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Next-Generation Ammonia Pretreatment Enhances Biofuel Production from Biomass via Simultaneous Cellulose Decrystallization and Lignin Extraction†

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A new liquid ammonia pretreatment methodology called Extractive Ammonia (EA) was developed to simultaneously convert native crystalline cellulose Iβ (CI) to a highly digestible cellulose IIII (CIII) allomorph and selectively extract up to ~45% of the lignin from lignocellulosic biomass with near-quantitative retention of all polysaccharides. EA pretreated corn stover yielded higher fermentable sugar yield compared to the older Ammonia Fiber Expansion (AFEX) process while using 60% lower enzyme loading. The EA process preserves extracted lignin functionalities, offering the potential to co-produce lignin-derived fuels and chemicals in the biorefinery. The single-stage EA fractionation process achieves high biofuel yields (18.2 kg ethanol per 100 kg untreated corn stover, dry weight basis), comparable to those achieved using ionic liquid pretreatments. The EA process achieves these ethanol yields at industrially-relevant conditions using low enzyme loading (7.5 mg protein/g glucan) and high solids loading (8% glucan, w/v).

1. Fundamentals of EA pretreatment process

Broader context
Replacing fossil-based feedstocks, particularly petroleum, to power a sustainable economy is a key challenge facing humankind. Lignocellulosic crop residues are a promising alternative feedstock for producing liquid fuels and chemicals for modern biobased economies. Biochemical conversion of lignocellulosic biomass to liquid fuels requires pretreatment and enzymatic hydrolysis of the biomass to yield fermentable sugars. To compete with traditional petroleum refineries, cellulosic biorefineries must achieve high carbohydrate-to-fuel yields with low enzyme inputs and facilitate lignin valorization to commodity products, beyond simply generating heat and power from lignin. Most pretreated feedstocks require high enzyme loadings to achieve high sugar yields under industrially-relevant processing conditions. Enzyme levels can be reduced by conducting pretreatment with expensive and difficult to recover chemicals (e.g., ionic liquids). Here, we propose using liquid ammonia to pretreat lignocellulosic biomass under conditions that allow conversion of native cellulose to a highly digestible cellulose allomorph and simultaneously extract a lignin fraction for downstream catalytic upgrading. Using this new method, enzyme loadings can be significantly reduced by removing lignin-caused inhibition of enzymes, increasing enzyme accessibility to structural carbohydrates and by enhancing cellulose reactivity. EA-pretreated biomass hydrolysates are readily fermentable due to removal of lignin-derived inhibitors while preserving the microbial nutrient availability. Lastly, upgrading recovered EA lignin can increase product yield akin to ‘bottom-of-the-barrel’ processing of crude oil.
Our previous study demonstrated that plant-derived native cellulose (CI) is amongst the least digestible crystalline allomorphic forms by fungal cellulases and that it is possible to increase enzymatic hydrolysis rates 2 to 5-fold by restructuring allomorphic forms by fungal cellulases and that it is possible to cellulose (CI) is amongst the least digestible crystalline forms by fungal cellulases. Cellulose treatment by liquid ammonia has been used industrially to improve textile fiber properties at sub-zero temperatures and low pressures. Effective CIII formation during ammonia pretreatment of lignocellulosic biomass requires the substrate to have low moisture content and the usage of high ammonia-to-biomass ratios to completely submerge the biomass in liquid ammonia, allowing the formation of an intermediate cellulose–ammonia complex. This complex subsequently converts to CIII following ammonia removal. (Fig. S1†). High water concentration during pretreatment impedes cellulose III formation and reverts the ammonia-cellulose complex back into the allomorph CI. Other ammonia-based pretreatments such as ammonia fiber expansion (AFEX™, trade mark of MBI International, Lansing, MI) and ammonia-recycle percolation (ARP) do not lead to CIII formation because they employ high moisture contents and/or low ammonia-to-biomass ratios. In the case of AFEX, heat generated by the exothermic reaction between ammonia and water is used to reach temperatures up to 140°C. AFEX pretreatment requires homogeneous dispersion of moisture in the biomass (moisture contents of about 60% of the biomass dry weight) allowing