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A chemical-recovery-free ammonium sulfite-based alkali pretreatment of corn stover for low-cost sugar production via fertilizer use of waste liquor

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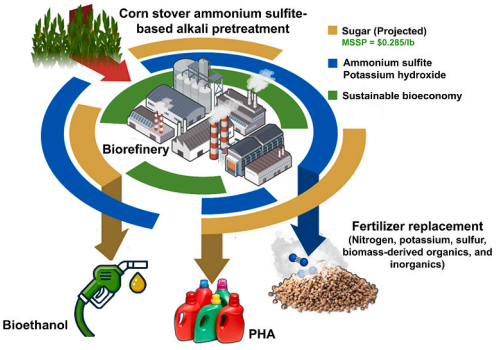
HIGHLIGHTS

- $(NH_4)_2SO_3$ showed limited biomass delignification and deacetylation at 80 °C.
- Alkali-facilitated sulfonation of biomass with AS occurs at low temperature.
- A combination of KOH and $(NH_4)_2SO_3$ achieved more than 95% total sugar yield.
- The spent liquor from the pretreatment showed significant potential as a fertilizer.
- The techno-economic analysis estimated a minimum sugar selling price of $0.285/lb.

GRAPHICAL ABSTRACT



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ABSTRACT

The production of cellulosic sugars is a pivotal strategy for advancing biomass bioconversion. This study evaluated the pretreatment of corn stover using ammonium sulfite and potassium hydroxide to develop comprehensive data on sugar, lignin, chemicals, and overall mass recovery profiles in a batch reactor at 80 °C. The results indicated significant improvements in delignification, deacetylation, enzymatic digestibility, and overall sugar yield. Specifically, pretreating corn stover with a solution of 40 wt% potassium hydroxide and 15 wt% ammonium sulfite at 80 °C for 2 h achieved 78.9 % lignin removal and 82.1 % acetyl removal, resulting in a total sugar yield exceeding 87.5 % with an enzyme loading of 12.5 mg protein/g-glucan plus xylan. The pretreated spent liquor, containing ammonium, potassium, sulfur, biomass-derived organics, and inorganics, demonstrated substantial potential as a fertilizer. The techno-economic analysis projected a minimum sugar selling price of $0.285 per pound, supporting the ongoing development and implementation of chemical-recovery-free pretreatment technology.

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1. Introduction

Biomass pretreatment is required to achieve high sugar yields and low costs essential for next generation biorefineries (Yang et al., 2018; Yang and Wyman, 2008). Pretreatment can decrease lignin inhibition on enzymatic saccharification of biomass carbohydrates by increasing cellulose accessibility and decreasing non-productive lignin binding to enzymes (Yang and Wyman, 2006). The formation of acetic acid from acetyl groups during removal hemicelluloses can further affect the microorganisms (e.g., *Zymomonas* and *Saccharomyces cerevisiae*) involved in downstream processing such as fermentation (Chen et al., 2016; Chen et al., 2011). Various pretreatment processes have been developed to address these challenges, focusing on delignification and deacetylation (Chen et al., 2016; Wu et al., 2020; Yang et al., 2018; Zhu et al., 2009). Delignification requires energy, water, and chemicals, the latter often needs expensive recovery operation, negatively affecting process economics and environmental impacts (Yang et al., 2018; Yang and Wyman, 2008). For example, the production and recovery of alkali in chemical pulping are energy intensive and have a negative environmental impact (Corcelli et al., 2018). Therefore, finding a pretreatment technology that is efficient and with low requirement of energy and chemical recovery is the key to the success of biorefinery industries (Yang and Wyman, 2008).

One promising approach is sulfite pretreatment, which is developed based on the sulfite pulping process (Zhu et al., 2009). This method dissolves lignin using mainly bisulfite (HSO_3^-) to produce water soluble lignosulfonate. Sulfonation also enhances the hydrophilicity and more importantly the surface charge of the residual lignin on the cellulosic substrate which can be mediated through pH to reduce nonproductive cellulase binding to lignin to improve enzymatic saccharification (Lan et al., 2013; Lou et al., 2013). The sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) process, has been extensively studied and has achieved remarkable cellulose conversion even for the most recalcitrance feedstocks (Zhu et al., 2015). However, under acidic conditions, hemicellulose degradation can lead to significant sugar loss at elevated temperatures, while alkaline sulfite pretreatment, conducted at pH values above 8, utilizes mainly SO_3^{2-} and OH^- ions for delignification (Wooley et al., 2020). Compared to acid and neutral sulfite pretreatments, alkaline sulfite pretreatment is less corrosive and does not generate HMF and furfural. It effectively removes lignin and acetyl groups from hemicelluloses, enhancing enzymatic hydrolysis of various lignocellulosic feedstocks (Monte et al., 2018; Silva et al., 2022).

Despite the efficacy of sulfite pretreatment, challenges persist, including the environmental impact and difficulties in chemical recovery. Here a chemical-recovery-free ammonium sulfite (AS) pretreatment was proposed. The idea is based on the historical use of sulfite pulping spent liquor for fertilizer by using ammonia as sulfite base to supply nitrogen to the soil (Phillips and Goss, 1936; Sears, 1976). A recent study also indicated that spent sulfite pulping liquor contains humic materials, an essential nutrient for microorganisms in soil (Li et al., 2017). Using ammonia not only provided nitrogen, but also eliminated metal based salt formation that is known to degrade soil. Therefore, using AS can eliminate the need for chemical recovery by directly utilizing spent liquor as fertilizer, especially with proper pH control during pretreatment and in the spent liquor itself. This study diverges from previous research on AS pretreatment (Qi et al., 2019; Zhang et al., 2019), which demonstrated the effectiveness of AS in delignifying biomass, such as wheat straw and bamboo, and enhancing sugar yields. In addition to sulfite, the ammonia released by AS can cleave C—O—C bonds in lignin as well as ether and ester bonds in lignin-carbohydrate complexes (Kim et al., 2003).

High-temperature sulfite pretreatment with the assistance of alkali has been widely verified, and commonly used alkalis include Na_2CO_3 and NaOH (Monte et al., 2018). Compared to these chemical reagents, potassium hydroxide (KOH) demonstrates comparable delignification and deacetylation capabilities at milder temperatures (Wu et al., 2020). Additionally, the spent liquor generated after KOH pretreatment has been shown to enhance soil microorganisms and promote plant growth (Xiao et al., 2006; Zahoor et al., 2021). Despite these advantages, research on AS and KOH pretreatment of lignocellulosic biomass under mild conditions, particularly for corn stover, remains limited.

The objective of this research is to explore the feasibility of pretreating corn stover with KOH and AS at low temperatures to produce fermentable sugars. The effects of different charges of KOH, AS, and their combinations on the removal of corn stover components were studied, and the sugar yields from the pretreatment and enzymatic hydrolysis stages were subsequently evaluated. The enzymatic hydrolysates of pretreated solids were compared to commercial glucose to assess their performance in polyhydroxyalkanoate (PHA) fermentation. Furthermore, the physical and chemical properties of the pretreated biomass and spent liquors were characterized to elucidate the chemical mechanisms of the pretreatment process. Finally, the feasibility of utilizing the pretreatment spent liquor as fertilizer was assessed through greenhouse bioassay experiments, and the economic viability of the process was evaluated using techno-economic analysis (TEA).

