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## Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass

--Manuscript Draft--

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| **Corresponding Author:** | Shishir Chundawat<br>Rutgers The State University of New Jersey<br>Piscataway, NJ UNITED STATES |
| **Corresponding Author's Institution:** | Rutgers The State University of New Jersey |
| **Corresponding Author E-Mail:** | spc120@soe.rutgers.edu |
| **Order of Authors:** | Shishir Chundawat |
| | Ramendra Pal |
| | Chao Zhao |
| | Timothy J Campbell |
| | Farzaneh Teymouri |
| | Josh Videto |
| | Chandra Nielson |
| | Bradley Wieferich |
| | Leonardo Sousa |
| | Bruce E Dale |
| | Venkatesh Balan |
| | Sarvada Chipkar |
| | Jacob Aguado |
| | Emily Burke |
| | Rebecca G Ong |
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Cover Letter


Dept. of Chemical & Biochemical Engineering
Engineering Building, Room C150A
Rutgers, The State University of New Jersey
98 Brett Road
Piscataway, NJ 08854-8058

http://chundawat.rutgers.edu
shishir.chundawat@rutgers.edu

p. 848-445-3678
f. 732-445-2421


Mar 30, 2020

To,
Nam Nguyen, Ph.D.
Editorial Department (Manager of Review)
JoVE, 1 Alewife Center, Suite 200, Cambridge, MA 02140

**Subject**: *Response to editor & reviewer comments for manuscript ID JoVE57488*

Dear Nam,

Please find below a response to editorial comments for our manuscript (ID: JoVE57488) titled "**Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass**". We have uploaded an updated video protocol and made some minor changes to our manuscript to reflect updated video protocol as requested by the editorial team. Updated video file titled 'JOVE 57488_R5.mp4' has been uploaded on JoVE's website (https://www.dropbox.com/request/LMKYhpFzdr3wHyqD7qUt).

On behalf of all authors, I would like to thank you very much for your consideration and look forward to finally publishing our updated manuscript.

Sincerely,


Prof. Shishir Chundawat (Lead Corresponding Author)
Assistant Professor, Chemical & Biochemical Engineering Department
Rutgers, The State University in New Jersey, 98 Brett Road, Piscataway, NJ 08854, USA
Email: shishir.chundawat@rutgers.edu
URL: www.rci.rutgers.edu/~spc120/
Phone: 848-445-3678
Fax: 732-445-2421


*Enclosed: Brief response to editorial team comments and updated manuscript pdf highlighting track changes made to the document.*

**Response to Editorial/Production Comments**

**Revisions regarding the written manuscript:**
1. Has reference 39 been published? If not, please remove it.
**Response**: Yes Reference 39 has been published and now has been updated.

**Revisions regarding the video:**
**Overall Response**: We have made all the changes requested by the editorial team for the updated video. A updated media has been now uploaded that should address all previous editorial team concerns (https://www.dropbox.com/request/LMKYhpFzdr3wHyqD7qUt). Individual responses are provided below.

1. 2:48 - Set up should be two words here.
**Response**: We have made all the changes requested by the editorial team for the updated video.

2. 6:43 - Shut down is two words here. Please make the change in the written manuscript as well.
**Response**: We have made all the changes requested by the editorial team for the updated video.

3. 7:48 - Please use correct chemical notation.
**Response**: We have made all the changes requested by the editorial team for the updated video.

4. 8:29 - There is a weird vocal glitch here.
**Response**: We have made all the changes requested by the editorial team for the updated video.

5. The cow images at 0:47 and 8:47 appear to be stock images. Is there a record of your license for these images?
**Response**: We have made all the changes requested by the editorial team for the updated video. We have replaced the earlier cow images with images from Pixabay, which does not require attribution:

Cow Image references:
https://pixabay.com/photos/cow-head-cow-head-animal-livestock-1715829/
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6. Audio: The audio in Rebecca Ong's on-screen introduction and conclusion has a large amount of room noise and what sounds like compression or noise removal. We may be able to clean this up a bit on our end. For the next submission, please remove any compression or processing from the audio.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7. Editing Style & Pacing: 1:13 - There is a jump cut here, followed immediately by a slow fade to an image of the pilot plant. I recommend cutting straight to this image before the jump cut is seen.

**Response**: We have made all the changes requested by the editorial team for the updated video.

8. Text/Graphics
"0:42, 0:47 - The images here could be scaled up to fill the frame. It isn't necessary, but it might look nicer and show more detail.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:20 - The white background of this image should be extended to fill in the thin black borders on the left and right sides of the frame.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:35 - It looks like half of the gray gradient background is missing here. This should be fixed.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:43, 8:04 - The gradient background disappears here. This should be fixed."
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:48 - The 3 in NH3 and 2 in H2O should be subscript to fit JoVE formatting
**Response**: We have made all the changes requested by the editorial team for the updated video.

**TITLE:**

# Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass

**AUTHORS & AFFILIATIONS:**
Shishir P S Chundawat[1], Ramendra Pal[1], Chao Zhao[1], Tim Campbell[2], Farzaneh Teymouri[2], Josh Videto[2], Chandra Nielson[2], Bradley Wieferich[3], Leonardo Sousa[3], Bruce E Dale[3], Venkatesh Balan[4], Sarvada Chipkar[5], Jacob Aguado[5], Emily Burke[5], Rebecca G Ong[5]

[1]Department of Chemical and Biochemical Engineering, Rutgers-State University of New Jersey, Piscataway, NJ, USA
[2]Michigan Biotechnology Institute (MBI), Lansing, MI
[3]Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, MI, USA
[4]Department of Engineering Technology, Biotechnology Division, School of Technology, University of Houston, Houston, TX, USA
[5]Department of Chemical Engineering, Michigan Technological University, Houghton, MI, USA

**EMAIL ADDRESSES:**
Shishir P S Chundawat (shishir.chundawat@rutgers.edu)
Ramendra Pal (rp954@scarletmail.rutgers.edu)
Chao Zhao (cz304@scarletmail.rutgers.edu)
Timothy J Campbell (campbell@mbi.org)
Farzaneh Teymouri (teymouri@mbi.org)
Josh Videto (videto@mbi.org)
Chandra Nielson (nielson@mbi.org)
Bradley Wieferich (wiefer16@msu.edu)
Leonardo Sousa (sousaleo@egr.msu.edu)
Bruce E Dale (bdale@egr.msu.edu)
Venkatesh Balan (vbalan@uh.edu)
Sarvada Chipkar (shchipka@mtu.edu)
Jacob Aguado (jraguado@mtu.edu)
Emily Burke (emilyb@mtu.edu)
Rebecca G Ong (rgong1@mtu.edu)

**CORRESPONDING AUTHORS**:
Shishir P S Chundawat
Venkatesh Balan
Rebecca G Ong

**KEYWORDS:**
Ammonia Fiber Expansion (AFEX) pretreatment, Lignocellulosic Biomass, Cellulosic Biofuels, Animal Feed, Cellulases, Enzymatic Hydrolysis, Biorefinery

**SUMMARY:**

Ammonia fiber expansion (AFEX) is a thermochemical pretreatment technology that can convert lignocellulosic biomass (e.g., corn stover, rice straw, and sugarcane bagasse) into a highly digestible feedstock for both biofuels and animal feed applications. Here, we describe a laboratory-scale method for conducting AFEX pretreatment on lignocellulosic biomass.

**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.

**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[1,2]. Unlike most other aqueous-based thermochemical pretreatments[3], 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[4,5]. 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[6–8], AFEX increases accessibility of the biomass to carbohydrate-active enzymes (CAZymes) and increases the rates of polysaccharides hydrolysis by several-fold[8,9], which also increases its digestibility by ruminant animals via their cellulolytic microbiome[4,10–12]. 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[10,11].

Anhydrous ammonia was first investigated for its potential to delignify wood in the 1950s and as a pulping chemical in the early 1970s[13–18]. In the early 1980s, pressurized, high-temperature, concentrated ammonia (>30% $NH_4OH$) under sub-critical conditions was first used in the Dale laboratory to enhance the enzymatic digestibility and microbial fermentability of lignocellulosic biomass[19]. 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[20–22]. In recent decades, there has been increased emphasis on using dilute aqueous ammonia solutions as a pretreatment reagent including ammonia recycle/percolation[23] (ARP), soaking in aqueous ammonia (SAA), or the Dow-DuPont process without ammonia recycle[24]. A few additional methods have looked at use of anhydrous ammonia (low-moisture anhydrous ammonia (LMAA), and low-liquid ammonia pretreatment[25] (LAA). In the last few years, two new advanced organosolv-type pretreatment technologies utilizing liquid anhydrous ammonia[26,27] and ammonia-salt based solutions[28] 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[29]. However, until recently[4], 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 $H_2O$/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 $NH_3$/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[30], and MBI has developed the packed bed AFEX reactor process (**Figure 1**)[4], 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[4,5]. 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 here 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 generated samples 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

1.1. 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**).

1.2. 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.

1.3. 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.

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

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

1.4. Weigh out this amount of biomass ($m_{wet}$) in a plastic container.

1.5. 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 $H_2O$ per g of dry biomass.

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

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

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

**2. Load and assemble the reactor**

2.1. 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 rachet.

2.2. Transfer the wet biomass to the assembled reactor base and place a plug of glass wool at the top of the biomass.

2.3. 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.

2.4. Bolt the clamp to the top of the reactor using a ratchet evenly on both sides.

2.5. Weigh the reactor ($m_{reactor}$) and record the weight.

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

3.1. Confirm that all equipment is plugged in and operable (temperature controller, temperature monitor, syringe pump, timers).

3.2. Set the timers to desired residence time for each reactor and sample to be run.

3.3. 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).

3.3.1. Save as the **AFEX** method to allow for easy reuse.

3.4. Verify that all valves into and out of the small ammonia cylinder are closed.

3.5. 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.

3.6. 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.

3.7. 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.

3.8. 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)**

4.1. Plug in the temperature monitor to the thermocouple and the heating tape to the temperature controller.

4.2. Manually adjust the temperature controller to bring the reactor up to 60 °C.

**5. Load the reactor with ammonia**

5.1. Turn on the syringe pump if not already on.

5.2. Calculate the volume of ammonia required based on the desired ammonia loading (g:g dry biomass) and a previously determined ammonia calibration.

$$m_{NH3} = (x_{NH3} * m_{dry}) \qquad [3]$$

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.

5.3. Set up the method to load the correct amount of ammonia:

5.3.1. Select the **AFEX** method from section 3.3.

5.3.2. Press **Step Definition | Step: 1 | Set Target Volume or Time**.

5.3.3. Key in the volume required in mL using the number pad and press the green checkmark.

5.3.4. 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.

5.3.5. Repeat steps 5.3.2 through 5.3.4 for "Step: 3".

5.3.6. Press the back button.

5.4. Open valve (D) on the bottom of the small ammonia cylinder towards the exhaust, and then close it once any residual ammonia has exited.

5.5. 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).

5.6. Disconnect the reactor from the temperature monitor and the temperature controller. Attach the reactor to the quick connect.

5.7. Open valve (D) towards the small ammonia cylinder and open valve (E) towards the small ammonia cylinder.

5.8. Press the green arrow on the pump to start the sequence and draw ammonia into the syringe.

5.9. 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.

