The synergetic effect of ultrafine grinding and alkaline hydrogen peroxide pretreatment for the enzymatic saccharification of corn stover

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ABSTRACT

Cellulose in lignocellulosic biomass is difficult to hydrolyze during enzymatic hydrolysis. To further simplify the pretreatment process in high solid loading on the premise of reducing the consumption of chemical reagent and energy, an effective one step mechanochemical (combines wet ultrafine grinding (WUG) with alkaline hydrogen peroxide (AHP)) composite pretreatment (CP) method for corn stover was developed. This new method could be performed at room temperature with low H_2O_2 dosages (0.05–0.15 g/g corn stover) and short processing time (0.5–0.1 h). The CP pretreatment removed lignin and acetyl groups, disrupted the cell structure of corn stover, effectively superimpose the enhancement effects of AHP and WUG. Its optimized enzymatic glucose yield was 254.23 g/kg with a low H_2O_2 dosage (0.10 g/g corn stover) and grinding time (0.5 h). The single pretreatment of WUG and AHP at the same condition could only achieve a glucose yield of 163.85 g/kg and 132.76 g/kg, respectively. The quantitative linear regression equation between cellulose content and enzymatic glucose yield (GY) for the CP pretreatment was found to be: GY= 31.78 × cellulose content - 979.40. The slope was higher than that for AHP pretreatment (23.26). This suggests that CP pretreatment with low H_2O_2 doses can improve the efficiency of enzymatic saccharification.

1. Introduction

Crop straw is one type of significant lignocellulosic biomass feedstock for second-generation ethanol production due to its wide distribution and high output (Chakraborty et al., 2024; Bhutto et al., 2017). However, cellulose in lignocellulosic biomass, which is wrapped in covalently linked lignin and hemicellulose, is extremely difficult to degrade during enzymatic hydrolysis, thus the yield of ethanol is greatly limited (Himmel and Picataggio, 2009). The pretreatment process plays a crucial role in enhancing the technical and economic viability of cellulosic ethanol production from lignocellulosic biomass by effectively reducing the recalcitrance of biomass (Karimipour-Fard et al., 2024; Kim, 2018).

Chemical pretreatment and mechanical pretreatment are two representative pretreatment methods for biomass and they have distinct mechanisms to improve the enzymatic efficiency of biomass. Chemical pretreatment of straw focuses on the removal or destruction of hemicellulose and lignin, as well as breaking down the network formed by lignin and carbohydrates (Rastogi and Shrivastava, 2017). Alkaline hydrogen peroxide (AHP) pretreatment operates by utilizing the free

radicals produced by H_2O_2 under alkaline conditions to target and depolymerize lignin. This process results in the creation of more pores in the cell wall, improving enzyme accessibility to cellulose and enhancing its cellulolytic efficiency (Li et al., 2019, 2018; Zhou et al., 2023; Gould, 1985a). The studies of Zhang (Zhang and Wu, 2023), Mittal (Mittal et al., 2017), Li (Li et al., 2018), and Berjee (Banerjee et al., 2011) on the pretreatment of straw with AHP have achieved satisfactory enzymatic saccharification results. However, it is worth noting that high temperatures during AHP pretreatment necessitate shorter durations but place greater requirements on the reaction vessel. Conversely, pretreatment at or near room temperature requires prolonged processing times. Additionally, the AHP pretreatment process has the disadvantages of low solids loading and high consumption of H_2O_2 (Mittal et al., 2017; Banerjee et al., 2011; Gould, 1984, 1985b; Gould and Freer, 1984; Pinheiro et al., 2021). On the other hand, mechanical pretreatment mainly destroys the dense network structure encompassing cellulose, hemicellulose, and lignin within straw biomass by reducing its particle size. This alteration enhances enzyme accessibility to cellulose. Preliminary research conducted in the authors' laboratory has indicated that ultrafine grinding could effectively dismantle the cellular structure

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of lignocellulosic substrates, exposing cellulose in the cell wall and partially disrupting the crystalline structure of cellulose (Zhang et al., 2017; Ji et al., 2016; Gao et al., 2021). Consequently, enzymatic saccharification efficiency towards cellulose is improved. Mechanical pretreatment does not require the use of chemical reagents, but has the disadvantage of high energy consumption.