2. Materials and methods

2.1. Materials

KOH and AS were purchased from Sigma Aldrich (St. Louis, MO). The air-dried corn stover was provided by the National Renewable Energy Laboratory (NREL) and was harvested in Hardin County, Iowa, the detail was reported by Li et al. (2023). The corn stover was hammer-milled and screened to a nominal size of 250–425 μm for subsequent experiments and analysis. Corn stover contained 35.6 ± 0.4 % of glucan, 23.9 ± 0.3 % of xylan, 4.7 ± 0.2 % of arabinan, 20.0 ± 0.2 % of lignin, and 3.5 ± 0.1 % of acetyl groups, respectively, which were determined by NREL standard methods (Sluiter et al., 2008).

2.2. Pretreatment

AS and KOH pretreatments were conducted in a 300 mL stainless-steel reactor (model 4848, Parr Instrument Company, USA) with a working volume of 200 mL (Yang et al., 2024). Before pretreatment, 10 g of dry corn stover was immersed in 100 mL of pretreatment solution at a specific concentration and presoaked at room temperature for 2 days. For the AS pretreatment, the corn stover was presoaked in 100 mL of water under the same conditions. The presoaking step aimed to enhance chemical penetration into the biomass, thereby improving the efficiency of the subsequent pretreatment process. After presoaking, the entire mixture, including both solid and liquid phases, was transferred to the reactor without undergoing solid–liquid separation. An additional 100 mL of pretreatment solution of the same concentration was then added to the reactor. The charges of KOH and AS were based on the weight of dry corn stover (wt.%), and labeled accordingly: 40 wt% KOH as 40KOH, 15 wt% AS as 15AS, and a combination of 40 wt% KOH with 15 wt% AS as 40KOH/15AS. The reactor was then heated and maintained at a temperature of 80 °C and a rotation speed of 100 rpm for 2 h. After pretreatment, the reactor was cooled to 30 °C using ice water, and the mixture was subjected to solid–liquid separation. The solid residue was thoroughly washed with 70 °C Milli-Q water until a neutral pH was achieved. The washed solid was then transferred into pre-weighed plastic bags to determine its moisture content. Both the pretreated liquor and the washed pretreated solids were stored in a refrigerator at 4 °C for subsequent enzymatic hydrolysis and analysis.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis experiments were performed in 100 mL flasks using enzymes Cellic CTec3 (322 mg protein/mL, 106 FPU/mL) and Cellic HTec3 (200 mg protein/mL), obtained from NREL Laboratory. For

pretreated solids, enzyme loading was carried out at 20 mg protein Ctec3 with 5 mg Htec3/g (glucan + xylan), and 1 % solids loading (w/v). For enzymatic hydrolysis of pretreated liquor, adjust its pH to 4.8 by H_2SO_4. Then, 10 mL of pretreated liquor was taken, and enzyme loading was performed at 100 mg protein Ctec3 with 25 mg Htec3/g (glucan + xylan) (based on acid hydrolysis sugar content). A solution of 0.1 M citrate buffer at pH 4.8 and 1 % (v/v) 2 % sodium azide was added based on the total working volume to avoid microbial growth. The mixtures were incubated at 50 °C and 150 rpm for 72 h.

2.4. Analytical methods

The standard NREL Klason-lignin analysis of the pretreated solid residues after pretreatment was performed (Sluiter et al., 2008). Monomeric sugars (glucose and xylose) in pretreated liquor after pretreatment and enzymatic hydrolysis were analyzed by high-performance liquid chromatography (HPLC, Thermo Fisher Scientific Inc., Waltham, MA). Acid hydrolysis of pretreated liquor was performed according to NREL's procedure to determine the monosaccharide and oligosaccharide content (Sluiter et al., 2006). Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet iS10) analysis was employed to identify changes in the functional groups of both untreated and pretreated corn stover. The concentration of total acid groups was measured following a modified conductometric titration method based on Katz and Beatson (1984). In brief, 0.15 g of pretreated corn stover was soaked in 15 mL of 0.1 mol/L HCl at room temperature for 24 h. The mixture was then filtered through a Buchner funnel and rinsed with 250 mL of deionized water. The washed corn stover was transferred to a beaker with 48.5 mL deionized water, 1 mL of 0.05 mol/L NaCl, and 0.5 mL of 0.01 mol/L HCl. The mixture was titrated with 0.05 mol/L NaOH.

Enzymatic digestibility and monomeric sugar yields of stage 1 (pretreatment) and stage 2 (enzymatic hydrolysis) were calculated as following (see Eqs. 1–5):

$$\text{Enzymatic digestibility } \% = \frac{W_{EG} + W_{EX}}{W_{TG} + W_{TX}} \times 100 \quad (1)$$

$$\text{Glucose yield in stage 1 } \% = \frac{W_{PG}}{W_B} \times 100 \quad (2)$$

$$\text{Xylose yield in stage 1 } \% = \frac{W_{PX}}{W_B} \times 100 \quad (3)$$

$$\text{Glucose yield in stage 2 } \% = \frac{W_{EG}}{W_B} \times 100 \quad (4)$$

$$\text{Xylose yield in stage 2 } \% = \frac{W_{EX}}{W_B} \times 100 \quad (5)$$

where W_{TG} and W_{TX} are the amounts of glucose and xylose in pretreated solids; W_{PG} and W_{PX} are glucose and xylose weight determined after acid hydrolysis of pretreated liquor; W_{EG} and W_{EX} are amounts of glucose and xylose in the enzymatic hydrolysate; and W_B is the mass of the original dry corn stover.

2.5. PHA fermentation

An engineered *Pseudomonas putida* KT2440 strain was used for PHA fermentation by overexpressing the genes *phaC1* and *phaJ4* (Zhang et al., 2022). The microbial colonies were activated by transferring them into 20 mL of sterile Luria-Bertani broth and incubating at 30 °C and 200 rpm for 24 h. Cells were then transferred into 50 mL sterile M9 mineral medium containing minerals, nitrogen, buffer, and glucose or enzymatic hydrolysate from 40KOH/15AS-pretreated solid residues as the carbon source. Fermentation was performed in 250 mL baffled flasks at 30 °C, pH 7, and 200 rpm, with an initial glucose concentration of 5 g/L and an initial optical density (OD600) of 0.2, and was stopped after 60 h. After fermentation, the cell biomass was recovered by centrifugation at 10,000 rpm for 10 min. The recovered biomass was freeze-dried for 24 h in a benchtop lyophilizer (Labconco Corporation, USA). For PHA analysis, ~10 mg of dried biomass was suspended in 2 mL chloroform, and 2 mL of 15 % v/v sulfuric acid in methanol was added. PHA monomers were converted to methyl esters at 100 °C for 140 min and analyzed via gas chromatography-mass spectrometry (GC–MS, QP2010SE, Shimadzu) after organic phase separation and filtration through a 0.45-μm PTFE membrane.

2.6. GC–MS analysis

The pretreated liquor was adjusted to neutral pH using 0.05 % H_2SO_4. Then, 2 mL of the liquor and 2 mL of ethyl acetate were mixed and shaken for 5 min. The extract was stored in GC vials for analysis. One microliter of the organic phase was injected into an Agilent GC–MS system (Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass spectrometer) with a DB-5MS column (30 m × 250 μm, 0.25 μm film). Helium was the carrier gas at 1.2 mL/min. The splitter/injector was at 300 °C in splitless mode. The oven temperature increased from 45 to 200 °C at 15 °C/min, then to 280 °C at 5 °C/min, with 5 min solvent delays for proper separation.

