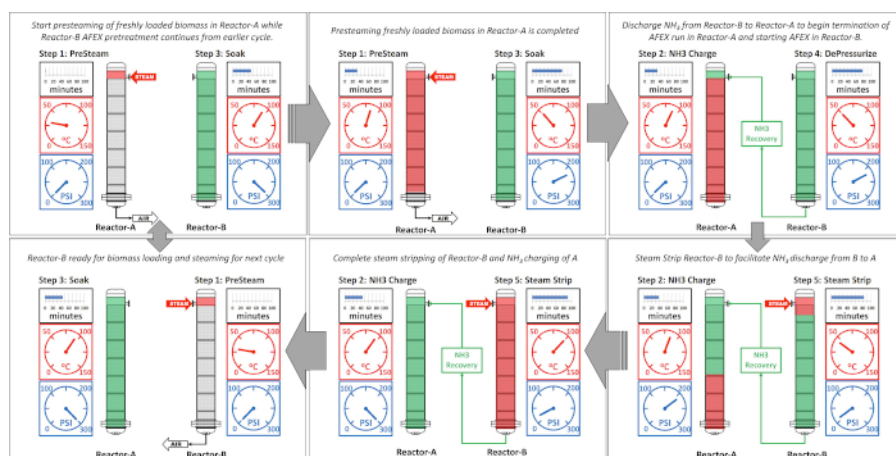
1. Plug in the temperature monitor to the thermocouple and the heating tape to the temperature controller.
  2. Record the initial temperature and pressure of the reactor following ammonia addition (the start of the residence time).
  3. Manually adjust the temperature controller to bring the reactor up to the set temperature. The goal is to reach the set point in <5 min.
  4. Record the pressure and temperature of the reactor every 3 min until the end of the residence time.
  5. At the end of the residence time, disconnect the reactor from the temperature controller and thermocouple, remove the reactor from the stand, and slowly open the ball release valve inside the fume hood.
- NOTE: Always wear a face shield during this step.

## 7. Shut down the system

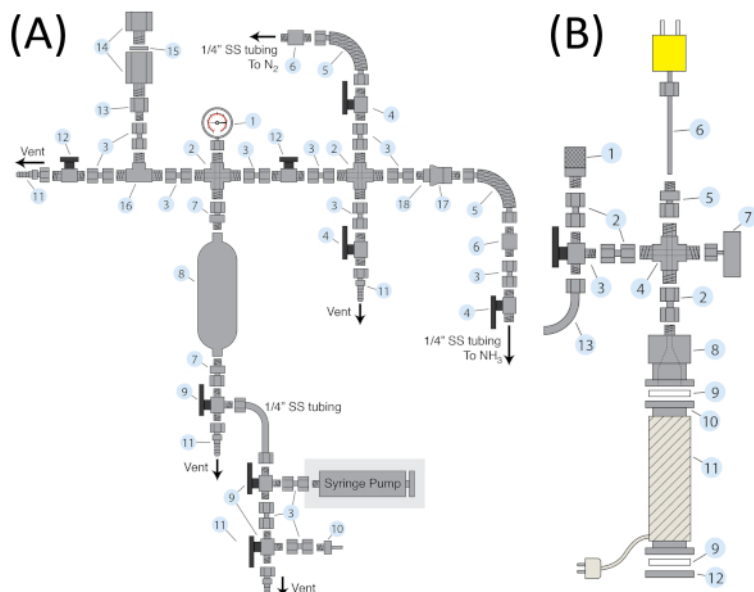
1. After allowing the reactor to cool for a few minutes, use a ratchet wrench to open the clamps on the reactor.
  2. Unload the biomass and glass wool from the reactor inside a fume hood. In order to prevent airborne contamination of the biomass as residual ammonia evaporates, it is best to dry inside an enclosed drying box inside a ventilated space.
  3. Clean the reactor with distilled water until the water runs clear and allow reactors to dry.
  4. If still open, close all valves on and connecting to the ammonia cylinder.
  5. Close all valves on nitrogen line.
  6. Turn off the temperature controller, temperature monitor, balance, syringe pump, and timer.
- CAUTION: If planning to run more reactions, it is not necessary to vent the small ammonia cylinder. However, if there is no plan to run more experiments, for safety it is best to vent the small cylinder into the hood at the end of the experiment. When doing this, it is important to leave the valves open as the release of ammonia can cause ice formation that may block some lines. As the lines thaw, additional ammonia can be released. Always make sure to have the ventilation functioning while allowing the system to vent. Any ammonia-treated biomass, even if it is not intended to be used, must be dried in the fume hood overnight to allow residual ammonia to evaporate. It cannot be immediately disposed of in the garbage.