5.10. If more than 85 mL of ammonia is required, repeat steps **Error! Reference source not found.** through 5.9.

5.11. 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).

5.12. Open valve (D) towards the exhaust, and then close it once the residual ammonia has left.

5.13. 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.

5.14. Start the timer for the appropriate reactor.

5.15. 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**

6.1. Plug in the temperature monitor to the thermocouple and the heating tape to the temperature controller.

6.2. Record the initial temperature and pressure of the reactor following ammonia addition (the start of the residence time).

6.3. 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.

6.4. Record the pressure and temperature of the reactor every 3 min until the end of the residence time.

6.5. 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**

7.1. After allowing the reactor to cool for a few minutes, use a ratchet wrench to open the clamps on the reactor.

7.2. 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.

7.3. Clean the reactor with distilled water until the water runs clear and allow reactors to dry.

7.4. If still open, close all valves on and connecting to the ammonia cylinder.

7.5. Close all valves on nitrogen line.

7.6. 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 $NH_3$:g dry biomass, 0.6 g $H_2O$:g dry biomass, for 30 min at 100 ± 5 °C. Pilot-scale AFEX[4] was carried out on the same material at 0.6 g $NH_3$:g dry biomass, 0.6 g $H_2O$:g dry biomass, for 30 min at 100 ± 5 °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 ± 10 °C from the set point, the experiment must be aborted. If the target temperature (within 5 °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 °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.**

**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.**

**Figure 3. AFEX pretreated biomass has a very similar gross morphology compared to untreated biomass, apart from being slightly darker in color.**

**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 (μ) reported here. Standard deviations (1σ) are reported here as error bars.

**Supplemental File 1: Additional protocols**

**Supplemental Table 1: Ammonia delivery system and strut frame**

## 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[31]. AFEX is much less effective on biomass derived from dicots and gymnosperms (hardwoods, softwoods, and native forbs)[32,33] 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[34].

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[6]. 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[35]. 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[36], all of which can be inhibitory for microorganisms[35,37]. 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[38], 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[39–45]. 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)[36]. 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 biomas. Pre-hydrolysis of ester linkages with alkali such as NaOH or $Ca(OH)_2$ 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 fluoroelastomer 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 delivery 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.

**ACKNOWLEDGMENTS:**

This material is based upon work supported in part by the Great Lakes Bioenergy Research Center, U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Numbers DE-SC0018409 and DE-FC02-07ER64494. Rebecca Ong acknowledges partial support from Michigan Technological University (startup funding). Shishir Chundawat acknowledges partial support from the US National Science Foundation CBET award (1604421), ORAU Ralph E. Powe Award, and Rutgers School of Engineering (Startup Funding). Bruce Dale acknowledges partial support from Michigan State University AgBioResearch office and also the USDA National Institute of Food and Agriculture. Venkatesh Balan acknowledges partial support from State of Texas and University of Houston (Startup Funding). MBI employees acknowledge partial support from the US Department of Energy and Michigan State University foundation. Lastly, we would like to dedicate this paper to our mentor and co-author Prof. Bruce Dale for inspiring us to collaboratively pursue our dream of making sustainable cellulosic biofuels.

**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. AFEX-treated biomass and its hydrolysates are available for sale as research-grade materials through 'Glydia Biotech LLC' with permission from MSU and MBI. Venkatesh Balan is the sole proprietor of Glydia Biotech LLC.

**REFERENCES:**

1. Dale, B. E., Henk, L. L. Response of Lignocellulosic Materials to Ammonia Freeze Explosion. *Abstracts of Papers of the American Chemical Society*. **190** (SEP), 78 MBD (1985).
2. Dale, B. E., Henk, L. L., Shiang, M. Fermentation of Lignocellulosic Materials Treated by Ammonia Freeze-Explosion. *Symposium: Bioconversion of Waste Materials to Useful Industrial Products*. 223–233 (1985).
3. Mosier, N. S. et al. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*. **96** (6(673–686 (2005).
4. Campbell, T. J. et al. A packed bed Ammonia Fiber Expansion reactor system for pretreatment of agricultural residues at regional depots. *Biofuels*. **4** (1), 23–34 (2013).
5. Bals, B., Teymouri, F., Campbell, T., Jin, M., Dale, B. E. Low temperature and long residence time AFEX pretreatment of corn stover. *BioEnergy Research* **5** (2), 373–379 (2012).
6. Chundawat, S. P. S. et al. Multi-scale visualization and characterization of plant cell wall deconstruction during thermochemical pretreatment. *Energy & Environmental Science*. **4** (3), 973–984 (2011).
7. Chundawat, S. P. S., Beckham, G. T., Himmel, M., Dale, B. E. Deconstruction of Lignocellulosic Biomass to Fuels and Chemicals. *Annual Review of Chemical and Biomolecular Engineering.* **2**, 121–145 (2011).
8. Chundawat, S. P. S. et al. Primer on Ammonia Fiber Expansion Pretreatment. *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. 169–200 (2013).
9. da Costa Sousa, L., Chundawat, S. P. S., Balan, V., Dale, B. E. "Cradle-to-grave" assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology*. **20** (3), 339–347 (2009).
10. Solaiman, S. G., Horn, G. W., Owens, F. N. Ammonium Hydroxide Treatment on Wheat Straw. *Journal of Animal Science*. **49** (3), 802–808 (1979).
11. Harbers, L. H., Kreitner, G. L., Davis Jr., G. V, Rasmussen, M. A., Corah, L. R. Ruminal Digestion of Ammonium Hydroxide-Treated Wheat Straw Observed by Scanning Electron

Microscopy. *Journal of Animal Science*. **54** (6), 1309–1319 (1982).

12. Dale, B. E., Bals, B. D., Kim, S., Eranki, P. Biofuels Done Right: Land Efficient Animal Feeds Enable Large Environmental and Energy Benefits. *Environmental Science & Technology*. **44**, 8385–8389 (2010).
13. Schuerch, C., Burdick, M. P., Mahdalik, M. Liquid Ammonia-Solvent Combinations in Wood Plasticization: Chemical Treatments. *Industrial & Engineering Chemistry Product Research and Development*. **5** (2), 101–105 (1966).
14. O'Connor, J. J. Ammonia explosion pulping: A new fiber separation process. *Tappi*. **55** (3), 353–358 (1972).
15. Yan, M. M., Purves, C. B. Extraction of a Lignin Fraction from Maple Wood by Liquid Ammonia. *Canadian Journal of Chemistry-Revue Canadienne De Chimie*. **34** (12), 1747–1755 (1956).
16. Yan, M. M., Purves, C. B. Attempted Delignifications with Sodium Bicarbonate - Carbon Dioxide, and with Anhydrous Liquid Ammonia, Under Pressure. *Canadian Journal of Chemistry*. **34** (11), 1582–1590 (1956).
17. Tarkow, H., Feist, W. C. A Mechanism for Improving the Digestibility of Lignocellulosic Materials with Dilute Alkali and Liquid Ammonia. *Cellulases and Their Applications*. (95), 197–217 (1969).
18. Peterson, R. C., Strauss, R. W. Chemi-mechanical pulping of hardwoods using ammonia vapor. *Journal of Polymer Science Part C: Polymer Symposia*. **36** (1), 241–250 (2007).
19. Dale, B. E., Moreira, M. J. A Freeze-Explosion Technique for Increasing Cellulose Hydrolysis. *Biotechnology and Bioengineering*. 31–43 (1982).
20. Weimer, P. J., Chou, Y.-C. T. Anaerobic Fermentation of Woody Biomass Pretreated with Supercritical Ammonia. *Applied and Environmental Microbiology*. **52** (4), 733–736 (1986).
21. Weimer, P. J., Chou, Y. C. T., Weston, W. M., Chase, D. B. Effect of supercritical ammonia on the physical and chemical structure of ground wood. *Biotechnol Bioeng Symp*. **17**, 5–18 (1986).
22. Chou, Y. C. T. Supercritical ammonia pretreatment of lignocellulosic materials. *Biotechnol Bioeng Symp*. **17**, 19–32 (1986).
23. Iyer, P. V, Wu, Z.-W., Kim, S. B., Lee, Y. Y. Ammonia recycled percolation process for pretreatment of herbaceous biomass. *Applied Biochemistry and Biotechnology*. **57**–**8**, 121–132 (1996).
24. Dunson, J. R., Elander, R. T., Tucker, M., Hennessey, S. M. Treatment of biomass to obtain fermentable sugars (USPTO 2007/0031918). **US 2007/00**, (2007).
25. Kim, T. H., Lee, Y. Y., Sunwoo, C., Kim, J. S. Pretreatment of corn stover by low-liquid ammonia recycle percolation process. *Applied Biochemistry and Biotechnology*. **133** (1), 41–57 (2006).
26. da Costa Sousa, L. et al. Next-generation ammonia pretreatment enhances cellulosic biofuel production. *Energy & Environmental Science.* **9**, 1215–1223 (2016).
27. da Costa Sousa, L., Foston, M. et al. Isolation and characterization of new lignin streams derived from extractive-ammonia (EA) pretreatment. *Green Chemistry*. **18** (15), 4205–4215 (2016).
28. Chundawat, S. P. S. et al. Ammonia-salt solvent promotes cellulosic biomass deconstruction under ambient pretreatment conditions to enable rapid soluble sugar

production at ultra-low enzyme loadings. *Green Chemistry*. **22**, 204–218 (2020).

29. Zhao, C., Shao, Q., Chundawat, S. P. S. Recent Advances on Ammonia-based Pretreatments of Lignocellulosic Biomass. *Bioresource Technology*. 122446 (2019).

30. Venkatesh, B., Dale, B. E., Chundawat, S., Sousa, L. Methods for pretreating biomass (US9644222 B2) (2011).

31. Garlock, R. J., Chundawat, S. P. S., Hodge, D. B., Keskar, S., Dale, B. E. Linking Plant Biology and Pretreatment: Understanding the Structure and Organization of the Plant Cell Wall and Interactions with Cellulosic Biofuel Production. *Plants and BioEnergy (Advances in Plant Biology*. **4**, 231–253 (2014).

32. Balan, V. et al. Enzymatic digestibility and pretreatment degradation products of AFEX-treated hardwoods (Populus nigra). *Biotechnology Progress*. **25** (2), 365–375 (2009).

33. Garlock, R. J., Bals, B., Jasrotia, P., Balan, V., Dale, B. E. Influence of variable species composition on the saccharification of AFEX pretreated biomass from unmanaged fields in comparison to corn stover. *Biomass and Bioenergy*. **37**, 49–59 (2012).

34. Wilkerson, C. G. et al. Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone. *Science*. **344** (6179), 90–93 (2014).

35. Tang, X. et al. Designer synthetic media for studying microbial-catalyzed biofuel production. *Biotechnology for Biofuels*. **8** (1), 1 (2015).

36. Chundawat, S. P. S. et al. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute-acid based pretreatments. *Search Results*. **101**, 8429–8438 (2010).

37. Lau, M. W., Dale, B. E. Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). *Proceedings of the National Academy of Sciences of the United States of America*. **106** (5), 1368–1373 (2009).