2.7. Fertilizer replacement experiment

Two bioassay experiments were conducted in a controlled environment greenhouse at the University of Connecticut from January 3 to March 15, 2024, to study the K and S fertilizer values of the pretreated spent liquor. Each experiment included six treatments with four replications: a control, and five different ratios of spent liquor to commercial fertilizers (0 %:100 %, 25 %:75 %, 50 %:50 %, 75 %:25 %, 100 %:0%) at nutrient requirements for either K or S, which were calculated based on typical recommendations based on soil tests and nutrient uptake of silage corn. The size of pots was 3.79 L and each pot is one experiment unit. The media used was a mixture of volume 50 % sand and 50 % soil by weight, with low K and S levels. Surface drip irrigation was set up for each pot. Two corn plants were grown in each 3.79 L pot. Irrigation schedules, temperature, and light were controlled to create an optimal corn growth environment. The fertilizer and liquor application rate was tailored to meet the corn's requirements, with 179 kg K/ha and 34 kg S/ha for the K and S experiments, respectively. The liquor application rate for the 100 % liquor treatment in the K experiment and the S experiment was 34 mL/pot and 40.7 mL/pot in each 3.78 L pot, respectively. The treatments were surface applied in the first 6 weeks of experiment. Corn plants were harvested 2.5 cm above the soil mixture to avoid contamination. After harvest, corn plants were oven-dried at 60 °C for 72 h and weighed for biomass per pot, and total plant tissue S and K concentrations were tested using high temperature dry oxidation and dissolution of the ash with hydrochloric acid described in the standard nutrient testing protocols for the western region of United States (Gavlak et al., 2005). This methodical approach aimed to provide a comprehensive evaluation of the agronomic value of spent liquor as a fertilizer alternative.

2.8. Techno-economic analysis methodology

TEA was conducted following the methods described in a previously published study (Chen et al., 2016; Chen et al., 2011), with the Aspen Plus models developed based on the NREL 2017 biochemical sugar model (BC1707A). The model was updated with the current chemical loadings and conditions, as well as new experimental results such as sugar yields. Experimental data using 1 % w/v solids for enzymatic hydrolysis were extrapolated to estimate similar performance with 20 % solid loading in the model. The outputs from this Aspen Plus model were then plugged into the Microsoft Excel-based economic model.

The cost of AS was factored in with other material inputs, assuming a

40 % increase compared to the price of ammonia and SO_2. The model includes a presoaking unit and removes the electricity costs associated with mechanical refining. Capital expenditures for the disc refiner and Szego mill are set to zero, as mechanical refining was deemed unnecessary.

3. Results and Discussion

3.1. Effect of AS and KOH pretreatments on corn stover cell wall chemical structure and sugar yield

AS loading at 30 wt% resulted in negligible amounts of lignocellulosic component removal at mild temperatures as shown in Fig. 1A-D. Increasing AS charge to 100 wt% slightly increased component removal, particularly noticeable lignin. However, meaningful acetyl group removal was not achieved at both AS loadings. Similar lignin removal (4.5 %) was reported after Na_2SO_3 pretreatment at 80 °C, albeit with the removal of 30 % acetyl groups (Wu et al., 2020), possibly attributed to an extended pretreatment time (3 h) and altered chemicals. Given that alkaline sulfite pulping is commonly operated above 140 °C to promote sulfonation reaction, it was expected that substantial biomass removal could not be achieved at 80 °C (Ingruber, 1985).

Hydroxide ions (OH^-) have the ability to cleave α-aryl ether and α-alkyl ether linkages within the phenolic framework of lignin, as well as disrupt β-aryl ether and methyl aryl ether linkages found in non-phenolic structures. Additionally, in an alkaline environment, the acetyl group's negative charge is neutralized, leading to the formation of acetate ions and the liberation of free hemicellulose groups. Therefore, KOH pretreatment resulted in significant removal of lignocellulosic components. The extents of both deacetylation and delignification positively correlate with KOH charge (Fig. 1A-D). KOH at 5 wt% was found not as effective as NaOH at 4.8 wt% in terms of deacetylation and delignification (Chen et al., 2011). This might be due to less molar amount of OH^- in KOH than NaOH under the same mass loading. On the other hand, the charge shielding of K^+ is greater than that of Na^+, which resulted in a weaker delignification ability of KOH than NaOH for the same molar amount (Melro et al., 2020). As the KOH charge increased, removal percentages of xylan, lignin, and acetyl groups continued to rise. Notably, deacetylation and delignification rates reached 88.1 % and 80.7 %, respectively, at 40 wt% KOH charge. However, 51.2 % of xylan was also degraded under alkaline hydrolysis conditions.

As depicted in Fig. 1E-G, the sugar yields by AS pretreatments at 30 wt% and 100 wt% were low from both pretreatment and enzymatic hydrolysis stages. Enzymatic digestibility ranged from 19.5 % to 24.1 % (Fig. 1H). This indicates that AS itself was unable to achieve satisfactory sugar yields at mild temperatures due to inefficiently removing lignin and acetyl groups. Therefore, subsequent experiments were conducted using AS loading of 30 wt% without increasing dosage. Conversely, with increasing KOH charge, sugar yield and enzymatic digestibility were increased. Notably, a remarkable 91.5 % glucose yield, 44.2 % xylose yield, and 94.2 % enzymatic digestibility were achieved from the enzymatic hydrolysis stage at 40 wt% KOH. Therefore, the charge of KOH was not increased beyond 40 wt% in further studies.

3.2. Effect of AS pretreatment with KOH on corn stover cell wall chemical structure and the corresponding sugar yield

Achieving a satisfactory sugar yield from corn stover through AS pretreatment at 80 °C is challenging based on data presented earlier. However, the addition of alkali has been widely demonstrated to enhance the effectiveness of sulfite pretreatment (Monte et al., 2018). Therefore, a subsequent pretreatment using 30 wt% AS supplemented with varying KOH charges to enhance the process (see supplementary materials). Increasing KOH charge resulted in higher removal of xylan, lignin, and acetyl groups. Lignin and acetyl group removal reached 61.7 % and 79.7 %, respectively at 40 wt% KOH and 30 wt% AS (40KOH/30AS). However, lignin removal was decreased, which resulted in lower total sugar yield and enzymatic digestibility (see supplementary materials) compared with pretreatment using KOH alone at 40 wt%.

The presence of 30 wt% AS clearly had a detrimental effect on the delignification performance of KOH. Therefore, it is essential to elucidate the specific role of AS charge in the AS and KOH pretreatment process. As depicted in Fig. 2D, AS exhibited no significant effect on acetyl group removal at low temperatures, suggesting that deacetylation was primarily attributed to KOH. Furthermore, low AS doses (5–15 wt%) had minimal impact on lignin removal. However, as the AS charge exceeded 20 wt%, lignin removal significantly decreased. Notably, at a 50 wt% AS charge, the lignin removal decreased by 27 % compared with delignification achieved using 40 wt% KOH alone. Additionally, AS notably reduced xylan removal, resulting in increased xylan retention in pretreated solids.