water to form a contact layer on the surface and in the inner voids of the biomass. Once gaseous ammonia is added to the system, heat is generated by the formation of liquid ammonium hydroxide in the solid-liquid interface with the biomass (Fig 1b). Here, reactions between ammonia/ammonium hydroxide and cell wall components take place. As high moisture levels are maintained near the cellulose fibers during AFEX, it is not possible to form cellulose III after ammonia leaves the system (in the gas phase) in the end of the pretreatment process. The gaseous ammonia is further recycled (in the gas phase) through steam stripping, a condenser and a compressor to another pretreatment reactor previously packed with moist untreated biomass. (Fig. S5†). During AFEX pretreatment, about 0.02 g ammonia/g dry biomass reacts with the biomass, which needs to be replenished after each pretreatment cycle. In summary, by combining high moisture content and low ammonia-to-biomass ratios of 1:1 or 2:1, AFEX allows high sugar yields after enzymatic hydrolysis with relatively low operation pressures (200 to 500 psig) at relatively high temperatures (up to 140°C) with minimal or no external heat. However, these conditions are not favorable to cellulose III formation. To take full advantage of ammonia’s potential to reduce cellulose recalcitrance, EA pretreatment was performed on biomass at low moisture levels, typically around 10% (total weight basis), in a three stage process that includes: 1) reaction, 2) extraction, and 3) product/solvent recovery. Stage 1 (reaction) is performed in the reactor vessel (Fig. 1a), in which liquid ammonia is contacted with biomass at a sufficiently high loading to fully immerse the biomass at a defined temperature and residence time. Unlike AFEX, external heat is required during EA pretreatment to increase reaction temperature due to the absence of high moisture levels. As temperature increases, ammonia pressure builds up until a new vapor-liquid equilibrium is established. It is important to control the reactor volume so that most of the ammonia is in the liquid phase, submerging the biomass, at equilibrium. During this stage, the cellulose-ammonia complex is formed, ester bonds are cleaved, and lignin is partly solubilized in the liquid ammonia phase, as demonstrated in this study. Similar to the AFEX process, EA pretreatment promotes ammonolysis of cell wall ester crosslinks that are particularly abundant in monocots and herbaceous dicots. These key reactions disrupt lignin-polysaccharide cross-links, thereby enabling biomass deconstruction by improving access of enzymes to embedded structural carbohydrates. In Stage 2, EA-pretreated biomass is filtered to separate the ammonia-soluble components from the residual solids (Fig. 1a). During this stage, lignin is extracted, and CIII is formed from the cellulose-ammonia complex as ammonia is Fig. 1 Process design differences between Extractive ammonia (EA) and AFEX pretreatment. (a) EA laboratory equipment setup and mass balances for pretreatment performed at 120 °C, 6:1 ammonia:biomass weight ratio (NH₃:BM) for 30 min residence time on corn stover with 10 % (w/w) moisture (dry weight basis). (b) AFEX laboratory equipment setup and mass balances for optimal AFEX conditions to pretreat corn stover.
continuously removed from the biomass into an extract-collection vessel. While ammonia-soluble components of the biomass are being extracted, nitrogen overpressure is used to maintain ammonia in the liquid state at constant temperature. During Stage 3, ammonia is evaporated from the extractives, which are subsequently recovered as a dark brown viscous liquid (Fig. 1a). During EA process, about 0.022 g ammonia per 100 g biomass input cannot be recycled due to reactions between ammonia and the biomass. The remaining ammonia is recoverable and can be recycled (see ESI†).

When EA pretreatment was applied to corn stover (CS) using 6:1 ammonia-to-biomass weight ratio (NH$_3$:BM) for 30 minutes at 120°C, 16 wt% of the biomass was extracted by ammonia (Fig. 2a). This ammonia-soluble fraction contained 44 wt% of the biomass lignin and less than 5 wt% of the principal carbohydrates (as soluble glucan, xylan, and arabinan) present in untreated corn stover (UT-CS). Unlike AFEX, EA pretreatment is highly selective toward solubilization of aromatic lignin vs. carbohydrate polymers. Lignin is a major barrier to polysaccharide accessibility by biomass degrading enzymes and microorganisms. The EA pretreatment leaves nearly all of the carbohydrates available in a single dry solid stream for subsequent processing and biofuel production.