38. Baumann, M., Borch, K., Westh, P. Xylan oligosaccharides and cellobiohydrolase I (TrCel7A) interaction and effect on activity. *Biotechnology for Biofuels*. **4** (1), 45 (2011).

39. Chundawat, S. et al. Shotgun approach to increasing enzymatic saccharification yields of Ammonia Fiber Expansion (AFEX) pretreated cellulosic biomass. *Frontiers in Energy Research*. **5**, 9 (2017).

40. Gao, D., Chundawat, S. P. S., Uppugundla, N., Balan, V., Dale, B. E. Binding Characteristics of Trichoderma reesei Cellulases on Untreated, Ammonia Fiber Expansion and Dilute-acid Pretreated Lignocellulosic Biomass. *Biotechnology and Bioengineering*. **108** (8), 1788–1800 (2011).

41. Gao, D., Chundawat, S. P. S., Krishnan, C., Balan, V., Dale, B. E. Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresource Technology*. **101** (8), 2770–2781 (2010).

42. Gao, D. et al. Strategy for identification of novel fungal and bacterial glycosyl hydrolase hybrid mixtures that can efficiently saccharify pretreated lignocellulosic biomass. *BioEnergy Research*. **3**, 67–81 (2010).

43. Banerjee, G. et al. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. *Bioresource Technology*. **101** (23(9097–9105 (2010).

44. Banerjee, G., Car, S., Scott-Craig, J. S., Borrusch, M. S., Aslam, N., Walton, J. D. Synthetic enzyme mixtures for biomass deconstruction: Production and optimization of a core set. *Biotechnology and Bioengineering*. **106** (5), 707–720 (2010).

45. Banerjee, G., Car, S., Scott-Craig, J., Borrusch, M., Walton, J. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. *Biotechnology for Biofuels*. **3** (1), 22 (2010).

Figure 1

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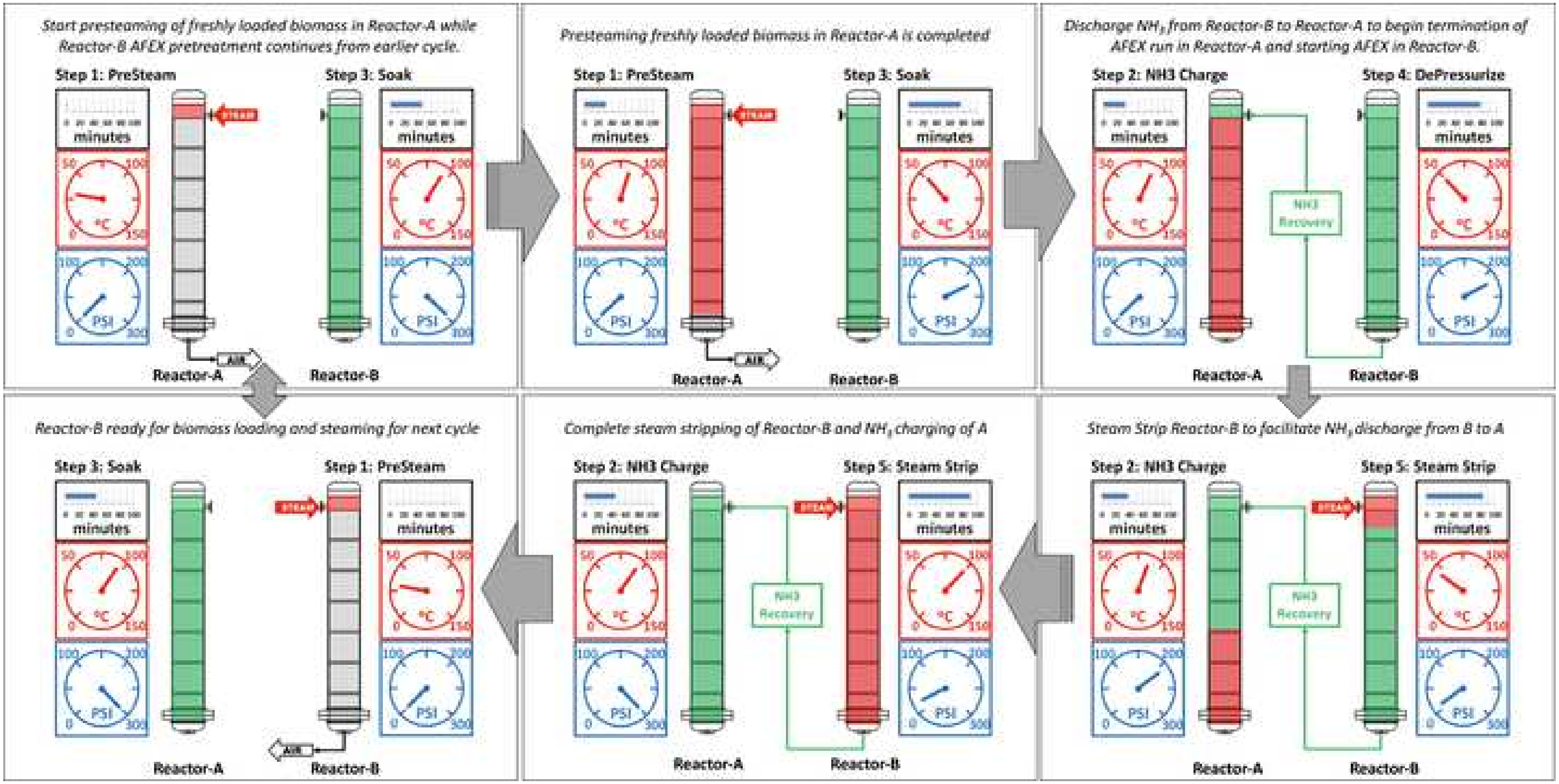


Figure 2

Click here to access/download;Figure;Fig-2.psd

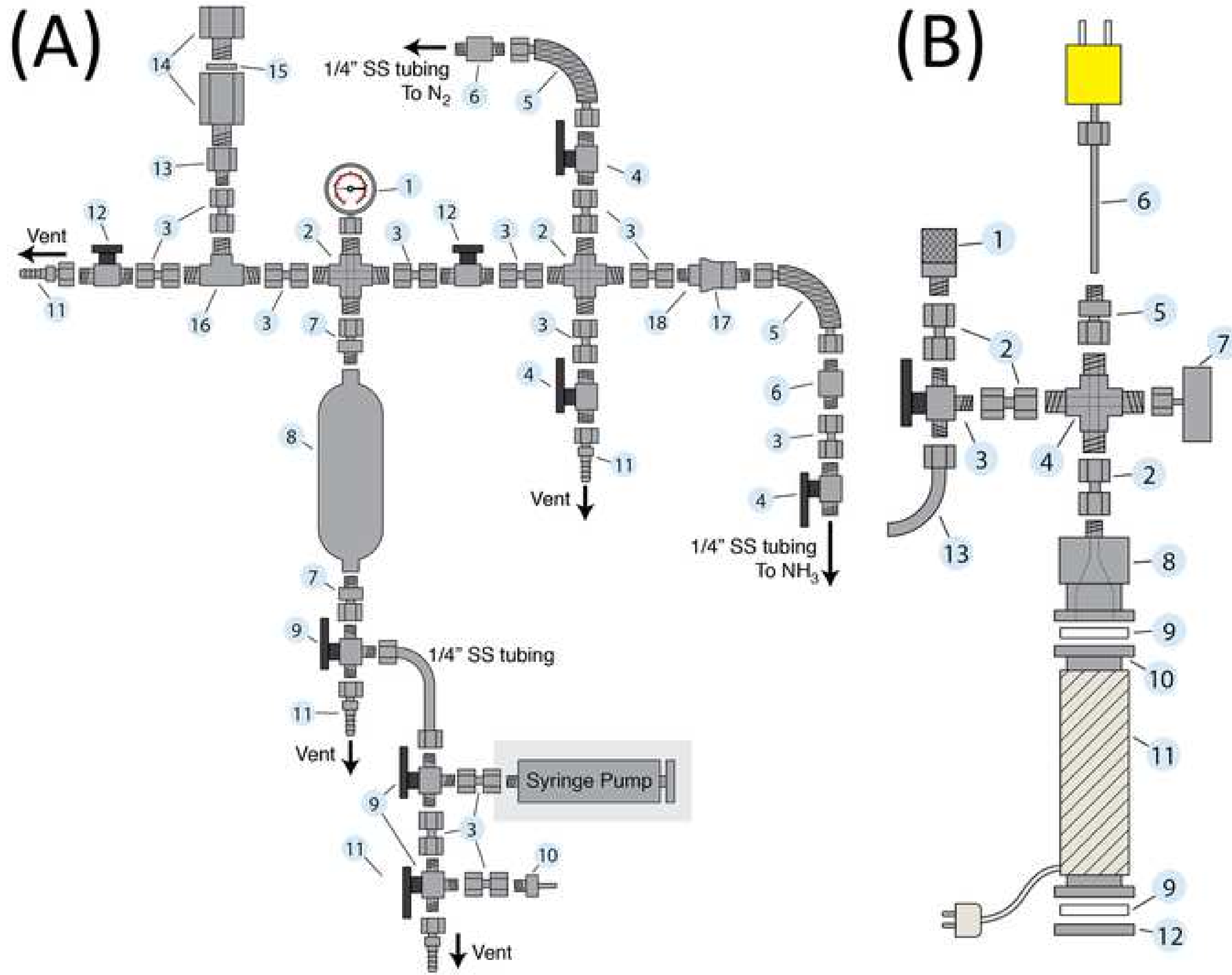
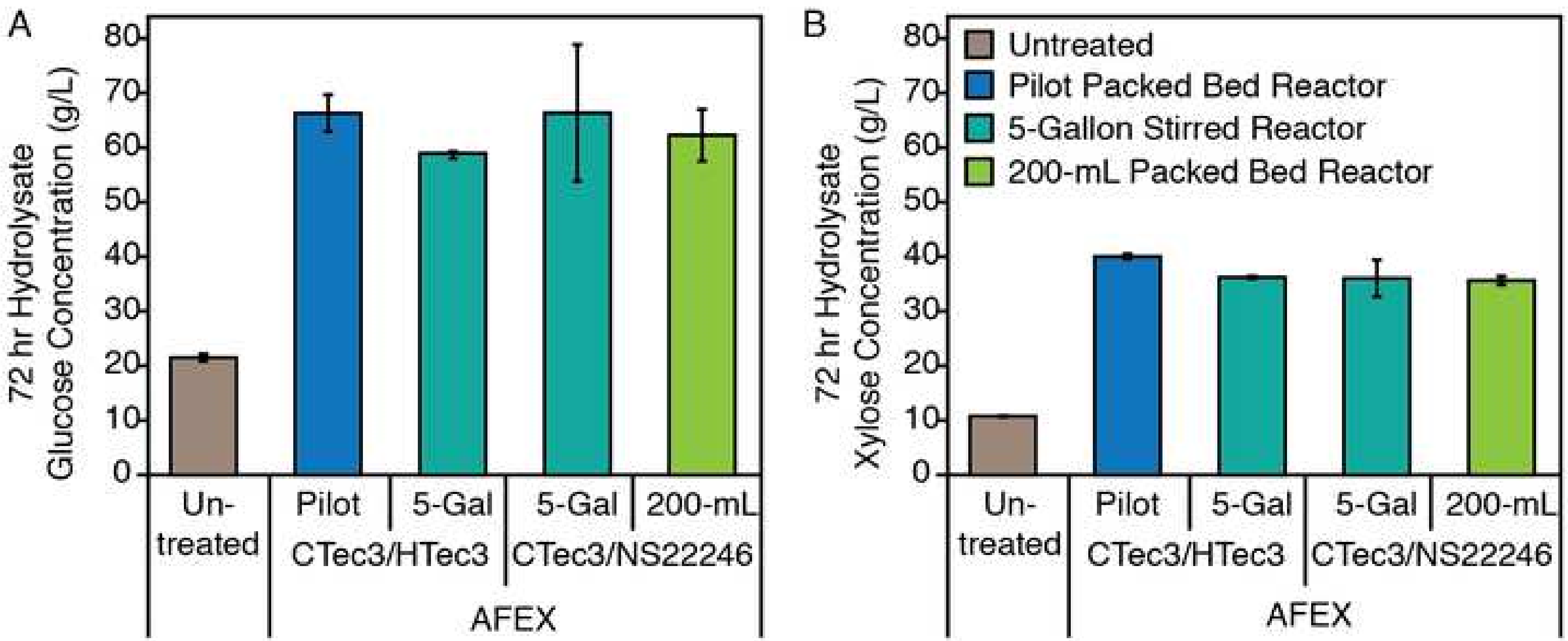