In recent years, researchers have been exploring composite pretreatment approaches to enhance enzymatic saccharification efficiency in breaking down lignocellulosic biomass. These methods combine mechanical (such as grinding) and chemical (such as dilute acid or alkali) treatments to target multiple effects simultaneously. The goal is to improve efficiency while reducing the use of chemical reagents (Elalami et al., 2022; Wang et al., 2018; Takada et al., 2020; Qi et al., 2019; Huang et al., 2019; Yu et al., 2024). However, most existing composite pretreatment studies involve sequential mechanical and chemical steps, often requiring high temperatures and specialized equipment, leading to complex processes. Huang et al (Huang et al., 2019). mixed sugarcane bagasse/pennisetum with NaOH solution (0.25 %, 1.0 %, and 4.0 %) at a ratio of 1:1 (w/v) in a planetary ball mill and ground it at 400 rpm for 2 h, then transferred the mixture to a reactor, added water to a solid-liquid ratio of 1:10 (w/v) and heated it at different temperatures (80°C and 100°C) for different time (20–60 min). The optimal reducing sugar yields of the mechanochemically pretreated sugarcane bagasse and pennisetum were 40.75 % and 55.74 %, respectively. Similarly, Ji et al (Yu et al., 2024). mixed rice straw with H_2SO_4 solution (1 mmol/g rice straw) at a ratio of 1:5 (w/v) for 30 min, then the mixture was ball milled at 50 Hz for 1 h after drying at 40°C.

Therefore, to further simplify the pretreatment process in high solid loading on the premise of reducing the consumption of H_2O_2 and energy, this study introduces a one-step mechanical-chemical composite pretreatment (CP) strategy based on previous research conducted in our groups (Li et al., 2018; Gao et al., 2021; Zhang et al., 2021a, 2023; Liu et al., 2019; Yang et al., 2022). This approach integrates alkaline hydrogen peroxide pretreatment with low H_2O_2 dosage and ultrafine grinding to simplify the pretreatment process and enhance the enzymatic saccharification of corn stover. In this study, the effects of the composite pretreatment on the main components and enzymatic hydrolysis efficacy of corn stover were systematically characterized, and the quantitative relationships between the cellulose content and enzymatic hydrolysis glucose yield of AHP-pretreated corn stover and CP-pretreated corn stover were analyzed to reveal the effects of CP pretreatment.

2. Materials and methods

2.1. Substrates and chemicals

Corn stover was collected from Shangzhuang Experimental Station of China Agricultural University (40º02'N, 116º20'E) in Beijing, China. It was air-dried, cut into short pieces, and further dried in a constant temperature oven at 40°C for 48 h. It was then pulverized using a hammer mill (RT-34, Hong Kong Rongchong Precision Technology Co., Ltd., China) until it passed through a 40-mesh vibrating sieve. The sieved samples were stored in sealed bags at room temperature in a dry and ventilated environment for future use.

Analytically pure reagents, including calcium carbonate, sodium carbonate, anhydrous ethanol, phosphoric acid, citric acid monohydrate, 3,5-dinitrosalicylic acid, phenol, sodium hydroxide, sodium metabisulfite, sodium potassium tartrate, sulfuric acid, and tetracycline hydrochloride, were purchased from Beijing Blue Eagle Chemical Products Company (China). Additionally, 4-nitrophenol was acquired from Beijing Bailinwei Technology Co., while No.1 filter paper was obtained from Whatman International Limited (UK). Chromatographically pure reagents like glucose, xylose, arabinose, and cellobiose were purchased from Sigma-Aldrich Corporation (USA). 4-nitrophenyl-D-glucopyranoside (pNPG), cellulase (Celluclast 1.5 L), and β-glucosidase

(Novozyme 188) were also obtained from Sigma-Aldrich Corporation. The enzyme activities of cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188) were measured to be 72.8 FPU/mL and 385.5 U/mL, respectively.

2.2. Wet ultrafine grinding (WUG) pretreatment of corn stover

To assess the feasibility and the optimal conditions of using CJM-SY-B vibratory ball mill crusher (Qinhuangdao Taichi Circular Nano Ltd., China) in the pretreatment of corn stover, the wet ultrafine grinding (WUG) of corn stover study was performed.