AS reacts with KOH to release ammonia, sulfonic acid groups, and water, resulting in a reduction in OH ions in the solution and consequently causing a decrease in pH value. Therefore, the effects of different AS charges on pH before and after pretreatment were explored (see supplementary materials). Excessively increasing AS charges (>20 wt%) in 40 wt% KOH solution decreased the pH value of the process liquor before and after pretreatment. Notably, the addition of 50 wt% AS charge in the 40 wt% KOH solution dropped the pH of the pretreatment solution to 10.4. Fig. 1 indicated that the concentration of OH^- in KOH at low temperatures played a crucial role in delignification and deacetylation. Sufficient OH^- also facilitated effective deacetylation. Lower AS charges (<20 wt%) did not excessively deplete OH^- in the solution, while higher charges (>20 wt%) significantly decreased OH^- levels, leading to a sharp decline in pH value and insufficient OH^- for good delignification.

With the addition of AS, total sugar yield was increased initially then decreased as shown in Fig. 2E-G. Almost over 95 % of the total sugar yield was obtained with the addition of 15 wt% AS from both the pretreatment and subsequent enzymatic hydrolysis stages. Because more xylan was retained, adding AS resulted in a greater xylose yield through enzymatic hydrolysis than that from KOH pretreatment alone. Furthermore, low charges of AS (less than 20 wt%) did not significantly decrease component removal but aided in improving the enzymatic hydrolysis performance of pretreated solids. Specifically, pretreatment with 40 wt% KOH and 15 wt% AS (40KOH/15AS) achieved over 99 % enzymatic digestibility, indicating that AS addition promoted enzymatic hydrolysis effectiveness, possibly due to the action of amino or sulfite groups released by the presence of KOH.

Under an optimal chemical loading (40KOH/15AS), over 95 % total sugar yield from the pretreatment and enzymatic hydrolysis stages was achieved. In the pretreatment stage (stage 1), sugar yield was determined by acid hydrolysis, wherein sulfuric acid hydrolysis converts all polysaccharides in the pretreated liquor into monosaccharides. However, it is essential to note that sugars quantified via acid hydrolysis in the pretreated liquor do not necessarily represent those readily recoverable through enzymatic hydrolysis, as inhibitory substances in the liquor may impede enzyme activity. To assess the potential recovery of fermentable sugars through enzymatic hydrolysis, a high enzyme loading (100 mg protein Ctec3 with 25 mg Htec3/g (glucan + xylan)) was performed. This resulted in the recovery of approximately 80.0 % glucose and 60.0 % xylose (Fig. 3A). The degradation products, particularly phenolic compounds from lignin in the pretreated liquor might hinder enzyme activity (Qin et al., 2016; Ximenes et al., 2010). Lignin-carbohydrate complexes might also be present in the spent liquor and hinder polysaccharide utilization by enzymes. GC–MS analysis was applied later to identify the degradation products in the spent liquor (see supplementary materials). Furthermore, the addition of 15 wt% AS led to a slight decrease in sugar yield compared to 40 wt% KOH, as sulfite has been reported to act as a reducing agent to regulate enzyme activity (Zhong et al., 2019).

The enzymatic digestibility of solid residues after 40KOH/15AS

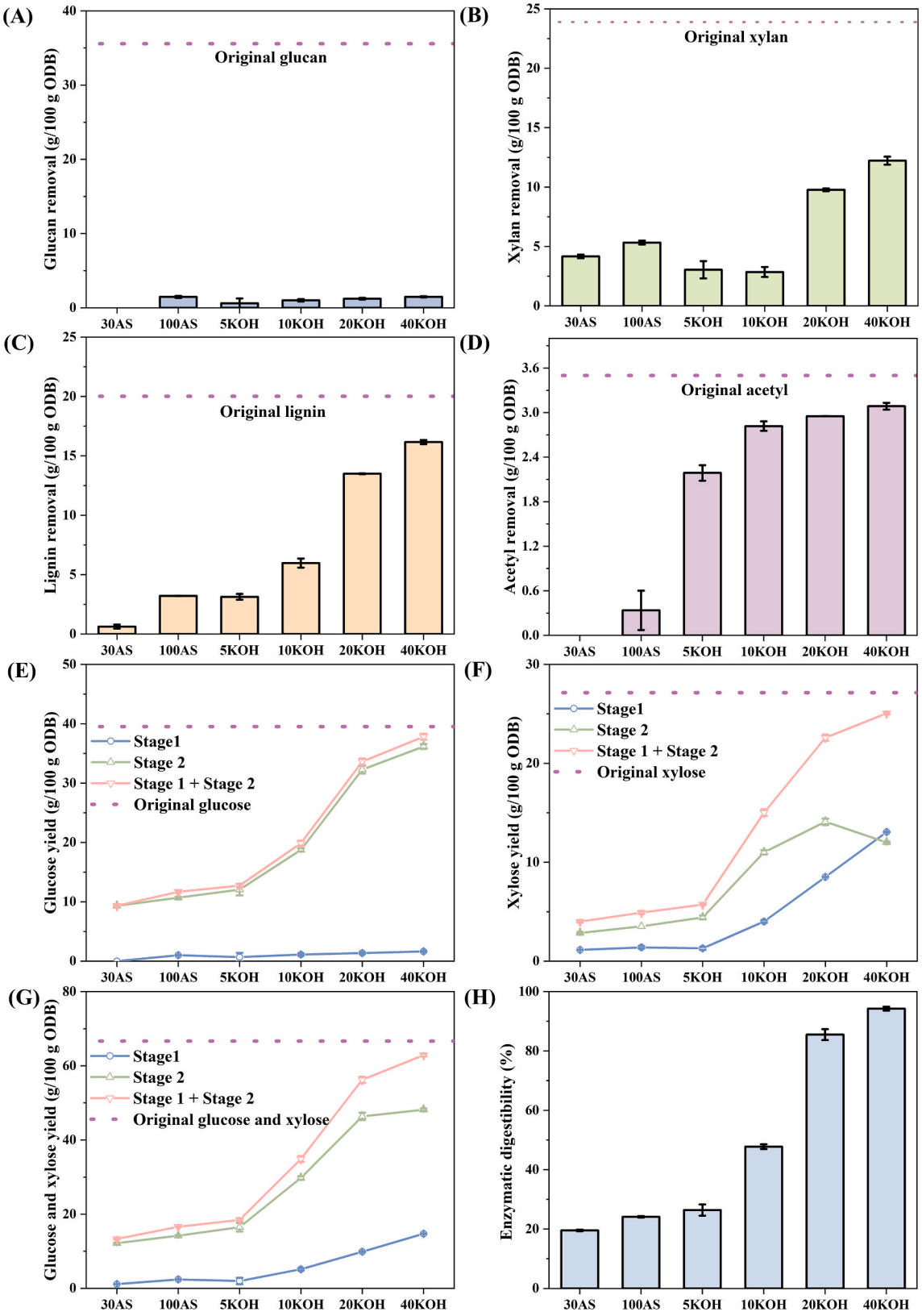


Fig. 1. Major component removal (% of original dry biomass (ODB)), sugar yields, and enzymatic digestibility of corn stover after AS and KOH pretreatment alone. Stage 1: sugar released during pretreatment (measured after acid hydrolysis); Stage 2: sugar released during enzymatic hydrolysis; Stage 1 + 2: sugar released after pretreatment and enzymatic hydrolysis, enzyme loading of 20 mg protein Ctec3 with 5 mg Htec3/g (glucan + xylan).