Figure 3



Figure 4

Click here to access/download;Figure;Fig-4.psd



| Name of Materials/Equipment | Company | Catalog Number/s |
|---|---|---|
| **Safety Equipment/PPE** | | |
| Ammonia Monitor | CanarySense | BW GAXT-A-DL |
| Cryogenic gloves | Amazon | B01L8WA238/B01L8WA1H0 |
| Ear muffs | 3M | H7A |
| Face shield | - | - |
| Heat protective gloves | Grainger | 2EWX1/2EWX2/2EWX3 |
| Nitrile gloves | - | - |
| | | |
| **Reagents** | | |
| Anhydrous Ammonia | - | - |
| Distilled water | - | - |
| Milled or Chopped Corn Stover | - | - |
| Nitrogen Compressed Gas | - | - |
| | | |
| **Equipment** | | |
| Ammonia Cylinder Adapter | - | - |
| Ammonia Delivery System | Swagelok | Misc. |
| Analytical Balance | Sartorius | CPA4202S |
| Chemraz O-rings | Harvard Apparatus | 5013091 |
| Custom Tubular Reactors (Figure | Parts were purchased | Misc. |
| Drying Box | - | - |
| High Pressure Syringe Pump | Harvard Apparatus | 70-3311 |
| Moisture Analyzer | Sartorius | MA35 |
| Nitrogen Delivery | Misc. | Misc. |
| Ratchet wrench and 7/8" socket | - | - |
| Retractable Thermocouple | Omega | RSC-K-3-4-5 |
| Stainless Steel Syringe | Harvard Apparatus | 702261 |
| Temperature Monitor | Omega | HH12B |
| Voltage Controller | McMaster-Carr | 6994K11 |
| | | |
| **Supplies** | | |

| | | |
|---|---|---|
| Metal Scoops, Spoons and/or | - | - |
| Plastic Bowls or Tubs | - | - |
| Spray Bottle | - | - |
| Wide-Mouth Funnel | - | - |
| Wooden Dowel | - | - |
| | | |
| **Consumables** | | |
| Glass Wool | Sigma-Aldrich | CLS3950-454G |
| Plastic Press-to-Close Bags | McMaster-Carr | 1959T24 |
| Plastic Tote | - | - |
| Plastic Weighboats or Metal | - | - |

| Comments/Description |
|---|
| Single gas detector, Ammonia (NH3), 0 to 100 ppm |
| Keep hands protected when handling liquid ammonia |
| Ear muffs to protect hearing when releasing ammonia at end of pretreatment |
| Wear while handling ammonia |
| Showa heat resistant gloves, max temperature 500°F |
| Wear while mixing biomass to prevent contamination |
| |
| |
| An anhydrous ammonia compressed gas cylinder with a dip tube is required for this process. The dip tube is |
| Used to add water to the biomass to achieve the desired water loading |
| Corn stover is not readily commercially available. Contact local farmers or agricultural extension if you wish |
| |
| |
| |
| CGA fitting that depends on the gas cylinder. Matheson is a good source. Some require teflon gaskets. This |
| Stainless steel pressure cylinder and components, valves, check valves, and gauges were used for all lines |
| Balance used for preparing biomass and weighing the reactors. Toploading balance, 4200g x 0.01g |
| Ammonia-resistant o-rings for the SS syringe |
| To be compatible with ammonia, the custom reactor was constructed from stainless steel components |
| Optional: an enclosed system for drying is necessary if planning to do microbial experiments to avoid |
| Infuse/Withrdraw PHD ULTRA HPSI Programmable Syringe Pump for transferring liquid ammonia |
| Moisture analyzer for determining moisture content of biomass prior to pretreatment. |
| Nitrogen compressed gas cylinder, inert gas regulator (at least 1000 psig max pressure rating), lines, and |
| Ratchet and socket to quickly tighten and open bolts on the sanitary clamp. Can be purchased anywhere. |
| Retractable thermocouple cable. You need one for each reactor. |
| Stainless steel syringe for tranferring ammonia to the reactors. |
| Dual input temperature monitor. You need one for every two reactors. |
| Variable-Voltage Transformer for controlling heating to the reactors. You need one for each reactor. |

For transferring biomass for weighing, mixing, transferring into the reactor and removing from the reactor
Used for mixing the biomass with the water. Any bowl or tub could be used.
Used to add water to the biomass to achieve the desired water loading
Any funnel that has a bottom opening 0.5-1.0 inches diameter.
1-1.5" diameter wooden dowel to assist with loading/unloading the reactor

For packing the top of the reactor to prevent biomass escape and clogging the tubing
Bags for storing processed samples and for transferring to drying box
Used to transfer pretreated biomass to an alternate location for drying
Used to catch the biomass when removing from the reactors, and for storing the samples while drying

RUTGERS
School of Engineering

**Dept. of Chemical & Biochemical Engineering**
Engineering Building, Room C150A
Rutgers, The State University of New Jersey
98 Brett Road
Piscataway, NJ 08854-8058

http://chundawat.rutgers.edu
shishir.chundawat@rutgers.edu

p. 848-445-3678
f. 732-445-2421

Mar 30, 2020

To,
Nam Nguyen, Ph.D.
Editorial Department (Manager of Review)
JoVE, 1 Alewife Center, Suite 200, Cambridge, MA 02140

**Subject**: *Response to editor & reviewer comments for manuscript ID JoVE57488*

Dear Nam,

Please find below a response to editorial comments for our manuscript (ID: JoVE57488) titled "**Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass**". We have uploaded an updated video protocol and made some minor changes to our manuscript to reflect updated video protocol as requested by the editorial team. Updated video file titled 'JOVE 57488_R5.mp4' has been uploaded on JoVE's website (https://www.dropbox.com/request/LMKYhpFzdr3wHyqD7qUt).

On behalf of all authors, I would like to thank you very much for your consideration and look forward to finally publishing our updated manuscript.

Sincerely,

Prof. Shishir Chundawat (Lead Corresponding Author)
Assistant Professor, Chemical & Biochemical Engineering Department
Rutgers, The State University in New Jersey, 98 Brett Road, Piscataway, NJ 08854, USA
Email: shishir.chundawat@rutgers.edu
URL: www.rci.rutgers.edu/~spc120/
Phone: 848-445-3678
Fax: 732-445-2421

*Enclosed: Brief response to editorial team comments and updated manuscript pdf highlighting track changes made to the document.*

**Response to Editorial/Production Comments**

**Revisions regarding the written manuscript:**
1. Has reference 39 been published? If not, please remove it.
**Response**: Yes Reference 39 has been published and now has been updated.

**Revisions regarding the video:**
**Overall Response**: We have made all the changes requested by the editorial team for the updated video. A updated media has been now uploaded that should address all previous editorial team concerns (https://www.dropbox.com/request/LMKYhpFzdr3wHyqD7qUt). Individual responses are provided below.

1. 2:48 - Set up should be two words here.
**Response**: We have made all the changes requested by the editorial team for the updated video.

2. 6:43 - Shut down is two words here. Please make the change in the written manuscript as well.
**Response**: We have made all the changes requested by the editorial team for the updated video.

3. 7:48 - Please use correct chemical notation.
**Response**: We have made all the changes requested by the editorial team for the updated video.

4. 8:29 - There is a weird vocal glitch here.
**Response**: We have made all the changes requested by the editorial team for the updated video.

5. The cow images at 0:47 and 8:47 appear to be stock images. Is there a record of your license for these images?
**Response**: We have made all the changes requested by the editorial team for the updated video. We have replaced the earlier cow images with images from Pixabay, which does not require attribution:

Cow Image references:
https://pixabay.com/photos/cow-head-cow-head-animal-livestock-1715829/
https://pixabay.com/photos/cow-allg%C3%A4u-cows-ruminant-2782461/

Pixabay License
Free for commercial use
No attribution required

6. Audio: The audio in Rebecca Ong's on-screen introduction and conclusion has a large amount of room noise and what sounds like compression or noise removal. We may be able to clean this up a bit on our end. For the next submission, please remove any compression or processing from the audio.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7. Editing Style & Pacing: 1:13 - There is a jump cut here, followed immediately by a slow fade to an image of the pilot plant. I recommend cutting straight to this image before the jump cut is seen.

**Response**: We have made all the changes requested by the editorial team for the updated video.

8. Text/Graphics
"0:42, 0:47 - The images here could be scaled up to fill the frame. It isn't necessary, but it might look nicer and show more detail.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:20 - The white background of this image should be extended to fill in the thin black borders on the left and right sides of the frame.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:35 - It looks like half of the gray gradient background is missing here. This should be fixed.
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:43, 8:04 - The gradient background disappears here. This should be fixed."
**Response**: We have made all the changes requested by the editorial team for the updated video.

7:48 - The 3 in NH3 and 2 in H2O should be subscript to fit JoVE formatting
**Response**: We have made all the changes requested by the editorial team for the updated video.