Following EA pretreatment, corn stover cell walls exhibit significant morphological differences with respect to the untreated control. Pretreated cell walls are swollen, delaminated and exhibit an overall lower contrast to safranin staining due to reduced lignin content compared to UT-CS (Fig. 2b). The intensity of safranin staining is greater in the vascular bundles of UT-CS, whereas for EA-pretreated cells walls, the staining intensity is reduced and is more uniform across the different cell types. Imaging with calcofluor (Fig. 2c), which stains crystalline and amorphous cellulose, shows higher intensity after EA pretreatment, suggesting that cellulose is more exposed and is therefore more accessible to the stain. Greater calcofluor staining does not necessarily mean higher enzyme accessibility, but this result indicates that cellulose fibers have been exposed following EA pretreatment, likely due to cell wall delignification, delamination, and swelling.

A similar effect was previously observed during AFEX pretreatment. AFEX dissolves lignin, creating delamination zones and cell wall swelling, and this dissolved lignin is deposited on the surface of pretreated cell walls when the ammonia evaporates. Consequently, cell wall porosity increases, and increased porosity is correlated with improved sugar yields during enzymatic hydrolysis of AFEX-pretreated corn stover (AFEX-CS)$^7$. Although both EA and AFEX pretreatments produce similar changes in overall cell wall morphology, EA also removes lignin and other decomposition products from the biomass while simultaneously producing CIII (Fig. 2d). These differences are crucial to the improved biological conversion of pretreated corn stover to fermentable sugars and a representative biofuel such as ethanol, as further demonstrated here.

2. Variables impacting EA performance during enzymatic hydrolysis

Sugar yields from EA-pretreated corn stover (EA-CS) are affected by pretreatment parameters, including temperature, the ammonia-to-biomass (NH$_3$:BM) weight ratio and reaction time. Contour plots describing the effect of pretreatment parameters on 24 h glucan and xylan conversion, at 1% glucan loading enzymatic hydrolysis, using 15 mg protein/g glucan enzyme loading, suggest that EA pretreatment is highly effective under a wide range of pretreatment conditions (Fig. 3a-b and Fig. S3†). Among these pretreatment parameters, temperature seems to be the most critical factor affecting fermentable sugar release during enzymatic hydrolysis. Fig. 3a and 3b show that EA-pretreated stover at 25 °C enables only 40-50% glucan and 20-30% xylan enzymatic conversion to glucose and xylose, respectively. Significant improvements in
carbohydrate digestibility are observed at higher temperatures, culminating with >90% glucan and >70% xylan conversion for temperatures greater than 115 °C, using NH₃:BM ratios higher than 4:1 and 30 min residence time. Examining the powder X-ray diffraction (XRD) spectra of EA-CS samples (Fig. S4†) we find that CIII is formed at both 25 °C and 115 °C, using 6:1 NH₃:BM ratio and 30 min residence time. However, the crystallinity of EA-CS pretreated at 115 °C is higher due to increased lignin solubilization and subsequent extraction. Ferulate cleavage by ammonia, as well as cleavage of other cross-linking esters in the monocot cell wall, are correlated to ease of cell wall deconstruction by hydrolytic enzymes¹⁰. A similar correlation can be seen for EA-CS (Fig. S2†). The rates of de-esterification reactions depend strongly on temperature, as the extent of ferulate depletion was negligible at 25 °C and 30 min reaction time (Fig. S2†). In contrast, more than 70% of the ferulate esters are removed from CS at 115 °C. These results suggest that CIII formed during EA pretreatment cannot be easily accessed by cellulases unless elevated temperatures that promote ester bond cleavage and lignin solubilization are employed to provide a more effective pretreatment.