The effect of the solid-to-liquid ratio on the WUG pretreatment was carried out first. Seived corn stover was thoroughly mixed with deionized water at the solid-to-liquid ratios of 1:0.5, 1:1.0, 1:1.5, 1:2.0, 1:2.5, and 1:3.0. The volume ratio of zirconia balls to corn stover was 2:1 and the filling ratio was 35 % (zirconia balls occupying 35 % of the ball mill tank volume). The WUG was performed for 1.0 h.

The effect of grinding time on the WUG pretreatment was then investigated. Seived corn stover was thoroughly mixed with deionized water at the solid-to-liquid ratio of 1:2.0; the volume ratio of zirconia balls to corn stover was 2:1 and the filling ratio was 35 % (zirconia balls occupying 35 % of the ball mill tank volume); the grinding times were 0.5 h, 1.0 h, and 1.5 h.

The ground samples were separated from zirconia balls right after the WUG pretreatment and kept in sealed bags at 4℃ for subsequent tests. Based on our preliminary results on the determination of pretreatment parameters in the WUG process, the solid-to-liquid ratio of 1:2.0 (w/w) was chosen for subsequent tests. Samples generated from the WUG process with 0.5 h and 1.0 h processing time were denoted as WUG-0.5 and WUG-1.0, respectively. The particle size distribution analysis revealed that WUG-0.5 had D_{10}, D_{50}, and D_{90} values of 6.07 μm, 23.80 μm, and 75.68 μm, while WUG-1.0 exhibited values of 4.82 μm, 17.30 μm, and 48.68 μm. The WUG-pretreated corn stover was effectively pulverized to the cellular scale (Zhang et al., 2021a; Ding et al., 2024).

2.3. Alkaline hydrogen peroxide pretreatment (AHP) of corn stover

The conventional AHP method typically utilizes H_2O_2 dosages ranging from 0.25 to 0.50 g/g of corn stover (Banerjee et al., 2011; Gould, 1984, 1985b). To investigate the impact of AHP treatment at lower H_2O_2 dosages, reduced H_2O_2 concentrations (0.05 g/g corn stover, 0.10 g/g corn stover, and 0.15 g/g corn stover) were used for the AHP pretreatment. Corn stover was thoroughly mixed with aqueous solutions containing varying H_2O_2 concentrations, with the pH adjusted to 11.5 using 10 M sodium hydroxide solution, at a solid-liquid ratio of 1:2 (w/w). The mixtures were allowed to stand for 0.5 h and 1.0 h at room temperature. These samples obtained were designated in the following manner: AHP-H_2O_2 dosage-grinding time, such as AHP-0.05–0.5, AHP-0.05–1.0, AHP-0.10–0.5, AHP-0.10–1.0, AHP-0.15–0.5, and AHP-0.15–1.0.

2.4. Composite pretreatment (CP) of corn stover

Corn stover was mixed with deionized water thoroughly at a solid-liquid ratio of 1:2 (w/w), and left to stand at room temperature for 0.5 h and 1 h, respectively as blank controls (recorded as BC-0.5 and BC-1.0). For composite pretreatment (CP), corn stover was thoroughly mixed with aqueous solutions containing varying H_2O_2 concentrations (0.05 g/g, 0.10 g/g, and 0.15 g/g) at a pH of 11.5 adjusted using 10 M aqueous sodium hydroxide at a solid-liquid ratio of 1:2 (w/w). Subsequently, the samples were mixed at the recommended ball material ratio (a volume ratio of zirconium oxide balls to corn stover of 2:1) and under specific filling conditions (35 % of the ball mill tank volume occupied by zirconia balls). The ultrafine grinding process was carried out using a CJM-SY-B vibrating ball mill pulverizer for 0.5 h and 1.0 h. The

experimental conditions were denoted as CP-0.05–0.5, CP-0.05–1.0, CP-0.10–0.5, CP-0.10–1.0, CP-0.15–0.5, and CP-0.15–1.0.

At the end of pretreatment (include BC, AHP, WUG, and CP), deionized water was added to the solid-liquid mixture, and the pH was neutralized with dilute hydrochloric acid. Following this, solid-liquid separation was promptly carried out in a Brinell funnel, and the solid residue was rinsed with deionized water until the filtrate became colorless and neutral. All the filtrate was collected and recorded as pretreatment supernatant for determination of monosaccharides, oligosaccharides, and by-products. A portion of the solid residue was selected and lyophilized using a vacuum lyophilizer (Alpha2–4 LD-Plus, Christ, Germany), then stored in sealed bags in a dry, ventilated area at room temperature for determination of wood fiber content. The remaining solid residue, which has not been dried, should be stored in sealed bags in a refrigerator at 4℃ for subsequent enzymatic saccharification tests.