Table



| Ammonia Delivery System & Strut Fram | |
|---|---|
| **Key** | **Part#** |
| 1 | PGI-63B-PG1000-LAQX-A |
| 2 | SS-400-4 |
| 3 | SS-401-PC |
| 4 | SS-42GS4 |
| 5 | SS-FM4TA4TA4-12 |
| 6 | SS-CHS4-EP-1 |
| 7 | SS-4-TA-1-4 |
| 8 | 304L-HDF4-500 |
| 9 | SS-42GXS4 |
| 10 | SS-QM2-S-200 |
| 11 | SS-5-HC-A-401 |
| 12 | SS-1RS4 |
| 13 | SS-400-7-2 |
| 14 | 15-61NMA |
| 15 | RD-1000 |
| 16 | SS-400-3 |
| 17 | SS-QC4-B-400KR |
| 18 | SS-QC4-S-400 |
| - | SS-T4-S-035-20 |
| - | 3310t59 |
| - | 3115t357 |
| - | 8920k115 |
| - | 3115t119 |
| - | CG-9250 |
| - | CG-9202 |

| AFEX Reactors (Parts list is for a single re | |
|---|---|
| 1 | SS-QM2-B-400KR |
| 2 | SS-401-PC |
| 3 | SS-42GXS4 |
| 4 | SS-400-4 |
| 5 | SS-200-R-4BT |
| 6 | KMQSS-125-G-9 |
| 7 | PGI-63B-PG2000-CAQX |
| 8 | SS-400-SC-16 |
| 9 | 40MP-G150 |
| 10 | 50485K75 |
| 11 | 4550T213 |
| 12 | 4322K221 |
| 13 | - |
| - | 4322K711 |

| e |
|---|
| **Item** |
| Pressure Gauge, 1000 psi, 63 mm dia., lower mount, 1/4" tube adapter |
| 1/4" SWG Cross Union |
| 1/4" Port Connector |
| 1/4" SS Ball Valve, Tube Fitting |
| 316L Stainless Steel Convoluted (FM) Hose, 1/4 in., 316L Stainless Steel Braid, 1/4 in. Tube Adapters, 12 in. (30.5 c |
| Stainless Steel Poppet 6000 psig (413 bar) Check Valve, 1/4 in. Swagelok Tube Fitting, 1 psig (0.07 bar), Ethylene P |
| Male Tube Adapter, 1/4 in. Tube OD x 1/4 in. Male NPT |
| 304L Stainless Steel Double Ended DOT-Compliant Sample Cylinder, 1/4 in. FNPT, 500 cm3, 1800 psig (124 bar) |
| 1/4" Tubing 3-Way Ball Valve |
| SS Miniature Quick Connect Stem without Valve, 0.06 Cv, 1/8 in. Swagelok Tube Fitting |
| SS Hose Connector, 1/4", Tube Adapter, 5/16" Hose ID |
| Stainless Steel Integral Bonnet Needle Valve, 0.37 Cv, 1/4 in. Swagelok Tube Fitting, Regulating Stem |
| 1/4" SS Tube Adapter x 1/8" FNPT |
| 1/8" MNPT Safety Head |
| 1/4" Rupture Disc, 1000 psi |
| 1/4" SS Union Tee |
| Quick Connect Body, 1/4" Tube Ends, Kalrez Seal |
| Quick Connect Stem, 1/4" Tube Ends |
| 316/316L Stainless Steel Seamless Tubing, 1/4 in. OD x 0.035 in. Wall x 20 Feet |
| Strut Channel, Green Powder-Coated Steel (various lengths for delivery system frame) |
| Strut-Mount Metal Routing Clamps, 2 1/8" ID (for bolting the NH3 transfer cylinder to the strut frame) |
| Low-Carbon Steel Rod, 1/4" OD, 3 ft length (for attaching the reactor clamps) |
| Strut-Mount Metal Routing Clamps, 1/4" ID (for bolting the steel rod to the frame) |
| Clamp Holders |
| Clamps, Two-prong extension |

| eactor) |
|---|
| Mini Quick Connect Body, 1/4" SWG, Kalrez |
| 1/4" Port Connector |
| 1/4" 3-Way SS Ball Valve |
| 1/4" SS Cross |
| 1/8" T x 1/4" TX Bore Through Reducer |
| Mini Thermocouple, K-type, SS sheath, 9" Length, 1/8" Dia |
| Pressure Gauge, 1/4" Tube Center Mount, 2000 PSI Max |
| 1/4"T x 1" Sanitary Clamp Flange |
| Sanitary Gasket, Teflon, 1.5" OD |
| High-Polish Quick-Clamp Sanitary Tube Fitting,316L Stainless Steel Straight Connector for 1-1/2" Tube OD, 8" Long |
| Ultra-High Temperature Heater with Plug, 120V AC, 2 Feet Long, 104W |
| 304 Stainless Steel Cap for 1" and 1-1/2" Tube OD High-Polish Quick-Clamp Sanitary Tube Fitting |
| 1/4" 316 Stainless Steel tubing bent to serve as a vent line (see ammonia delivery system) |
| 304 Stainless Steel Clamp with Bolt for 1 and 1-1/2" Tube OD Sanitary Fittings |

| | | |
|---|---|---|
| # | Unit | Store |
| 1 | each | Swagelok |
| 2 | each | Swagelok |
| 6 | each | Swagelok |
| 4 | each | Swagelok |
| 2 | each | Swagelok |
| 2 | each | Swagelok |
| 2 | each | Swagelok |
| 1 | each | Swagelok |
| 3 | each | Swagelok |
| 1 | each | Swagelok |
| 4 | each | Swagelok |
| 2 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | High Pressure Equipment Company |
| 1 | each | High Pressure Equipment Company |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| - | - | McMaster-Carr |
| 2 | each | McMaster-Carr |
| 1 | each | McMaster-Carr |
| 2 | each | McMaster-Carr |
| 3 | each | Chemglass |
| 3 | each | Chemglass |

| | | |
|---|---|---|
| 1 | each | Swagelok |
| 3 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| 1 | each | Omega |
| 1 | each | Swagelok |
| 1 | each | Swagelok |
| 2 | each | Motion Industries |
| 1 | each | McMaster-Carr |
| 1 | each | McMaster-Carr |
| 1 | each | McMaster-Carr |
| - | - | - |
| 2 | each | McMaster-Carr |

**SUPPLEMENTARY PDF FILE FOR**

***Ammonia Fiber Expansion (AFEX) Pretreatment of Lignocellulosic Biomass***

*Shishir P S Chundawat[1], Ramendra Pal[1], Chao Zhao[1], Tim Campbell[2], Farzaneh Teymouri[2], Josh Videto[2], Chandra Nielson[2], Bradley Wieferich[3], Leonardo Sousa[3], Bruce E Dale[3], Venkatesh Balan[4], Sarvada Chipkar[5], Jacob Aguado[5], Emily Burke[5], Rebecca G Ong[5]*

[1]Department of Chemical and Biochemical Engineering, Rutgers-State University of New Jersey, Piscataway, NJ, USA

[2]Michigan Biotechnology Institute (MBI), Lansing, MI

[3]Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, MI, USA

[4]Department of Engineering Technology, Biotechnology division, School of Technology, University of Houston, Houston, TX, USA

[5]Department of Chemical Engineering, Michigan Technological University, Houghton, MI, USA

**SUMMARY OF SUPPLEMENTAL DOCUMENT CONTENTS:**

Here, we present here additional supplementary protocols for conducting AFEX pretreatment of corn stover at the lab-scale using a 0.6-liter or 5-gallon Parr reactors by either delivering the anhydrous liquid ammonia to the reactor gravimetrically or volumetrically using a pump, respectively. In addition, we have provided a rough outline of the protocol used to conduct packed bed AFEX using MBI's custom-built, commercial scale, 1-ton AFEX reactor system with integrated ammonia recovery. Bench-scale or lab-scale AFEX (using either the 5 gallon or 0.6 liter Parr reactors) was carried out at 1.0 g ammonia per g dry biomass, 0.6 g water per g dry biomass, for 30 min at 100 ± 5 °C. Pilot-scale packed bed AFEX was carried out at 0.6:1 ammonia-to-biomass loading, 60% moisture loading, for 30 min at 100 ± 5 °C based on packed bed AFEX method outlined in detail elsewhere.[1]

Our goal is to provide supplemental operating procedures for the safe and consistent operation of pressurized reactors for performing AFEX pretreatment on cellulosic biomass like corn stover. These supplemental protocols can be used to scale-up the AFEX process using larger Parr-type stirred stainless-steel reactors based on user requirements.

## SUPPLEMENTAL PROTOCOL 1 TO PERFORM AFEX USING 5-GALLON PARR REACTOR WITH AMMONIA DELIVERY USING PUMP:

**PROTOCOL STEPS:**

### 1. Adjusting Biomass Moisture Content

1.1 See supplemental **Table S1** below outlining all major equipment and materials necessary to perform bench or lab scale AFEX pretreatment.

1.2 Estimate total moisture content of biomass using a moisture analyzer, or an oven set at 103 °C. The moisture content of the biomass used here was estimated to be 10% (on total weight basis).

1.3 Combine 750 g of total weight biomass (or 675 g of dry weight of biomass) and water in the amounts according to the desired AFEX pretreatment conditions in a plastic container, mix well using spatula or by hand wearing nitrile gloves. Here, to achieve final desired moisture content of 40% on total weight basis, add 195 g of water to the original biomass.

### 2. Pretreatment Reactor and Auxiliary Equipment Setup

2.1 Make sure that the following equipment is securely plugged in by careful visible inspection: Lewa pump skid, Parr controller, and Julabo water bath (Set temperature to 4 °C). Check that the fume hood ventilation system is turned on. Directly underneath the pump head, the inlet side of the pump, fill the plastic pail reservoir with crushed ice. Do not run pump until the temperature gauge reads 40 °C or below.

| **Table S1** Major equipment and materials necessary to perform AFEX benchscale pretreatment using 5 gallon Parr reactor with volumetric ammonia delivery capabilities | | | |
|---|---|---|---|
| **Name of Materials/Equipment** | **Company** | **Catalog Number/s** | **Comments/Description** |
| 5 Gallon Pressure Reactor | Parr Instrument Company (Moline, IL) | NS4557 | 5 Gallon high pressure mixing reactor made with 316 Stainless Steel, equipped with an ammonia inlet valve and an exhaust valve. Maximum pressure of the vessel is 1900 psig at 225 °C. |
| Reactor PID Controller | Parr Instrument Company (Moline, IL) | 4843 | Temperature and mixing speed controller. |
| Respirator with filters | Northern Safety Co., Inc. (Utica, NY) | 161706 and 3943 | Full-face respirator fitted with unexpired methylamine filter to prevent exposure to anhydrous ammonia |
| Diaphragm Pump | American Lewa, Inc. (Rochester, NY) | EKM5-1-20MM | Diaphragm pump for ammonia delivery. Power: 0.5 HP; RPM: 1730 min-1; Diaphragm material: PTFE. |
| Julabo recirculating chiller/water bath | Julabo GmbH (Allentown, PA) | CD-200F | We need a recirculating chiller water bath at 4 C to keep ammonia pump skids cooled down. |
| Portable single gas direct readout ammonia monitor | Honeywell Analytics Ltd. (Lincolnshire, IL) | ToxiPro 544521VD Single Gas Polycarbonate Ammonia (NH3) Detector monitors | For detection of ammonia concentration in lab to prevent accidental exposure |
| Coriolis Mass Flow Measuring System | Endress + Hauser (Chalfont, PA) | Model Proline Promass 80 | Mass flow meter for liquid ammonia. |
| Microprocessor Control | Kessler-Ellis Products Co. (Eatontown, NJ) | Batch Control II - BT 28A3AZE Ver. 8.8 | Liquid ammonia mass flow controller. |
| Back Pressure Regulator | Tescom Corporation (RJM Sales, Scotch Plains, NJ) | 26-2300 Series - 26-2363-24 | Back pressure regulator installed downstream of the mass flow meter. Operation range: 10-250 psig. Body: Stainless Steel. Seals: Teflon. |
| Corn Stover | National Institute of Standards and Technology | Standard Reference Material RM 8412 | Cellulosic biomass feedstock that needs to be pretreated. Corn stover can be procured from other sources if not available at NIST. |
| Nalgene Plastic Utility Boxes | VWR | 36212-361 | To be used for mixing biomass with water |
| Heat protective gloves | VWR | 75836-506 | Kevlar® Nomex® heat resistant gloves protect up to 260°C during handling of hot reactor vessel |
| Cold protective gloves | VWR | 89217-722 | Keep hands warm and dry in temperatures as low as –31°C when handling liquid ammonia |
| Crushed ice | - | - | Need crushed ice to keep Lewa pump skids cool |
| Earplugs | VWR | 55533-030 | Ear plugs to protect hearing when releasing ammonia at end of pretreatment |
| Spatulas with PVC Handles | VWR | 82027-516 | Large spatula to mix and handle biomass |
| Crescent and open wrench | VWR | 62510-100 | Suitable tool kit to open/close Parr reactor etc |
| Distilled water | - | - | To mix into biomass to adjust moisture content |

2.2 Open the utility cold water valve to the reactor internal heat transfer coils and verify that water is flowing through the reactor and out to the drain. Put the reactor in the carrier. Align the reactor base with an agitator and raise the reactor base by pressing up on the control stick until there is a four-inch gap between the upper and lower part of the reactor bomb.