## Representative Results

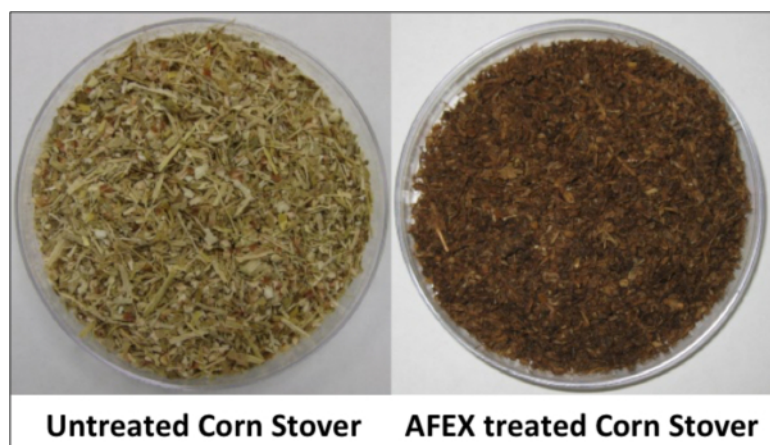
Following AFEX pretreatment, the biomass is darker in color, but otherwise visually unchanged (**Figure 3**). AFEX process generates a highly digestible material at a variety of scales besides the one outlined in this protocol. Here, we pretreated the same corn stover sample in our small 200 mL, packed-bed, bench-scale system; a larger 5 gallon, stirred Parr reactor; and MBI's pilot reactor. The conditions used for the two smaller reactors (i.e., 200 mL and 5 gallon scale) were 1.0 g  $\text{NH}_3$ :g dry biomass, 0.6 g  $\text{H}_2\text{O}$ :g dry biomass, for 30 min at  $100 \pm 5^\circ\text{C}$ . Pilot-scale AFEX<sup>4</sup> was carried out on the same material at 0.6 g  $\text{NH}_3$ :g dry biomass, 0.6 g  $\text{H}_2\text{O}$ :g dry biomass, for 30 min at  $100 \pm 5^\circ\text{C}$ . Details regarding the protocols used for conducting AFEX pretreatment at larger scales are provided in the supporting information (see **Supplemental File 1**). The following 'Quality Control Criteria' have been established based on target temperature for AFEX pretreatment. If after reaching the set point, the reactor temperature goes outside  $\pm 10^\circ\text{C}$  from the set point, the experiment must be aborted. If the target temperature (within  $5^\circ\text{C}$ ) is not reached within 5 min after ammonia pumping, abort the experiment. In addition, pretreatment efficacy for the AFEX process can be tested using cellulolytic enzyme cocktails to hydrolyze the accessible polysaccharides into fermentable sugars. Samples were enzymatically hydrolyzed for 72 hours at 6% glucan loading, pH 5.0,  $50^\circ\text{C}$ , and 250 rpm in a shaking incubator. A commercial cocktail of enzymes consisting of 60% cellulase (CTec3):40% hemicellulase (HTec3 or NS22246) on a fixed total protein loading basis loaded at 15 mg enzyme/g glucan was employed for all saccharification assays. The results (**Figure 4**) demonstrate that AFEX pretreatment significantly increases the yield of fermentable sugars in all cases. Furthermore, the cellulose/xylan hydrolysis yields for biomass pretreated using the lab-scale AFEX process is comparable to the larger 5-gallon Parr reactor and MBI's pilot-scale packed bed AFEX process.