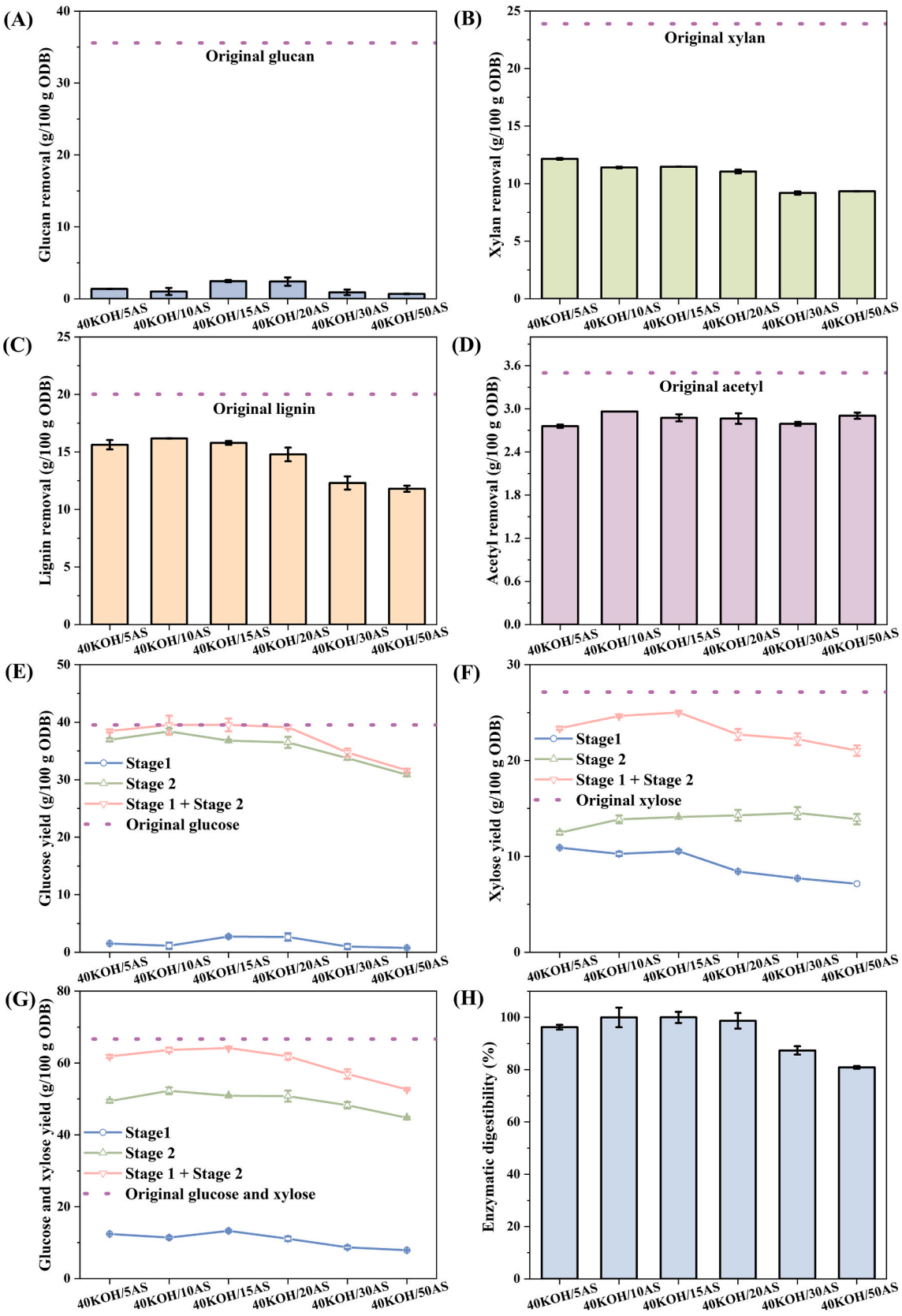


Fig. 2. Effect of AS dosage on major component removal (% of ODB), sugar yields, and enzymatic digestibility after alkaline AS pretreatment. Stage 1: sugar released during pretreatment (measured after acid hydrolysis); Stage 2: sugar released during enzymatic hydrolysis; Stage 1 + 2: sugar released after pretreatment and enzymatic hydrolysis, enzyme loading of 20 mg protein Ctec3 with 5 mg Htec3/g (glucan + xylan).

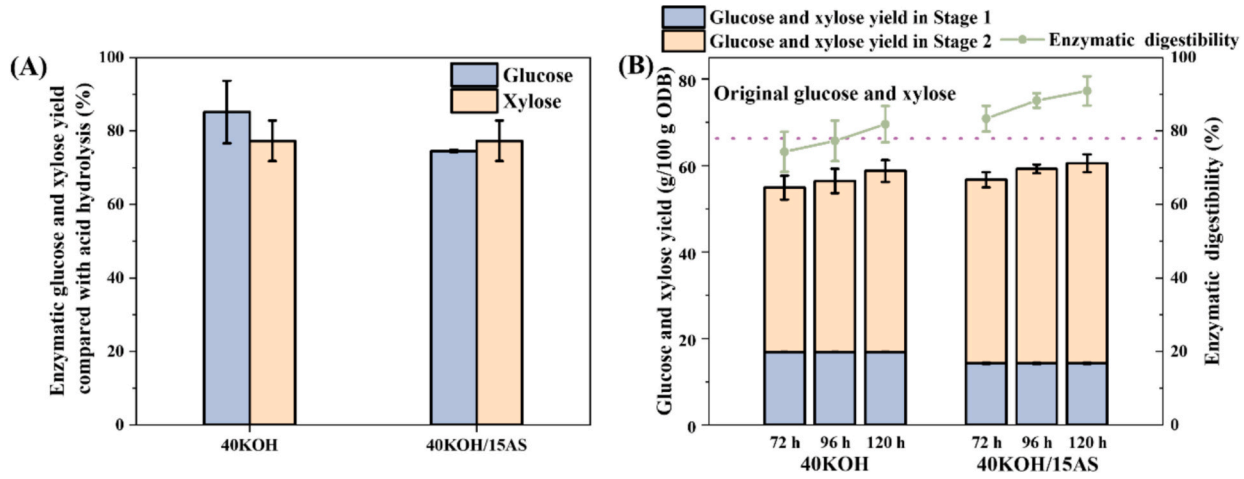


Fig. 3. (A) Glucose and xylose yield from pretreated liquor via enzymatic hydrolysis (at high enzyme loading (100 mg protein Ctec3 with 25 mg Htec3/g (glucan + xylan))) compared with acid hydrolysis glucose and xylose yield; (B) Glucose and xylose yield after alkaline AS pretreatment at low enzyme loading (10 mg protein Ctec3 with 2.5 mg Htec3/g (glucan + xylan)). Stage 1: sugar released during pretreatment (measured after acid hydrolysis); Stage 2: sugar released during enzymatic hydrolysis.

pretreatment was slightly higher than that of residues pretreated with 40KOH alone. To highlight the effect of AS addition, enzymatic hydrolysis was conducted on corn stover pretreated with 40KOH and 40KOH/15AS using a low enzyme loading (10 mg protein Ctec3 with 2.5 mg Htec3/g (glucan + xylan)). All these treatments achieved glucose and xylose yields exceeding 57.2 % after 72 h in the enzymatic hydrolysis stage (stage 2), which further increased to 63.0 %-69.6 % when the hydrolysis duration was extended to 120 h (Fig. 3B). A total sugar yield exceeding 87.5 % was also obtained after 120 h of enzymatic hydrolysis from the 40KOH/15AS pretreatment. Notably, the enzymatic digestibility of 40KOH/15AS was 11.1–14.2 % higher than that of 40KOH pretreatment. This further demonstrates that while the addition of AS does not increase biomass removal, the combination of KOH with AS improves the sugar yield from enzymatic hydrolysis.