To better understand the potential benefits of EA pretreatment on enzymatic hydrolysis, it is necessary to evaluate pretreated feedstocks under industrially-relevant conditions. Thus EA-CS pretreated at 120 °C for 30 min at 6:1 NH₃:BM was subjected to enzymatic hydrolysis at 15% and 25% solids loading for 72 h at 7.5 mg protein/g glucan and 30 mg protein/g glucan enzyme loadings. Fig. 3c compares the monomeric sugar release from EA-CS and AFEX-CS under identical enzymatic hydrolysis conditions. EA-CS is more digestible compared to AFEX-CS for all conditions tested. However, the benefits of EA become more evident at lower enzyme loadings, showing that the EA-pretreated substrate is highly digestible even under enzyme-limiting conditions. Similar 72 h sugar conversions were achieved for EA-CS at 7.5 mg protein/g glucan as when AFEX-CS was hydrolyzed at 18.75 mg protein/g glucan, i.e., EA-CS was as effective with a 60% reduction in net enzyme needed. As enzymes can contribute up to 50% of the biorefinery operating costs, enzyme usage reductions are crucial to cost-effective biofuel production. Uncertainties surrounding high enzyme costs tend to lower the confidence of potential investors in biofuels, further highlighting the benefit of the EA process.

For EA performed using 6:1 NH₃:BM ratio and 30 min pretreatment time, delignification is effective at temperatures above 115 °C, reaching up to 44% delignification at 120 °C (Fig. 4a). Lower ammonia loadings reduce lignin extraction and therefore, we chose 6:1 NH₃:BM ratio to achieve high levels of cell wall delignification and evaluate the full potential of this technology during downstream processing. To evaluate the effect of lignin extraction on enzymatic digestibility of EA-CS, EA pretreatment was conducted with and without lignin extraction, i.e., the cell wall extractives were re-deposited on the biomass and ammonia gently evaporated from the reactor at the end of Stage 2 of the process described above. AFEX-CS was used as a control, as AFEX does not physically remove lignin and does not generate CIII. Even when lignin is not separated from the biomass during EA, CIII was formed (data not shown). During AFEX pretreatment, under conditions that are typically mass-transfer limited, lignin is partially solubilized and re-deposited back on the surface of the biomass upon ammonia evaporation⁷. EA without lignin extraction improved glucan conversion by 21 percentage points compared to AFEX, most likely due to CIII formation, but perhaps also due to more effective lignin solubilization/re-deposition at higher ammonia-to-biomass loading. Lignin extraction during EA further increased glucan conversion by 6 percentage points compared to an already highly digestible material (i.e., EA without lignin extraction) to yield 89% overall glucan conversion (Fig. 4b).

3. Chemical properties of EA extracted lignin

To better understand the chemical composition of EA-solubilized lignin, 2D-NMR was performed on native lignin isolated from CS as well as on the crude EA lignin-enriched
extract from CS (Fig. 3c and Table S1†). Extracts from the EA process contain most of the native lignin functionalities typically found in native CS. However, the relative abundance of those functionalities and also the syringyl-to-guaiacyl (S:G) ratios change, as only a fraction of the lignin is removed during EA pretreatment. The β-aryl ether linkages remain intact after EA pretreatment, without degradation or condensation and polymerization reactions, unlike those occurring during steam explosion or acid-based pretreatment of lignin. Lignin depolymerization and condensation reactions are dominant lignin modifications during acid pretreatments. These reactions lead to the formation of C-C bonds, between lignin monomers. As these chemical linkages require more energy to be cleaved, lignins with high degree of condensation tend to be less susceptible to depolymerization via chemical catalysis. Therefore, pretreatment methods that preserve the dominant ether linkages between monolignols are preferable, if the goal is to valorize lignin to fuels and chemicals. Various methods have been proposed to depolymerize lignin via chemical catalysis. For example, some of the most promising methods to depolymerize lignin for fuel applications involve hydrogenolysis of ether linkages, which can be performed by hydrogen transfer from alcohols. By this approach, the fuel value is increased due to the addition of H₂ equivalents during lignin depolymerization via ether linkage cleavage. As EA-derived lignin preserves ether linkages, it suggests that it can be more easily utilized for subsequent chemical upgrading using methods as the ones described above. Other alternative processes involve conversion of lignin to lignosulfonates, which are currently used for production of plasticizers, etc.

During EA, the major chemical modifications to lignin occur via ammonolysis of ester-linked ferulate and coumarate linkages. The EA extract contains some unreacted coumarate esters but not residual ferulate esters (primarily because these remain with the insoluble polysaccharide-rich fraction). Coumaroyl amide and feruloyl amide were identified in the EA extract as the major products of ammonolysis reactions. Preliminary work also showed that about 70% of the ammonia-extracted lignin is ethanol soluble, which may become an important factor to improve the rate of hydrogenolysis reactions, compared with insoluble lignin. The ultimate impact of these lignin properties on lignin upgrade still needs to be experimentally determined in order to have a comprehensive assessment of the value of EA-derived lignins compared to other available lignins.