2.3 Check that the O-ring located in the top part of the reactor is free of debris and thinly coated with vacuum grease. Reapply grease, if needed, to ensure reactor lid seals properly.

**3. Loading Biomass into Pretreatment Reactor**

3.1 Add the moist and well-mixed biomass into the reactor. Once the biomass has been added, raise the reactor base until the bottom of the reactor is sealed with the top of the reactor.

3.2 When the reactor lid is seated against the O-ring, place the collar around the Parr reactor, and seal the reactor using a wrench. Use the rotational control stick to rotate the vessel from the vertical position to the horizontal position.

3.3 Ensure that all of the following manual valves on the reactor head or vessel are closed to begin with (based on reactor head design): (a) for ammonia release after run is completed, (b) for loading ammonia into the reactor, (c) for loading nitrogen into the reactor, and (d) for removing contents from the underside of the reaction vessel.

3.4 Connect the nitrogen tank line into the nitrogen inlet. Open the nitrogen tank valve. Charge the vessel with high pressure regulated nitrogen to 65 psi or $4.4 \times 10^5$ Pa to prevent the lines from freezing during addition of ammonia. Open the manual valve connecting the tank to reactor. Adjust regulator until the desired pressure has been reached; then close the valve and disconnect. Close the nitrogen tank valve.

3.5 Open the valve located directly on top of the ammonia tank and the ammonia outlet/inlet valves to the reactor. Check all fittings for tightness by visual inspection.

3.6 Record the temperature and pressure readings prior to heating. Preheat the mantle with the set point at 105 °C, on full power for 30 min prior to addition of ammonia to reactor. If step is skipped, the targeted temperature during pretreatment after ammonia addition will likely not be achieved.

3.7 Turn on and off the mantle power (manually or using temperature controller if available) depending on the temperature during the course of the process.

3.8 Turn on the Parr controller display and heater. Press button I and press the Reset button on the back of the controller. Set heater to desired mantle temperature, raise mantle, and begin preheating.

**Table S2** Lewa pump settings to be used to transfer desired ammonia to AFEX reactor

| Desired Ammonia Loading (g) | Pump Setting |
|---|---|
| 100 | 0.22 |
| 150 | 0.33 |
| 200 | 0.44 |
| 250 | 0.55 |
| 300 | 0.66 |
| 350 | 0.77 |
| 400 | 0.88 |
| 450 | 0.99 |
| 500 | 1.1 |
| 600 | 1.32 |
| 700 | 1.54 |
| 800 | 1.76 |

**4. Loading Ammonia into Pretreatment Reactor**

4.1 For addition of liquid ammonia into the reactor, use a pre-calibrated Lewa pump skid. See supplementary **Table S2** outlining details on pump settings to be used to transfer desired ammonia. The amount of ammonia to be added will depend on the AFEX pretreatment process conditions to be tested.

4.2 To program the pump skid equipment; first press the menu button on the pump display to PRESET option and then press enter. Next, enter the desired amount of ammonia to be batched (in grams) and press enter again.

4.3 If the PREWARN setting is equal to or greater than the PRESET, observe a display of PREWRONG indicating that the user must increase the PREWARN setting. To change this, press the menu button until it reads PREWARN, then press enter and adjust the PREWARN value so it is less than the preset value. Press enter.

4.4 To load ammonia into the reactor, turn the three-way valve towards the 5-gallon reactor (Valve midway down line from pump skid). To start the Run Mode, press "A" and then press the START button.

Note: When started, the pump turns on and the counter begins to calculate the amount of ammonia being administered in grams per min. When complete, the pump will automatically turn off and display the amount that was dispensed for that batch run.

4.5 To stop a batch, press "B", to temporarily suspend the current batch process by de-energizing the PRESET and the PREWARN switches. Press start, "A", to continue process.

4.5.1 To repeat a batch, press the CLR button then repeat step.

4.5.2 To change the batch size – with the current PRESET flashing on the display, type a new set point using the keypad. This number becomes the PRESET.

4.5.3 To display the batch total or rate – with the current PRESET flashing, press "ENT" to place the PRESET value in memory and use the "C" button to toggle between the batch total and the rate.

**5. AFEX Pretreatment Operation & Biomass Recovery**

5.1 After the desired amount of ammonia has been delivered, close the bonnet and needle valves on the ammonia line. Turn the agitator on by rocking the switch to the on position. Slowly ramp speed up to the second hash mark on the speed knob to set the desired agitation speed of 100 rpm (rotations per minute).

5.2 When the reactor is within 5 °C of the set point, start the reaction time. If unable to reach the target temperature within 5 min of the ammonia pump stopping, abort the run.

5.3 If the target temperature is obtained within the time criteria, record the temperature and pressure of the system. This is the initial temperature reading. Record the temperature and the pressure of the reactor every three min.

5.4 To regulate the temperature of the reactor, turn the mantle off and on, and raise and lower the mantle as needed during the run (if automatic temperature controller is not available). 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 ± 10 °C from the set point, the experiment must be aborted. If the target temperature (within 5 °C) is not reached within 5 min after ammonia pumping, abort the experiment.

5.5 When the desired pretreatment reaction time is complete, set the agitation to zero and turn off the heater jacket.

5.6 To arrest the reaction, slowly open the ball valve to release the ammonia directly into the fume hood. Wear earplugs to prevent damage to hearing. To access the reactor inside a walk-in fume hood, wear a respirator.

5.7 Flush the reactor vessel with nitrogen to remove bound ammonia from the vessel before opening the reactor to recover the pretreated samples. Wait for sufficient period of time. Note that this time depends on reactor size. For example, a 5-gallon reactor will likely require waiting for 15-30 min to purge most of the ammonia from the vessel.

5.8 Before opening reactor, verify that the reactor is fully depressurized. Using thermo-protective gloves, as needed, loosen and remove the collar. Lower the reactor cylinder using the pneumatic control stick. While wearing a respirator, remove the biomass from the reactor cylinder inside the fume hood.

**6. Reactor Shutdown and Cleanup**

6.1 Clean out the reactor and lid.

6.2 Close all the ammonia valves. Turn off the Parr reactor controller. Turn off the pump skid controller. Turn off the cooler for the pump skid. Close the utility cold water valves.

**REPRESENTATIVE RESULTS & DISCUSSION FOR SUPPLEMENTARY PROTOCOL 1:**

**Figure S1.** Nine different steps involved in producing AFEX (or Ammonia Fiber Expansion) treated biomass in a lab-scale 5-gallon pressure reactor. 1) Weighing biomass; 2) Adding water; 3) Mixing water with biomass; 4) Loading biomass in the reactor; 5) Applying vacuum and loading ammonia; 6) Running the reaction; 7) Removing the reactor head; 8) Unloading the AFEX-treated biomass; 9) Bagging biomass once dry.

In **Figure S1**, the following nine steps are summarized. (1) Add the desired amount of biomass into a pre-weighed plastic tub. Details regarding moisture analysis of biomass are available online on the National Renewable Energy Laboratory (NREL, Golden, Colorado, USA) website highlighting commonly used laboratory analytical procedures for standard biomass analysis (https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html). The moisture content of the biomass used here was estimated to be 10% (on total weight basis). Corn stover stalks were finely milled using standard hammer/knife mill to pass through a 5 mm sized screen, as outlined elsewhere[2], prior to performing bench-scale AFEX. (2) The required amount of water has to be mixed with biomass to raise the moisture content. Based on the moisture loading needed, the required amount of water is mixed with biomass. Here, to achieve final desired moisture content of 40% on total weight basis, we add 195 g of water to 750 g of total weight biomass (or 675 g of dry weight of biomass). (3) The biomass is mixed well to evenly distribute the moisture. (4) Moist biomass is loaded immediately into the 5-gallon pressure reactor. (5) The reactor head is tightened to seal the reactor and vacuum is applied for few min to remove air from inside the reactor. Next, an appropriate amount of ammonia is loaded into the reactor using the pump skid. Here, we are looking to add 1 kg of ammonia per kg of dry weight biomass. Therefore, the pump setting was set to deliver 675 g of ammonia to the reactor. (6) The reactor is then tilted from vertical position to horizontal position and mixed using a motorized auger for even heating of biomass using heating jacket. The operator user can use vertical mixing if access is limited to upright positioned reactor. At the end of the reaction time, the pressure is rapidly released, and ammonia is vented inside a fume hood. (7) The reactor bolts are loosened, and the head is removed from the reactor base. (8) AFEX treated biomass is then transferred into a plastic tub and left inside the fume hood to remove residual traces of ammonia overnight. (9) Lastly, AFEX treated biomass dry (<10% moisture) is then packaged the next day into zip-lock bags for storage at room temperature (or deli fridge for long-term storage) until further use. Representative enzymatic hydrolysis results for the 5-gallon AFEX reactor treated corn stover are highlighted in Figure 4.

In order to operate this larger bench scale AFEX reactor equipment, there should always be two operators present at all times for sake of safety. Operators must wear safety glasses at all times when operating the reactor and ear protection (ear plugs or protective ear muffs) during venting. Operators must wear protective heat resistant gloves when opening the reactor following the reaction. Cold-protective gloves must be available for use in the event of liquid ammonia leaks. Operators must be certified for full-face portable respirators. These should be equipped with unexpired methylamine cartridges and worn when inside the walk-in hood and opening the reactor following a run or unloading biomass from the reactor. Any residual ammonia in the reactor should be allowed to evaporate inside a fume hood. Prior to entering the fume hood, the operator should insert the portable ammonia monitor behind the plastic screen to determine the atmospheric concentration of ammonia. At concentrations above 35 ppm (STEL or Short Term Exposure Limit which is the employee's 15 minute time-weighted average exposure which shall not be exceeded at any time during a work day), operators must wear a respirator when entering the hood. At concentrations above 50 ppm, operators should not enter the hood. In the event of exposure to anhydrous ammonia, the operator first evacuates the premises for access to fresh air and immediately flush the affected area with water for at least 15 min. In the event of a catastrophic failure in which ammonia levels exceed the Immediately Dangerous to Life/Health or IDHL (IDHL is maximum concentration of a substance from which one could escape within 30 min without escape-impairing symptoms or irreversible effects) of 300 ppm (or the upper limit of sensor detection) inside the main work area, operators must evacuate the room and not attempt to contain the leak.