2.5. Enzymatic saccharification of corn stover after different pretreatments

Enzymatic saccharification of pretreated corn stover was conducted in 50 mM citrate buffer at pH 4.8 with a 5 % solid loading (w/v, DM). Cellulase was added at a loading of 20 FPU/g DM, and β-glucosidase was added at a loading of 40 CBU/g DM. To avoid microbial interference during the enzymatic tests, tetracycline hydrochloride was added at a concentration of 0.08 g/L. The enzymatic saccharification was carried out at 50℃ and 150 rpm in a water-bath constant temperature oscillator (Jintan Keran Instrument Co., Ltd., China) for 72 h. After enzymatic saccharification, the samples were removed and subjected to a 10-minute treatment in boiling water to deactivate the enzymes. Solid-liquid separation was then performed by vacuum filtration, with the solid residue being rinsed three times with deionized water. The collected filtrates were combined and recorded as the enzymatic hydrolysis liquid. A portion of the sample was adjusted to pH 5–6 using calcium carbonate and stored for glucose yield testing. The enzymatic saccharification was conducted in three parallel groups for accuracy and consistency.

2.6. Particle size and chemistry analysis

2.6.1. Particle size analysis

Particle size analysis was conducted using a Mastersizer 3000 laser particle size analyzer (Malvern, UK) covering a range of 0.01–3500 μm. Before testing, the samples were dispersed in deionized water and ultrasonicated in an ultrasonic cleaner (KQ-500DV, Kunshan Ultrasonic Instrument Co., Ltd., China) at 50 % ultrasonic power for 0.5 h. During analysis, the dispersed samples were injected into the wet analysis module with a pipette, and the results were averaged for each sample based on five replicates. Parameters were set for non-spherical particles with 10–20 % shading, stirring speed of 3000 rpm, and ultrasonic intensity of 50 %. The instrument provided D_{10}, D_{50}, and D_{90} values representing the particle sizes corresponding to cumulative size distribution percentages of 10 %, 50 %, and 90 %, respectively.

2.6.2. Determination of the composition of pretreated samples

The lignocellulosic composition of each pretreated solid residue was determined, including glucan, xylan, arabinoglycan, lignin, and acetyl group contents, following the NREL/TP-510–42618 method (Sluiter et al., 2012). Samples were dried to absolute dryness in an oven at 105℃ before the determination, with cellulose content measured as glucan, hemicellulose content as the sum of xylan and arabinoxylan, and lignin content as the sum of acid-soluble lignin and acid-insoluble lignin. Two replicates were analyzed for each sample. For monosaccharides, oligosaccharides, and by-products (acetic acid, furfural, and hydroxymethylfurfural) in the supernatant of each pretreatment, the content was determined using the NREL/TP-510–42623 method (Sluiter et al., 2008).

2.6.3. Determination of glucose concentration after enzymatic saccharification

The concentration of glucose in the enzymatic solution was determined using an HPLC (Hitachi L-7000 series, Japan) equipped with a differential refractive detector (Hitachi L-2490, Japan). The column used was a Benson BP-800 Pb^{++} type carbohydrate analyzer column with ultrapure water as the mobile phase at 0.6 mL/min. The column temperature was maintained at 80℃, with an injection volume of 20 μL and an elution time of 40 minutes. The glucose yield after enzymatic saccharification was calculated using the following equation:

$$GY = \frac{c \times V}{m} \tag{1}$$

GY——glucose yield at 72 h of enzymatic hydrolysis (g/kg)

c——glucose concentration in enzyme hydrolysate (g/L)

V——volume of enzyme hydrolysate (L)

m——total weight of substrate added for enzymatic saccharification (kg)

2.7. Statistical analysis

Except for particle size analysis (5 replicates) and the lignocellulosic composition determination (2 replicates), all experiments were triple-measured. The results were displayed as mean ± standard deviation. Statistical analyses, including comparison of means tests, correlation analyses, and regression analyses, were conducted using SPSS 20.0. ANOVA analysis was performed based on Duncan's test at the 99 % level. Scatter plots were generated using Origin 8.5.