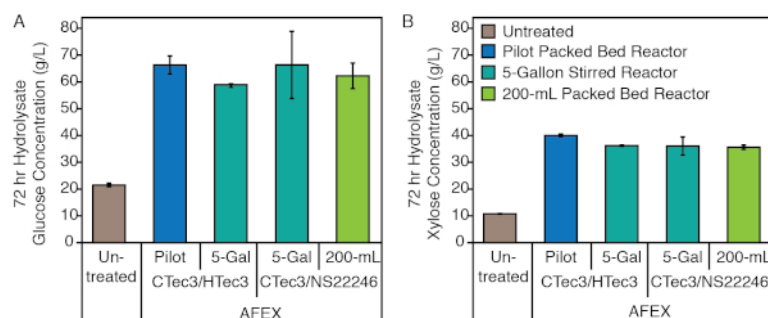
**Figure 1.** Schematic outline of steps involved in the pilot scale operation of MBI's AFEX reactor for pretreating lignocellulosic biomass fully-integrated with efficient ammonia recycle. [Please click here to view a larger version of this figure.](#)



**Figure 2. Schematics of lab-scale of A) ammonia delivery system and B) small 200 mL AFEX pretreatment reactor utilized to perform AFEX process outlined in the video protocol. Please click here to view a larger version of this figure.**



**Figure 3. AFEX pretreated biomass has a very similar gross morphology compared to untreated biomass, apart from being slightly darker in color. Please click here to view a larger version of this figure.**



**Figure 4. Glucose and xylose yields obtained after 72 h enzymatic hydrolysis of 6% glucan loading AFEX treated corn stover is shown here. All saccharification assays were carried out in duplicate with mean values (m) reported here. Standard deviations (1s) are reported here as error bars. Please click here to view a larger version of this figure.**

**Supplemental File 1: Additional protocols** Please click here to download this file.

**Supplemental Table 1: Ammonia delivery system and strut frame** Please click here to download this file.



## Discussion

The AFEX protocol describes how to process plant materials in the presence of anhydrous ammonia and water at elevated temperatures to increase the digestibility of the pretreatment material by cellulolytic enzymes and/or microbes. AFEX is highly effective on graminoid monocot species (e.g., corn stover, switchgrass, miscanthus, rice straw, wheat straw, and sugarcane bagasse) due to the efficiency of the process to cleave ester linkages that are naturally abundant in these materials<sup>31</sup>. AFEX is much less effective on biomass derived from dicots and gymnosperms (hardwoods, softwoods, and native forbs)<sup>32,33</sup> due to the smaller proportion of lignin-carbohydrate based ester linkages. However, when these linkages are introduced into woody cell walls using plant biotechnology, the AFEX pretreatment process becomes much more effective<sup>34</sup>.

Cleavage of ester linkages allows certain biomass components to be removed from the material, but redeposited as extractives on the outer cell wall surfaces, resulting in the formation of nanoscale holes that facilitate penetration and action of the cellulolytic enzymes<sup>6</sup>. AFEX pretreated corn stover showed a roughly 3-fold increase in glucose and xylose release rate following enzymatic hydrolysis under high solids conditions compared to the untreated material. Ammonia pretreatments also produce fewer and far less inhibitory degradation products compared to dilute acid pretreatment<sup>35</sup>. A previous comparison of AFEX and dilute acid-treated corn stover showed that dilute acid pretreatment produces 316% more acids, 142% more aromatics, and 3,555% more furan aldehydes than AFEX<sup>36</sup>, all of which can be inhibitory for microorganisms<sup>35,37</sup>. As AFEX is a dry-to-dry process, there is also no loss of sugars as a dilute liquid stream that cannot economically be utilized during enzymatic hydrolysis. However, this does lead to complications as enzymes with both cellulose-degrading and hemicellulose-degrading capability are required to fully break down the cell wall polysaccharides during enzymatic hydrolysis into mixed fermentable sugars like glucose and xylose. Hemicellulosic oligomers have been reported to inhibit cellulase activity<sup>38</sup>, which could necessitate a higher enzyme loading to maintain a high final sugar yield. However, optimization of suitable enzyme cocktails can reduce overall enzyme usage during saccharification of AFEX pretreated biomass<sup>39,40,41,42,43,44,45</sup>. During AFEX pretreatment process the hydrolysis and ammonolysis of ester linkages leads to the formation of acid and amide products in the pretreated biomass (e.g., acetic acid/acetamide, ferulic acid/ferulamide, coumaric acid/coumarylamide)<sup>36</sup>. Though formation of amides has been shown to help the fermentation process, their presence at very high concentrations in pretreated feedstock could be a concern if feeding animals pretreated biomass. Pre-hydrolysis of ester linkages with alkali such as NaOH or Ca(OH)<sub>2</sub> prior to AFEX pretreatment can be used to address the issue.