Having positive pressure in the reactor after loading the biomass and sealing the reactor will hamper the ammonia loading. De-pressurizing the reactor by applying vacuum (or adding steam to wet the biomass to targeted moisture content and purge air) prior to loading ammonia will help remove air from the reactor and subsequently make it easier to load liquid or gaseous ammonia. Temperature and pressure sensors are very important for monitoring the system. If any of those sensors fail, they must be replaced prior to beginning experiments. An operator should be present close to the reactor and ammonia delivery system during the course of pretreatment process to oversee the operation. Stainless steel vessels (conventional alloys) are highly recommended. Mild steel or aluminum reactors will corrode over time when exposed to ammonia. If the operator realizes during the course of the pretreatment that too much or too little ammonia was added, that pretreatment run should be aborted. It may be dangerous to add or try to remove ammonia during the course of the reaction.

**SUPPLEMENTAL PROTOCOL 2 TO PERFORM AFEX USING MBI's PACKED BED 1-TON PILOT SCALE REACTOR SYSTEM WITH INTEGRATED AMMONIA RECYCLE:**

Summary of critical equipment specifics for performing packed bed AFEX process on a pilot scale is provided in **Table S3** below. Additional details can be made available by MBI/MSU[1] and a representative image of the MBI pilot AFEX equipment is shown in **Figure S2A** below.

**PROTOCOL STEPS:**

**1. Biomass preparation**

Ground corn stover with particle size around 1-2 inches and the required amount of water for adjusting the moisture to 20 ±2% (based on total weight) are added to a ribbon mixer. The contents of the mixer are then dispensed into baskets (**Fig. S2B**) and packed to the target density (80-100 kg/$m^3$) using the in-house fabricated basket packer device (**Fig. S2C**). Baskets were fabricated by MBI team using stainless steel perforated sheet, with 16.5"diameter and 14-3/4 inch height. Seven baskets are needed for each reactor.

**2. Load biomass**

The tops of MBI's pilot-scale reactor vessels are equipped with hinged T-Bolt closures and the packed baskets are lowered in from the top. Baskets' outer diameters are ¼" to 1/8" smaller than the reactors' inner diameters, so that the baskets fit snugly within the reactors. The baskets sit on top of each other. Once all seven baskets are loaded into the reactor, the T-Bolt closure is sealed.

**3. Pre-steaming**

Once the reactor is sealed, the pre-steaming step starts by opening the bottom valve on the reactor and introducing a low pressure (15 psig) steam to the reactor from the top. During the pre-steaming process, air is forced out of the reactor through the bottom port and displaced by saturated water vapor. At the same time, condensation of steam heats and moistens the biomass. The process continues until a temperature sensor at the bottom of the reactor reaches 80°C, at which point the steam is turned off and the valves at both the top and bottom of the reactor are closed. The average temperature in the reactor is approximately 95°C at this point, and the pressure remains at or near atmospheric.

**4. Ammonia addition**

After pre-steaming, the reactor bottom valve is closed and ammonia vapor is added to the reactor through a port located above the top of top basket. Ammonia added to the reactor may be compressed vapor recovered from depressurization and steam stripping of the opposite reactor, or may be fresh vapor generated by pumping anhydrous liquid ammonia from a storage tank into a steam-jacketed vaporizer. As fresh ammonia vapor is added, the pressure in the reactor vessel rises. When the pressure reaches 300 psig, the ammonia pump is turned off and the pressure in the reactor is allowed to decrease until the pressure drops to 250 psig. At that point, the pump is turned back on and more ammonia is added. This process continues until the desired amount of ammonia is added. When recovered ammonia vapor obtained from the opposite reactor is added, the pressure typically rises to between 200 and 250 psig, then fresh ammonia vapor is added to reach 300 psig as described above.

| **Table S3** Major equipment and materials necessary to perform AFEX pilotscale pretreatment using packed bed AFEX reactor with volumetric ammonia delivery capabilities | | | |
|---|---|---|---|
| **Name of Material/ Equipment** | **Company** | **Catalog Number** | **Comments/Description** |
| **Major equipment of AFEX pilot plant** | | | |
| Pilot plant Packed bed AFEX reactor | Kennedy Tank and manufacturing Co, Indianapolis, IN | Custom made | Diameter: 18", Height: 144", Material: 304 stainless steel, Maximum pressure: 490psig@400˚F, Top closure type: Sypris tube turns T-bolt, Bottom head: Standard weight pipe cap, Tank shell: 1/2" thick rolled and welded stainless steel plate |
| Compressor | Fisher Refrigeration , INC | Frick model RXF 15H | Type: Screw compressor, Suction pressure: 0 psig, Discharge pressure: 300psig, flow 3lb/min |
| Vaporizer | Enerquip, LLC | Custom made | Inlet: Liquid anhydrous ammonia, 300 psig , Ambient temperature of TK-1A1, lowest temperature of -18˚ C; Outlet:Gasseous anhydrous ammonia, 300 psig, Minimum 55 °C; Flow Rate: 2.35 kg/min; Utility: Saturated 75 psig steam |
| Ribbon mixer | Colorado Mill Equipment | RB-2000 | Mixing capacity: $60ft^3$, Motor: 7.5 HP, Agitator RPM: 18 |
| Baskets | Designed and fabricated by MBI | Custom made | 304 stainless steel perforated sheet, with 16.5"diameter and 14-3/4 inch height |
| Basket packing device | Designed and fabricated by MBI | Custom made | Capable of compressing the biomass to density abount 100 kg/m3 |
| Ammonia pump | Hydra-Cell | M-03 | with metalic head, Model#M03EASJHFEHA |

**Figure S2.** (A) Picture showing original packed bed AFEX reactor constructed at MBI-MSU. Next, picture shows the type of wire mesh basket used to load untreated biomass, before loading in to the pilot plant tubular reactor. Here, (B) depicts baskets fabricated by MBI, and (C) depicts manual basket packing device designed and fabricated by MBI.

**5. Soak time**
Once all ammonia is added to the reactor, it is allowed to soak. Valves at the top and bottom of the reactor are closed, and the biomass becomes pretreated. During this time, the pressure within the reactor gradually decreases, as ammonia vapor enters the liquid phase. Likewise, the temperature gradually decreases as heat is lost to the ambient air. The residence time of this soaking period can be between 30 min and several hours. In pilot scale operations, the residence time is approximately one hour, which is the amount of time needed to allow the second reactor to cool sufficiently to open and remove the treated biomass baskets and add new untreated baskets.

**6. Depressurization**
After soaking, the reactor is depressurized. Typically, depressurization of a reactor takes place just as the opposite reactor has finished pre-steaming. A flow control valve at the bottom of the reactor vessel is opened slowly and the pressure is released by allowing the ammonia vapor to flow into the top of the opposite reactor, until the two reactor vessels are at equal pressure. At this stage, a compressor is engaged, drawing the pressure in the reactor to below 20 psig. A condenser and liquid trap located on the suction side of the compressor minimizes intake of water vapor into the compressor. Ammonia vapor removed from the reactor during depressurization is re-pressurized by the compressor and is added to the opposite reactor.

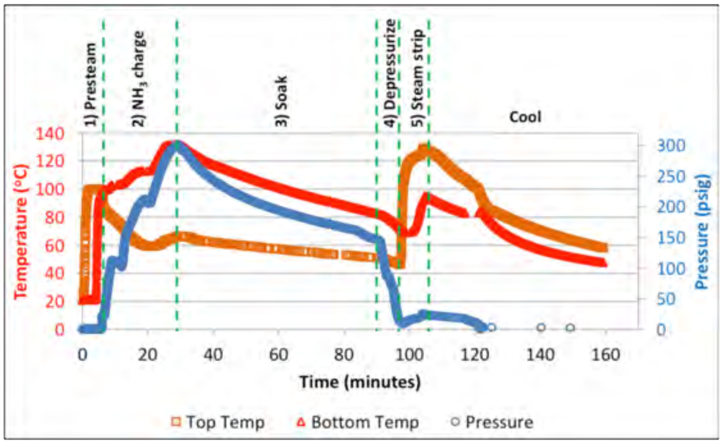
**7. Steam stripping**
Ammonia remaining in the reactor vessel after depressurization is removed via steam stripping. During steam stripping, steam is admitted to the top of the reactor vessel. Condensation of steam on the biomass releases heat which drives evaporation of ammonia vapor. The stripped ammonia vapor has lower temperature and higher density than the incoming steam, and is thus pushed out the bottom of the reactor as steam penetrates downward through the biomass. Ammonia vapor is drawn from the bottom of the reactor by the compressor, and the compressed ammonia vapor is added to the opposite reactor. Once the temperature at the bottom of the reactor reaches 100°C, it is assumed that all ammonia has been stripped. At this point, the steam flow is stopped and the bottom valve is shut off. The pressure in the reactor remains below 20 psig during steam stripping.

**8. Unloading the reactor**
At the end of steam stripping the baskets are too hot (about 100°C) for manual handling and are allowed to cool with air sweeping through the reactor for approximately 1-1.5 hours. The reactor is allowed to cool to 60°C before opening the T-Bolt closure, at which point the baskets can be removed using a hoist. The AFEX treated biomass is approximately 40 to 45 percent moisture at this stage and must be dried prior to storage.

**REPRESENTATIVE RESULTS & DISCUSSION FOR SUPPLEMENTARY PROTOCOL 2:**
Pretreatment efficacy for the pilot-scale AFEX reactor has been demonstrated to match the 5-gallon and 200 mL lab-scale batch reactions, achieving comparable yields of hydrolyzed sugars using commercial cellulase cocktails (Figure 4). **Figure S3A** shows the temperature and pressure profile within the packed bed pilot-scale reactor during a typical AFEX run. All steps except for

loading the reactor and unloading the reactor are shown. This custom designed system enables control for two reactors, a condenser, compressor, and ammonia vaporizer, as well as inlet ports for steam, ammonia, and air and an outlet port to a scrubber connected to a waste vessel. This control panel is used to open or close each of the valves that are involved in the processing steps for both reactors. Overall bed cycle time at pilot scale is approximately 110 minutes to perform a single cycle of packed bed AFEX pretreatment. Duration of each step is presented in **Figure S3B**. Lastly, **Figure S3C** shows representative glucose and xylose hydrolysis yields from more than 500 pilot scale AFEX treatment runs of corn stover.