3. Results and discussion

3.1. Determination of pretreatment parameters in the wet ultrafine grinding (WUG) process

After being processed in the ball mill for 1 h, corn stover samples with different solid-to-liquid ratios (1:0.5–1:3.0) showed notable variations in morphology as shown in Fig. 1. At solid-to-liquid ratios of 1:0.5 and 1:1.0, due to the lack of water as a dispersing agent, a significant portion of the corn stover agglomerated during the WUG pretreatment, resulting in numerous large granularity and uncrushed samples. In contrast, at solid-liquid ratios of 1:1.5 and 1:2.0, the corn stover was uniformly dispersed among the zirconium oxide spheres, with no large size of aggregates or samples of significant granularity present. However, at solid-liquid ratios of 1:2.5 and 1:3.0, while the corn stover remained evenly distributed between the zirconia balls, a higher viscosity was observed, leading to severe sticking issues that hindered sample collection after WUG pretreatment.

The particle size changes of the WUG-pretreated samples at different solid-to-liquid ratios are summarized in Fig. 2. It can be seen that the D_{10} value of the samples decreased slowly with the decrease of the solid-to-liquid ratio. In contrast, the D_{50} and D_{90} values showed different behaviors. For samples with solid-to-liquid ratios of 1:0.5 and 1:1.0, the D_{50} and D_{90} values were larger, measuring 39.68 μm and 253.00 μm (for the 1:0.5 ratio), and 43.82 μm and 318.20 μm (for the 1:1.0 ratio), respectively. These values are significantly higher than those of samples with lower solid-to-liquid ratios. This indicates that a portion of the samples are still in the tissue scale (100–500 nm) (Ji et al., 2016; Barakat et al., 2015). At a solid-liquid ratio of 1:1.5, the D_{50} and D_{90} values decreased to 18.52 μm and 54.14 μm. As the solid-liquid ratio continued to decrease, the changes in the D_{50} and D_{90} values began to stabilize and decrease at a slower rate.

Combining the results shown in Figs. 1 and 2, it can be seen that when the solid-to-liquid ratio during WUG pretreatment exceeds 1:1.5, the ball mill crusher is unable to sufficiently grind the corn stover, resulting in a higher quantity of larger-sized particles. This suggests that energy efficiency is lower under these solid-to-liquid ratio conditions.
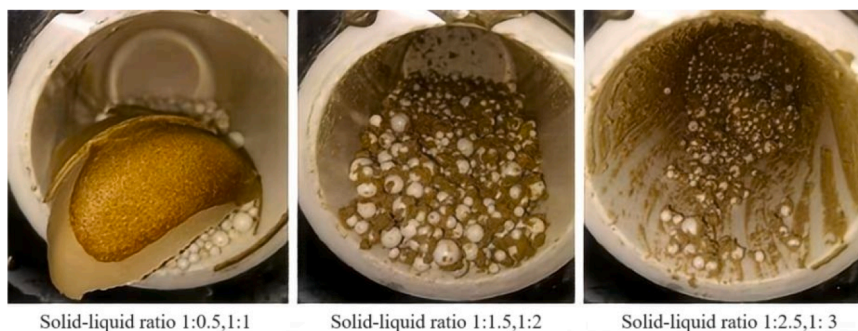
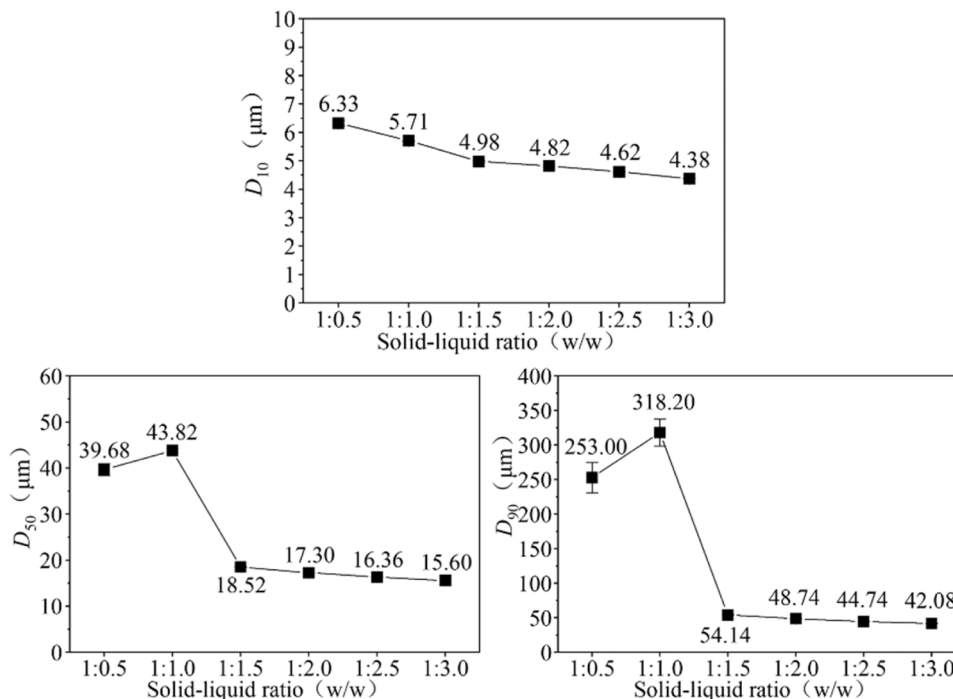**Fig. 1.** Morphology of corn stover after WUG pretreatment with different solid-to-liquid ratios.