The fermentability of recovered sugar from the pretreatment by an engineered strain of *Pseudomonas putida* was evaluated to produce medium-chain-length PHA. During the fermentation process, two carbon sources were tested: the enzymatic hydrolysate of solid residues pretreated with 40KOH/15AS and commercial glucose (see supplementary materials). The results showed that using the enzymatic hydrolysate as a carbon source achieved a PHA yield of 0.072 g PHA/g glucose with a conversion efficiency of 18.82 %, which was 99 % similar to the performance achieved with commercial glucose. Additionally, the composition of PHA produced from the enzymatic hydrolysate was nearly identical to that obtained using commercial glucose. These findings suggest that the enzymatic hydrolysate of pretreated solid residues exhibits comparable PHA fermentation performance to commercial glucose, indicating its potential as an alternative carbon source for industrial PHA production.

3.3. Characterization of pretreated solid and liquor

FTIR analyses indicate that the peak at 2910 cm^{-1} representing C—H stretching was enhanced with KOH and KOH/15AS pretreatments (see supplementary materials), suggesting increased cellulose content, in agreement with compositional analysis. The peak at 1516 cm^{-1} corresponds to aromatic C═C stretching in lignin, while signal around 1720 cm^{-1} is related to the C═O functional group, characteristic of ester bonds between hemicellulose and lignin (Barmana et al., 2014; Domanski et al., 2016). These peaks nearly vanished after KOH and alkaline ammonium sulfite pretreatment, signifying extensive lignin and acetyl group removal. The absorption peak at 1027 cm^{-1} represents S═O stretching (Fitria et al., 2023). No change occurred after KOH pretreatment alone, but after the addition of 15 wt% AS, these peaks significantly intensified, indicating sulfonation reaction.

The total acid groups on the pretreated solid were quantified by conductometric titration (see supplementary materials). The conductivity changed with the amount of base added, and the total acid groups were calculated based on the volume of base required to reach the second inflection point (Katz and Beatson, 1984). It was observed that KOH pretreatment alone slightly increased the total acid groups compared to untreated corn stover. In contrast, AS pretreatment significantly increased the total acid groups to approximately 140 mmol/kg, closely linked to the sulfonation modification. A study similarly observed that corn stover pretreated with sodium sulfite at low temperatures exhibited high total acid groups (147 mmol/kg) (Wu et al., 2020). However, despite the increase in total acid groups, 30 wt% AS alone could not effectively remove lignin (Fig. 1C), resulting in unsatisfactory sugar yields. With the addition of 15 wt% AS, the total acid groups in the corn stover pretreated with 40KOH/15AS significantly increased compared to the 40KOH pretreatment, reaching 126.6 mmol/kg.

Under alkaline conditions, pretreatment with 40 wt% KOH alone achieved substantial removal of lignin and acetyl groups, forming the foundation for high sugar yields. Although the addition of 15 wt% AS did not further enhance lignin removal, FTIR analysis and changes in total acid groups confirmed the occurrence of sulfonation under these low-temperature alkaline conditions. Furthermore, KOH may induce the cleavage of phenolic hydroxyl groups in lignin, potentially leading to the formation of a methylene quinone structure. This structure is readily sulfonated by the free nucleophilic sulfite ion, which is beneficial for the subsequent enzymatic hydrolysis process (Zhong et al., 2019). The sulfonation modification of the remaining lignin reduces the nonspecific adsorption of cellulase onto the substrate, allowing more enzymes to effectively target cellulose (Zhang et al., 2013; Zhou et al., 2013). This leads to a further increase in saccharification performance, particularly at low enzyme loading (10 mg protein Ctec3 with 2.5 mg Htec3/g (glucan + xylan)) (Fig. 3B). These results suggest that the combination of sulfonation modification and high delignification are critical factors contributing to the extremely high sugar yields observed with this pretreatment.

Under alkaline conditions, lignin undergoes degradation to form various monomers. GC–MS analysis was utilized to identify the types of lignin degradation products present in pretreated liquor under strong alkaline conditions (see supplementary materials). Pretreatment spent liquor contains a large number of phenolic substances, particularly

styrenes such as 4-vinylphenol and 2-methoxy-4-vinylphenol. The addition of 15 wt% AS did not induce significant changes in the pretreated liquor. However, the presence of these hydrolysis products inhibited enzyme activity, a challenge to the recovery of fermentable sugars from the whole slurry enzymatic hydrolysis process (Zha et al., 2014). Nonetheless, these phenolics in the spent liquor have economic value with efficient separation. For example, 4-vinylphenol and its derivatives have a wide range of applications in the food, pharmaceutical, and polymer industries (Liu et al., 2024). Furthermore, lignin components in spent liquor have been shown to promote plant growth (Savy et al., 2018). The addition of lignin helped to diminish the conversion of urea by inhibiting the activity of urease, consequently enhancing the sustained release and utilization of urea (Zhang et al., 2010). Thus, in conjunction with the nitrogen, sulfur, and potassium elements introduced during the pretreatment process, the feasibility of substituting chemical fertilizers with pretreatment spent liquor was subsequently investigated in this study.

3.4. Mass balance

The total mass balance was determined based on 100.0 kg of corn stover as depicted in Fig. 4. Initial water insoluble solids (WIS) yield was 61.2 %. Following enzymatic hydrolysis, this pretreated WIS yielded 50.9 kg of fermentable sugars, consisting of 36.8 kg of glucose and 14.1 kg of xylose. Additionally, the pretreatment spent liquor contained 11.8 kg of polysaccharides and a substantial amount of lignin (15.8 kg), along with N, K, and other elements inherent to the corn stover.

3.5. Fertilizer replacement value of the pretreatment spent liquor

After pretreatment 40 wt% KOH and 15 wt% AS, the nutrients N, K, and S from both the treatment chemicals and the organic (polysaccharides and lignin) and inorganic components (N and S) of the corn stover remained in the pretreatment spent liquor. The total N, K_2O, and S contents in the spent liquor were 0.03 %, 1.32 %, and 0.17 %, respectively (see supplementary materials). The relatively low nitrogen content in the spent liquor might be due to the volatilization of ammonia during the pretreatment process and subsequent storage. Subsequent experiments assessed the fertilizer replacement ability of the pretreatment spent liquor by comparing the growth effects of various ratios of spent liquor to commercial fertilizer (ranging from 0 %:100 % to 100 %:0%) on corn. Statistical analysis comparing the means of the no-K control, commercial fertilizer as a K source, and replacing 25–100 % of commercial fertilizer K with the spent liquor showed that replacing fertilizer K at any rate from 25 % to 100 % significantly increased corn biomass, whereas applying commercial fertilizer K alone did not significantly increase corn biomass compared to the control (Fig. 5A and 5B). Although replacing commercial S fertilizer with spent liquor showed similar trends, the differences in biomass were not statistically significant (Fig. 5C and 5D). These results indicate that the K and S in the liquor are as effective as commercial fertilizers and using the spent liquor at any rate (replacing commercial fertilizers by 25–100 %) significantly benefited corn biomass yield. Previous field studies have confirmed the beneficial effects of land application of value-added spent liquor, which not only recycles nutrients back into the fields but also improves the chemical, physical, and biological properties of the soil, thereby enhancing crop growth (Tao et al., 2019; Xiao et al., 2006). Notably, the complex structural characteristics of lignin improve soil aggregate structure, increase nutrient retention capacity, and enhance soil water retention capacity (Ahmad et al., 2021). Additionally, soluble organic matter transferred from the biomass to the liquid fraction during pretreatment positively affects the activity of soil microorganisms (Xiao et al., 2006). These comprehensive benefits underscore the potential of utilizing spent liquor from 40KOH/15AS pretreatment as a sustainable alternative to commercial fertilizers in agricultural practices.