Depending on the pretreatment method used in the biorefinery, lignin can be recovered in different unit operations. For example, in biorefineries based on AFEX and dilute acid (DA) pretreatments, lignin is always recovered in the solid residue after enzymatic hydrolysis and fermentation. Based on previous reports, lignin from AFEX pretreated biomass has similar chemical functionalities to the EA-extracted lignin. This observation was expected, as

![Fig. 4 Importance of biomass delignification for the Extractive Ammonia (EA) process and structural profiling of EA-derived lignin for downstream chemical upgrading. (a) Extent of delignification of EA-CS after pretreatment at 6:1 ammonia:biomass (NH₃:BM) ratio, 30 min residence time, varying temperature from 25 to 120 °C. (b) Impact of lignin extraction on glucan conversion to glucose. AFEX was used as a control to evaluate the benefits of CIII conversion and lignin extraction to enzymatic digestibility of corn stover. (c) 2D-NMR of I) Native lignin extracted from corn stover and II) Crude lignin extracts resulting from EA pretreatment of corn stover. Legend: C-I, cellulose internal unit; C-NR, cellulose non-reducing end unit; C-Rα, cellulose reducing end unit, α-anomer; C-Rβ, cellulose reducing end unit, β-anomer; X-I, xylose internal unit; X-NR, xylan non-reducing end unit; X-Rα, xylan reducing end unit, α-anomer; X-Rβ, xylan reducing end unit, β-anomer; R, reducing end; NR, non-reducing end. NMR spectra have correlation contours color-coded to match those of the aromatics and lignin structures shown here.](image-url)
ammonolysis reactions occur both during EA and AFEX pretreatment processes. In contrast, lignin derived from DA pretreatment has reduced levels of β-O-4 linkages and increased molecular weight, suggesting repolymerization reactions and formation of C-C bonds. In biorefineries using ionic liquid (IL) pretreatments, it is also possible to fractionate the biomass into a lignin-rich and a carbohydrate-rich fraction before enzymatic hydrolysis, similarly to EA pretreatment. A previous report shows that [C$_2$ mim][OAc] can be used for lignin extraction during pretreatment and/or after enzymatic hydrolysis, depending on pretreatment conditions. That report showed that pretreatment conditions can be used to depolymerize lignin and, consequently, the level of β-O-4 linkages present in both lignin effluents. Similarly to AFEX and EA pretreatments, no condensation of lignin was observed during pretreatment with [C$_2$ mim][OAc]. For this reason, lignins from both ammonia-based and IL-based pretreatment processes offer good potential for further lignin valorization in lignocellulosic biorefineries.

4. Potential of EA pretreatment ethanol production, comparing with DA and IL pretreatments.

EA pretreatment was also compared to ILs and DA pretreatments using the same feedstock and optimized enzyme cocktails as described by Uppugundla et al. (2014). Condition 1 used 7.5 mg protein/g glucan enzyme loading 8% glucan loading for 96 h enzymatic hydrolysis; Condition 2 used 30 mg protein/g glucan enzyme loading, 6% glucan loading for 72 h enzymatic hydrolysis. DA and IL pretreatment results for Condition 1 were from Uppugundla et al. (2014) for direct comparison with EA. (c) Fermentable sugar yield in the basis of 100 kg of untreated biomass input for the three pretreatments. (d) Total process yield for production of ethanol after pretreatment, enzymatic hydrolysis and fermentation, on the basis of 100 kg of untreated biomass input. The potential ethanol yield from available fermentable sugars in each hydrolysate is also presented. In this work, fermentation of sugars present in the liquid stream generated by DA pretreatment was not performed experimentally, but those sugars were accounted for calculating the potential ethanol yield from available fermentable sugars.
Saccharomyces cerevisiae 424A (LNH-ST) without detoxification and exogenous nutrient supplementation (Fig. 5d and Table S2†). Detoxification and nutrient supplementation are typically required for hydrolysates generated from both the IL and DA pretreatments. The liquid stream generated by DA pretreatment contains a considerable amount of sugars that could potentially be fermented. However, that stream, which was not fermented in this study, requires neutralization, detoxification and nutrient supplementation to be effectively fermented to biofuel23.