There are a number of safety considerations to keep in mind when working with anhydrous ammonia during the AFEX process. Anhydrous ammonia reacts with copper, brass, aluminum, carbon steel, and common fluor elastomer polymers used in seals (e.g. Viton, etc.). Any tubing or reactor components that may come in contact with ammonia should be made from stainless steel, and gaskets, valve seats, and quick connect seals should be made from Teflon or Kalrez when possible. Ammonia is not considered a toxic chemical, but it is still dangerous due to its hygroscopic and cryogenic properties. It readily targets and can severely damage mucous membranes in the eyes and respiratory system. Ammonia is a cryogenic fluid and ammonia leaks can cause severe frostbite due to direct contact with the gas stream or chilled equipment. Ammonia is immediately dangerous to life and health (IDLH) at concentrations above 300 ppm. Workers should evacuate immediately in the event the concentration exceeds 50 ppm. It is recommended that operators wear a calibrated ammonia monitor to warn of hazardous concentrations in their vicinity. Installing sensors with alarms in the main work area is also advisable. Workers who handle ammonia should be properly trained and wear protective gear such as escape respirators equipped with methylamine cartridges, and cryogenic and heat protective gloves, and be prepared to handle emergency situations. In the event of exposure to anhydrous ammonia, the operator should move to safety and immediately flush the affected area with water for at least 15 min. The ammonia pretreatment process should be conducted inside a fume hood, and the ammonia cylinder should either be stored in a fume hood or ventilated cabinet. Following the experiment, pretreated biomass will have some residual free ammonia and should be either dried in the hood overnight or in a custom ventilated drying box before storage in plastic bags at room temperature for follow-up experiments. Some other key safety considerations include installing an ammonia delivery system with a flow meter that will help to precisely deliver ammonia to the reactor and a reactor designed to handle at least 1.5 times the pressure that the pretreatment process will undergo (e.g., for handling AFEX process at  $2 \times 10^6$  Pa pressure, the minimum pressure rating of the reactor should be  $3 \times 10^6$  Pa).

AFEX pretreatment is a promising method to produce highly digestible plant biomass that can be used directly as animal feed or as a feedstock to generate fuels and chemicals. Beyond these two industries, AFEX might find use in other areas such as a bio-renewable feedstock for making biomaterials, or as a feedstock for producing biogas. The laboratory-scale process can be conducted in a laboratory equipped with proper ventilated space and safety precautions, and our current work confirms that this scaled-down AFEX process shows similar results to material generated in a scaled-up and/or pilot AFEX reactor. The lab-scale AFEX process can be used to test feedstocks, processing conditions, and applications in a higher throughput manner, while providing a reasonable expectation of how the process would perform at pilot or industrial scales.

## Disclosures

Several authors (namely Shishir P S Chundawat, Tim Campbell, Farzaneh Teymouri, Leonardo Sousa, Bruce E Dale, Venkatesh Balan) are inventors/co-inventors on multiple patents filed on ammonia pretreatment and reactor design at MSU/MBI.

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