3.6. Techno-economic analysis

The production process of lignocellulosic sugars necessitated TEA to evaluate its commercial feasibility and identify potential technical barriers. A discounted cash flow calculation was employed to determine the minimum sugar selling price (MSSP) that would achieve an after-tax

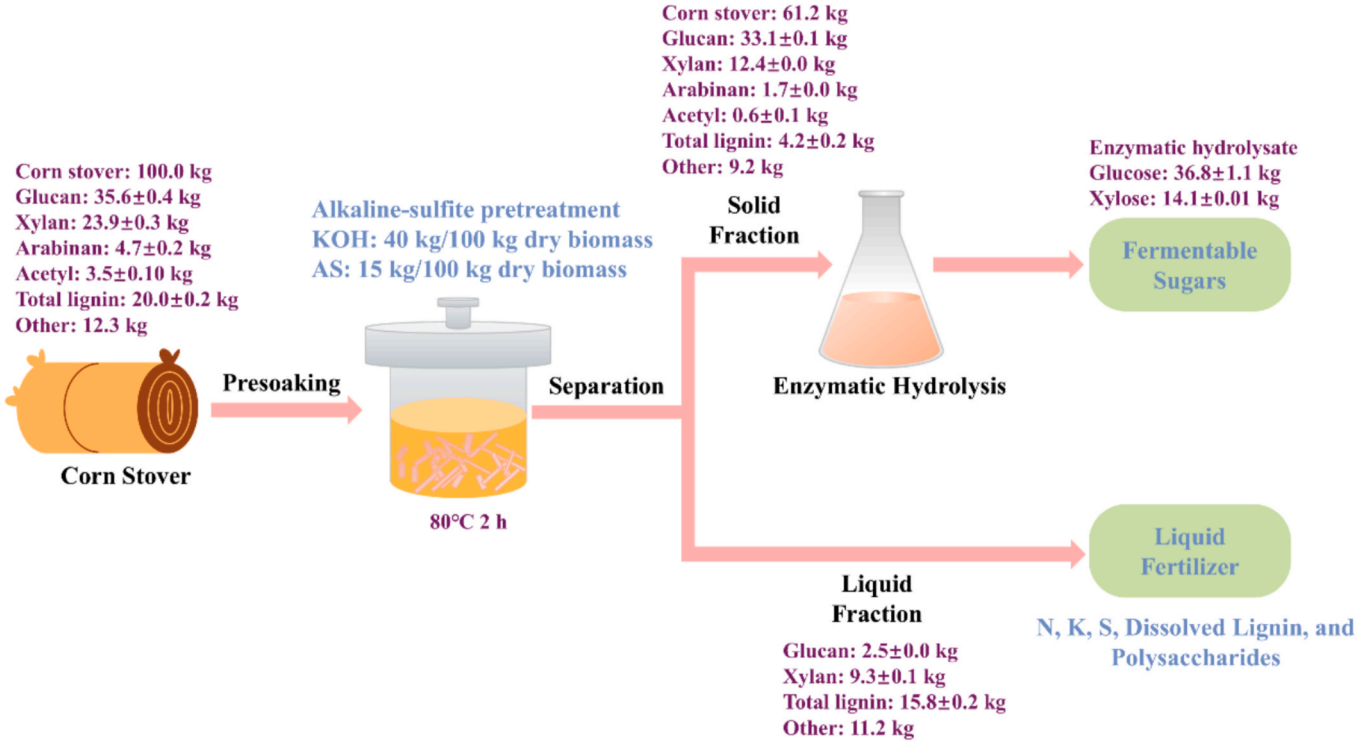


Fig. 4. Overall mass balance of sugar production from corn stover through pretreatment.