For the more industrially relevant Condition 1, the ethanol yield from EA-CS was comparable to that obtained from IL-CS. Although IL-CS hydrolysate produced under Condition 1 offers a slightly higher biofuel potential based on the apparently available sugars, only 19% of the xylose in IL-CS hydrolysate could be consumed by S. cerevisiae 424A (LNH-ST) (Table S2†), whereas 94% of the xylose in EA-CS hydrolysate was consumed. Therefore, xylose consumption during fermentation is a major factor limiting the performance of the IL pretreatment. Low xylose consumption can be minimized by adding nutrients to the hydrolysate24, but this represents an additional cost that is not necessary for EA pretreated feedstock. Both IL and EA are pretreatments that remove lignin and modify the cellulose crystalline structure. IL converts C1 to amorphous cellulose and cellulose II (CII)24, whereas EA converts C1 to CIII. Neither the DA nor the AFEX pretreatments modify the native cellulose allomorph. These differences significantly affect cellulose degradation by fungal enzyme cocktails, as observed in the current study and elsewhere (Fig. 4)22,1.

5. Process considerations for EA pretreatment
There are several key aspects to consider when evolving the EA pretreatment technology for industrial use. In the first place, this process currently operates under high pressure (~1250 psi), which is required to maintain most of the ammonia in the liquid-phase at temperatures near 120°C (see ESI†). Such operating pressures lead to high capital costs associated to pretreatment. High operating pressure also raises concerns about feasibility of EA pretreatment in a continuous mode vs batch process. A continuous operation requires feeding solid biomass against a high pressure gradient, which remains challenging using current commercial equipment. However, technologies have been rapidly evolving in this area, as new low-cost pump designs for solid feedings are being developed for the coal/biomass gasification industry, which can be used across pressure gradients up to 1200 psi25.

A continuous process has various advantages. For example, it avoids process scheduling and biomass unloading/reloading time, leading to lower volume requirements for the pretreatment reactors. Also, nitrogen usage becomes unnecessary during continuous EA pretreatment, as the operating pressure generated by ammonia gas remains constant at steady state. Nitrogen is only required in a batch setup to maintain the pressure constant, while ammonia is removed from the system in the liquid-phase. Commercial scale EA pretreatment is also possible in a batch process, similarly to what has been used in this work. For example, efforts are being made by MBI International (Lansing, MI) to implement a commercial scale AFEX pretreatment in batch mode6.

Another important consideration when evolving EA pretreatment for commercial biorefineries is the reduction of ammonia loading during operation. High NH3:BM translates into high energy costs of ammonia recycling. A preliminary analysis of the energy required to recycle ammonia during EA pretreatment is presented and discussed in ESI†. In order to effectively recycle ammonia with minimal energy costs and recover the extracted lignin, liquid ammonia must be evaporated under high pressure and temperature in a flash tank. Gaseous ammonia and water vapor requires to be further separated using high-pressure distillation (~512 psi), for example. Keeping ammonia in gas-phase at high pressure facilitates ammonia condensation using cooling water or air at room temperature. This allows the usage of a pump for feeding ammonia to the pretreatment reactor instead of a compressor (unlike what is used for AFEX), thus saving power to the biorefinery. However, it is important to note that operating at higher pressure increases the capital cost of the pretreatment unit.