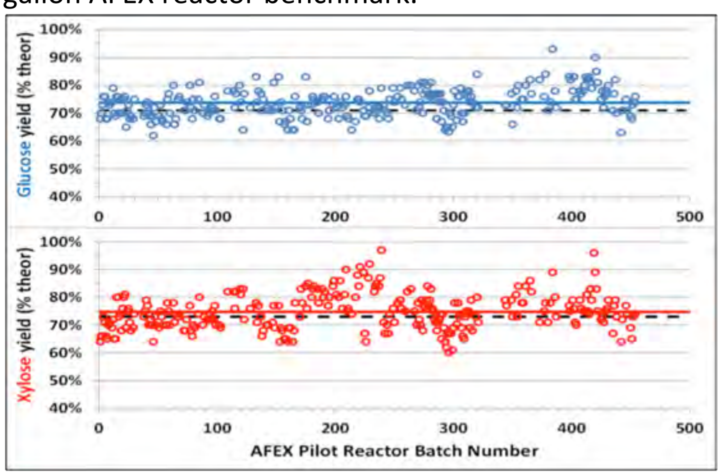
**Figure S3.** (A) Temperature and pressure profiles within a pilot-scale reactor during a typical run.




(B) Cycle time based on packed bed AFEX pilot plant runs.

| Steps | $NH_3$ Charge | Makeup $NH_3$ Add → Soak | DePressurize → Steam Strip | Unload → Reload → PreSteam |
|---|---|---|---|---|
| Time (minutes) | 19 | 36 | 19 | 36 |
| | ← Reactor Cycle Time = 110 minutes → | | | |

(C) Trend showing the glucose and xylose yield from more than 500 pilot scale runs of corn stover. The solid line represents the average while the dashed line represents the laboratory scale 5-gallon AFEX reactor benchmark.



**SUPPLEMENTAL PROTOCOL 3 TO PERFORM AFEX USING 0.6-LITER PARR REACTOR:**

In the absence of a suitable pump to transfer of ammonia from the bulk ammonia cylinder to the reactor, anhydrous ammonia can be transferred gravimetrically. The following protocol summarizes this approach using standard Parr reactors (ranging in volume from 0.1-0.6 L), and details are similar to what has been published previously[3,4]. Here a 0.6 L reactor was operated at Rutgers University to demonstrate how to perform AFEX pretreatment. Equipment details are summarized in **Table S4** and representative pictures of the reactor presented in **Figure S4**.

1. **Adjusting Biomass Moisture Content**

1.1 Estimate the total moisture content of biomass, as illustrated in the 5-gallon reactor protocol.
1.2 Add 28.3 g of water to the original biomass (e.g., 60 g of total weight biomass or 55.2 g of the dry weight of biomass with 8% initial moisture) to achieve a final desired moisture content of 60% on a total weight basis. Mix well by hand wearing nitrile gloves.

2. **Pretreatment Reactor and Auxiliary Equipment Setup**

2.1 Ensure that the Parr controller is securely plugged in, and heating coils are functional. Check that the fume hood ventilation system is operating correctly.
2.2 Check that the captured O-ring located in the top part of the reactor is free of debris and thinly coated with vacuum grease. Reapply grease, if needed, to ensure reactor lid seals properly.

3. **Loading Biomass into Pretreatment Reactor**

3.1 Add the moist and well-mixed biomass into the reactor. Once the biomass has been added, put the reactor lid on the reactor vessel.
3.2 When the reactor lid is seated against the O-ring, place the collar around the Parr reactor, and seal the reactor using a wrench.
3.3 Ensure that all of the following manual valves on the reactor head or vessel are closed, to begin with (based on reactor head design): (a) for ammonia release after run is completed, (b) for loading ammonia into the reactor, (c) for loading nitrogen into the reactor, and (d) for removing contents from the underside of the reaction vessel.
3.4 Leak test of the reactor. Connect the nitrogen tank line into the inlet of the reactor. Open the nitrogen tank valve. Charge the vessel with high pressure regulated nitrogen to 200 psi. Isolate the reactor by closing the valve and disconnect. Close the nitrogen tank valve. Wait for 15 minutes to see the pressure drop. If the pressure drops less than 5 psi, continue the move to the next step. Otherwise, depressurize the reactor, dismantle the reactor, clean the seals and check the valves. Repeat steps from 3.2 to 3.4 again.
3.5 Depressurize the reactor opening the vent valve slowly.
3.6 Connect the reactor inlet to the ammonia tank. Open the valve located directly on top of the ammonia tank and the ammonia outlet/inlet valves to the reactor. Check all fittings for tightness by visual inspection.
3.6 Record the temperature and pressure readings before heating. Preheat the mantle with the setpoint at 105 °C, on full power for 30 min before addition of ammonia to the reactor. If the step is skipped, the targeted temperature during pretreatment after ammonia addition will likely not be achieved.
3.7 Turn on and off the mantle power (manually or using a temperature controller if available)

depending on the temperature during the process.
3.8 Turn on the Parr controller display and heater. Press button I and press the Reset button on the back of the controller. Set heater to desired mantle temperature, raise mantle and begin preheating.

4. **Loading Ammonia into Pretreatment Reactor**
4.1 Before ammonia addition, weigh the reactor filled with biomass to find the initial weight.
4.2 For the addition of liquid ammonia into the reactor, use a time-based withdrawal. The amount of ammonia to be added will depend on the AFEX pretreatment process conditions to be tested. This approach is based on a trial-and-error method and the user will need to run some preliminary experiments to determine the optimal time for obtaining the desired ammonia loading in their respective pretreatment system setup.
4.3 Stop the loading of ammonia after a specified time. Weigh the reactor. Subtract the initial weight to find out ammonia addition into the reactor. If the charged amount of ammonia is less than the required amount. Calculate the ammonia loaded per minute to approximate the time required for further addition. Add ammonia to the reactor for up until the calculated time.
4.4 If the ammonia is loaded more than the required amount. Adjust the ammonia loading by venting the excess ammonia through the vent valve. Here, for a 1:1 ammonia to dry weight biomass loading a total of 55.2 g of ammonia needs to be added to the reactor.

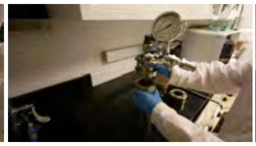
5. **AFEX Pretreatment Operation & Biomass Recovery**
5.1 After the desired amount of ammonia has been delivered, close the needle valves on the ammonia line.
5.2 When the reactor is within 5 °C of the setpoint, start the reaction time. If unable to reach the target temperature within 5 min of the ammonia addition, abort the run.
5.3 If the target temperature is obtained within the time criteria, record the temperature and pressure of the system. This is the initial temperature reading. Record the temperature and the pressure of the reactor every three min.
5.4 To regulate the temperature of the reactor, turn the mantle off and on, and raise and lower the mantle as needed during the run (if automatic temperature controller is not available). 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 ± 10 °C from the setpoint, the experiment must be aborted. If the target temperature (within 5 °C) is not reached within 5 min after ammonia loading, abort the experiment.
5.5 When the desired pretreatment reaction time is complete, turn off the heating mantle.
5.6 To arrest the reaction, slowly open the ball valve to release the ammonia directly into the fume hood.
5.7 Before opening the reactor, verify that the reactor is fully depressurized. Using thermo-protective gloves, as needed, loosen the collar and remove the collar. Remove the biomass from the reactor cylinder inside the fume hood.

6. **Reactor Shutdown and Cleanup**
6.1 Clean out the reactor and lid.
6.2 Close all the ammonia valves. Turn off the Parr reactor controller.

| Table S4 Major equipment and materials necessary to perform AFEX benchscale pretreatment using 0.1-0.6 L Parr reactor with gravimetric ammonia delivery option | | | |
|---|---|---|---|
| **Name of Materials/Equipment** | **Company** | **Catalog Number/s** | **Comments/Description** |
| 0.1-0.6 L Pressure Reactor System | Parr Instrument Company (Moline, IL) | Series 4560 Mini Reactor system with integrated heating mantle and controller | 100-600 mL range high pressure mixing reactor made with 316 Stainless Steel, equipped with an ammonia inlet valve and an exhaust valve. Maximum pressure of the vessel is 2000 psig at 225 °C. Temperature and mixing speed controller integrated with reactor system. |
| Weighing Balance | OHAUS | e.g., OHAUS Model D31P60BR5 | Weighing balance to weigh reactor before and after ammonia addition |
| Portable single gas direct readout ammonia monitor | Honeywell Analytics Ltd. (Lincolnshire, IL) | ToxiPro 544521VD Single Gas Polycarbonate Ammonia (NH3) Detector monitors | For detection of ammonia concentration in lab to prevent accidental exposure |
| Corn Stover | National Institute of Standards and Technology | Standard Reference Material RM 8412 | Cellulosic biomass feedstock that needs to be pretreated. Corn stover can be procured from other sources if not available at NIST. |
| Nalgene Plastic Utility Boxes | VWR | 36212-361 | To be used for mixing biomass with water |
| Heat protective gloves | VWR | 75836-506 | Kevlar® Nomex® heat resistant gloves protect up to 260°C during handling of hot reactor vessel |
| Cold protective gloves | VWR | 89217-722 | Keep hands warm and dry in temperatures as low as –31°C when handling liquid ammonia |
| Earplugs | VWR | 55533-030 | Ear plugs to protect hearing when releasing ammonia at end of pretreatment |
| Distilled water | - | - | To mix into biomass to adjust moisture content |

**Figure S4.** Different steps involved in producing AFEX (or Ammonia Fiber Expansion) treated biomass using a lab-scale 0.6 L Parr reactor using gravimetric method for ammonia delivery.

1. Adjust corn stover biomass moisture and mix contents well prior to addition into standard 0.6L Parr reactor



2. Add wet biomass to Parr reactor and seal reactor lid shut along with all ammonia entry/exit valve ports

3. Weigh the sealed reactor with loaded biomass prior to charging ammonia using a weighing balance

4. Pre-heat biomass in reactor to ~60 $^0$C prior to charging in ammonia for target temperatures > 100 $^0$C

5. Charge the liquid ammonia directly from ammonia cylinder (with dip-tube) into 0.6L-Parr reactor

6. Weigh reactor after charging ammonia and adjust by venting or adding ammonia according to desired loading

7. Pretreatment begins at desired temperature and ammonia loading for fixed total residence time

8. Monitor reactor pressure to check for any leaks during the entire process prior to ending the AFEX pretreatment run

9. Discharge ammonia carefully inside fume hood from reactor using vent valve to end AFEX pretreatment run

10. AFEX pretreated biomass is next removed from reactor for drying in fume hood overnight to ensure ammonia removal

**REFERENCES:**


1. Campbell, T. J., Teymouri, F., Bals, B., Glassbrook, J., Nielson, C. D. & Videto, J. A packed bed Ammonia Fiber Expansion reactor system for pretreatment of agricultural residues at regional depots. *Biofuels* **4**, )1(23–34 (2013).
2. Chundawat, S. P. S., Balan, V. & Dale, B. E. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. *Biotech Bioeng* **96**, )2(219 – 231 (2007).
3. Balan, V., Bals, B., Chundawat, S. P., Marshall, D. & Dale, B. E. Lignocellulosic biomass pretreatment using AFEX. *Methods in molecular biology (Clifton, N.J.)* **581**, (2009).
4. Teymouri, F., Laureano-Perez, L., Alizadeh, H. & Dale, B. E. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresource Technology* **96**, )18(2014–2018 (2005).