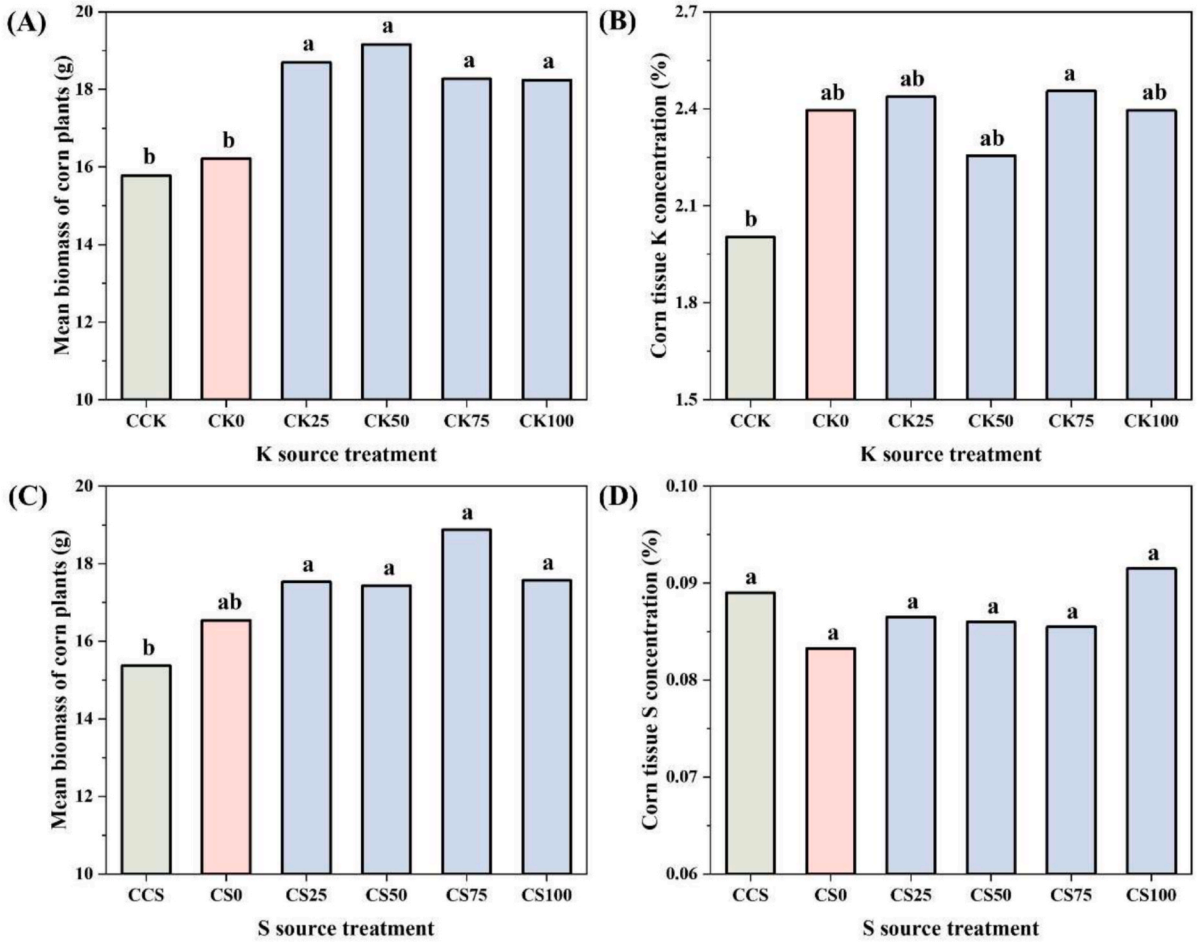


Fig. 5. Results of the K replacement experiment: (A) Means of total biomass (measured as dry weight of two corn plants per pot); and (B) Tissue total K concentration (measured as % based on dry plant tissue). Results of the S replacement experiment: (C) Means of total biomass (measured as dry weight of two corn plants per pot); (D) Tissue total S concentration (measured as % based on dry plant tissue). CCK = no K fertilizer application control; CCS = no S fertilizer application control; CK0 = fertilizer K was applied; CS0 = fertilizer S was applied; CK25, CK50, CK75, CK100 = 25 %, 50 %, 75 %, and 100 % fertilizer K was replaced by the liquor; CS25, CS50, CS75, CS100 = 25 %, 50 %, 75 %, and 100 % fertilizer S was replaced by the liquor. Different letters above the bars in each figure indicate significant differences of the means at $\alpha = 0.05$ significance levels.

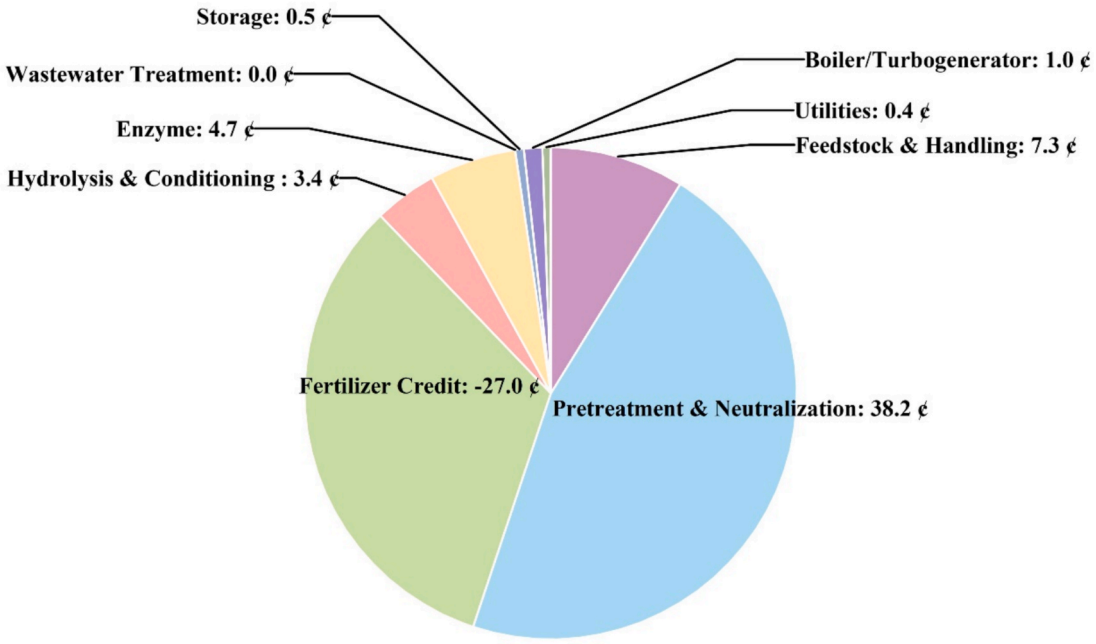
internal rate of return of 10 %. In this TEA model, it was assumed that wastewater treatment costs would be eliminated, as the spent liquor was to be used directly for fertilizer applications. Additionally, it was assumed that 80 % of the chemical inputs in the spent liquor would be recovered and made bioavailable as fertilizer. AS inputs were converted to an "ammonia equivalent" credit, while KOH was credited on a potassium sulfate basis. Given that the current value of the liquor is somewhat below the retail fertilizer value, the credits were calculated based on an industrial wholesale rate for ammonia and potassium sulfate. This approach ensures that the biorefinery is not credited for more than the actual expenditure on the original chemical inputs. The analysis revealed that this process could achieve a low MSSP of 0.285 $/lb (2020 dollars). Fig. 6 shows a breakdown of the contributions to MSSP by process area in cents per pound of sugar, where fertilizer credit is a negative contributor, nearly offsetting the cost of pretreatment and neutralization (the single largest contributor to MSSP). For all the process areas, this contribution to MSSP was calculated by adding the capital-related expenses, raw materials and waste, grid electricity, and fixed costs (labor, maintenance, and property insurance plus tax) attributed to each respective area of the process. As discussed above, wastewater treatment is assumed to not be required, and thus contributes zero cents to the overall MSSP. Additionally, enzyme costs remain a significant factor, accounting for 16.5 % of the MSSP, which aligns with the reported ranges in other studies (Baral and Shah, 2017; Boakye-Boaten et al., 2017).

4. Conclusion

A chemical-recovery-free ammonium sulfite-based alkali pretreatment process for producing high quality fermentable sugar from corn stover at an estimated MSSP of 0.285 $/lb has been demonstrated. The spent liquor containing nitrogen, potassium, sulfur, and other inorganics will be directly used along with lignin and other organics as fertilizer to replenish the soil nutrients for the agriculture industry. In the future, the low cost and high-yield sugar produced from the ammonium sulfite-based alkali pretreatment process will be validated with industrial microorganisms to produce various products, including ethanol, lipids, lactic acid, and jet fuel intermediates.

CRediT authorship contribution statement

Shuaishuai Ma: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Shiva:** Investigation. **Haiying Tao:** Writing – review & editing, Investigation. **Jacob Dempsey:** Writing – review & editing, Investigation. **Xiaowen Chen:** Writing – review & editing, Supervision, Funding acquisition. **Jinxia Yuan:** Investigation. **J.Y. Zhu:** Writing – review & editing, Supervision. **Joshua S. Yuan:** Writing – review & editing. **Le Zhou:** Investigation.



Area	Type	Area Totals	Labels
None	Feedstock & Handling	$0.073	7.3 ¢
A200	Pretreatment & Neutralization	$0.382	38.2 ¢
A200	Fertilizer Credit	-$0.270	-27.0 ¢
A300	Hydrolysis & Conditioning	$0.034	3.4 ¢
A400	Enzyme	$0.047	4.7 ¢
A600	Wastewater Treatment	$0.000	0.0 ¢
A700	Storage	$0.005	0.5 ¢
A800	Boiler/Turbogenerator	$0.010	1.0 ¢
A900	Utilities	$0.004	0.4 ¢
	Net Summation	$0.285	28.5 ¢

Fig. 6. Contribution to minimum sugar selling price by process area.

Bin Yang: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132402.

Data availability

Data will be made available on request.

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