Our preliminary analysis shows that the heat and power required for recycling ammonia in a 6:1 NH3:BM ratio is 0.07 MMBTU/ gallon ethanol and 0.99 kWh/ gallon ethanol, respectively. If we assume a comparable ethanol yield after reducing ammonia loading to 3:1, the heat and power required to recycle ammonia is reduced to 0.05 MMBTU/ gallon ethanol and 0.51 kWh/ gallon ethanol, respectively. Assuming a cost of heat comparable to the cost of natural gas ($2.06/ MMBTU) and a cost of electrical power of $0.08/ kWh, the overall cost of recycling ammonia is $0.22/ gallon ethanol for 6:1 ammonia loading and $0.13/ gallon ethanol for 3:1 ammonia loading. Both these costs are higher than the energy costs required to recycle ammonia during AFEX pretreatment ($0.11/ gallon ethanol). However, higher ammonia loading during EA also translates into enzyme savings during enzymatic hydrolysis. If we consider ammonia makeup costs, energy costs for ammonia recycling and enzyme costs, AFEX pretreatment requires a total of $0.77/ gallon ethanol. In contrast, EA pretreatment requires $0.64/ gallon ethanol or $0.55/ gallon ethanol, depending if pretreatment is done at 6:1 or 3:1 ammonia loading, respectively.

Though the operating costs associated to EA pretreatment can be lower than for AFEX, it is still important to evolve EA pretreatment technology and reduce energy inputs for the following reasons. Assuming that two thirds of the heat used to produce electricity are not recovered as electrical power, the overall energy used to recycle ammonia during EA pretreatment (6:1 NH3:BM ratio) is about 94% of the high heating value (HHV) of the ethanol produced. By using lower ammonia loadings of 3:1 during EA pretreatment, this number can be reduced to 60% of the HHV from the ethanol produced. In contrast, the energy required to recycle ammonia during AFEX is about 36% of the HHV from the ethanol produced. A more thorough analysis is required to evaluate the total...
energy savings during downstream processing (including energy savings during enzyme production) when EA pretreatment is applied, compared to AFEX pretreatment. However, it is clear that EA pretreatment technology must evolve toward ammonia loading reduction in order to achieve higher standards of environmental and economic sustainability. Our target is to perform EA pretreatment effectively using NH\textsubscript{3}/BM below 2:1 (ideally 1:1), thereby achieving energy requirements comparable to AFEX pretreatment. Various strategies may be adopted to achieve this goal in future research. One possible scenario is to use cheap, volatile organic co-solvents (e.g., ethanol) during EA pretreatment to help submerge the biomass in an ammonia-solvent solution. This technique would allow the usage of lower ammonia loadings, cellulose III formation and lignin extraction during EA pretreatment. Moreover, volatile organic solvents are easily recoverable and can be reused. This process would be different from existing ammonia-catalyzed organosolv processes\textsuperscript{26,25}, as the concentration of ammonia must be high enough to convert native cellulose I to cellulose III, under low moisture conditions.

**Conclusions**

In summary, EA pretreatment was developed to selectively extract lignin from lignocellulosic biomass, while simultaneously converting recalcitrant C1 to a highly digestible CIII allomorph. Though CIII can be produced at room temperature, EA pretreatment is more effective at higher temperatures, which are required to maximize ester bond cleavage, lignin solubilization, and thereby improve enzyme accessibility to CIII. These cell wall modifications during EA pretreatment contribute to enzyme reductions of about 60% during saccharification, compared to a leading ammonia-based pretreatment – AFEX. The lignin extracted by the EA process preserves most lignin functionalities, including \(\beta\)-aryl ether bonds, and offers great potential for chemical upgrading to value-added aromatic/phenolic products. Lignin valorization is critical to the biorefinery techno-economic feasibility, as a range of chemical products can be generated by chemical or biological conversion from the same biomass input, as opposed to only heat and power in traditional lignin-utilization scenarios. EA pretreated corn stover generated ethanol yields comparable to IL pretreatment without either nutrient supplementation or detoxification, and achieved up to 18.2 kg of ethanol per 100 kg of untreated biomass (dry weight basis), at low enzyme loading of 7.5 mg protein/g glucan and at 8% glucan loading enzymatic hydrolysis. The EA process offers a key advantage of using ammonia as a pretreatment chemical. Ammonia is a widely available, inexpensive commodity chemical which enables comparatively easy recycling due to its high volatility compared to other more exotic pretreatment chemicals (see ESIF). Ultimately, the work presented here lays the foundation to understand the potential of ammonia as a pretreatment chemical for achieving high ethanol yields at low enzyme loadings, beyond what was possible with AFEX pretreatment. The fundamental knowledge described in this work can now be used to design EA process adjustments and achieve similar sugar yields while reducing ammonia loading and operating pressures.

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**Notes and references**