Fig. 2. Particle size distribution of corn stover samples after WUG pretreatment with various solid-to-liquid ratios.

Conversely, when the solid-liquid ratio is less than or equal to 1:1.5, the corn stover is more effectively ground. At the solid-to-liquid ratio of 1:2.0, the D_{90} of the samples reached 48.74 µm, indicating that 90 % of the corn stover was reduced to the cellular scale (< 50 µm) (Zhang et al., 2021a; Ding et al., 2024). As the solid-to-liquid ratio continued to decrease, sample collection became increasingly challenging, even though the particle size gradually decreased. Therefore, a solid-liquid ratio of 1:2.0 was selected for the subsequent WUG pretreatment.

Fig. 3 illustrates the particle size distribution of corn stover samples with different WUG pretreatment times (0.5–1.5 hrs). It is evident that as the grinding time increased, the corn stover was progressively reduced to finer particles. Notably, at the 1.0 h mark, 90 % of the corn stover was successfully crushed to the cellular scale, indicating that most of the cellular structure had been disrupted. This disruption allows the cell wall components to fully participate in the reaction when combined with the AHP pretreatment (Zhang et al., 2021b). The theoretical enhancement of the crushing effect through the combination of AHP pretreatment further supports this approach. Consequently, the processing times for the subsequent WUG and CP pretreatments were set at 0.5 h and 1.0 h, respectively.

3.2. Glucose yield from enzymatic hydrolysis of corn stover with different pretreatments

The glucose yields obtained after 72 h of enzymatic hydrolysis of corn stover with different pretreatments were summarized in Fig. 4. Compared with raw corn stover (BC), three pretreatment methods (AHP, WUG, and CP) could increase the glucose yield of corn stover to a different extent (P < 0.01).

It can be observed from Fig. 4 that after AHP pretreatment, the enzymatic glucose yield of corn stover increased from 60.87 ± 1.94 g/kg (BC-0.5) and 63.01 ± 1.18 g/kg (BC-1.0) to 163.89 ± 3.01 g/kg (AHP-0.10–0.5) and 164.76 ± 0.64 g/kg (AHP-0.10–1.0) (P < 0.01). WUG pretreatment achieved glucose yields of around 150 g/kg, which was similar to the AHP pretreatment. On the other hand, the CP pretreatment could result in a substantial increase in enzymatic glucose yield for corn stover, reaching 259.30 ± 9.57 g/kg (P < 0.01). This represented an increase of 196.29 g/kg compared to BC-1.0 and slightly exceeded the combined increase in enzymatic glucose yield for AHP-0.15–1.0 and WUG-1.0 (93.91 g/kg and 91.74 g/kg, respectively). These results suggest that the CP pretreatment effectively superimposed the enhancement effects of AHP and WUG pretreatment on the enzymatic hydrolysis of corn stover, leading to an increased glucose yield
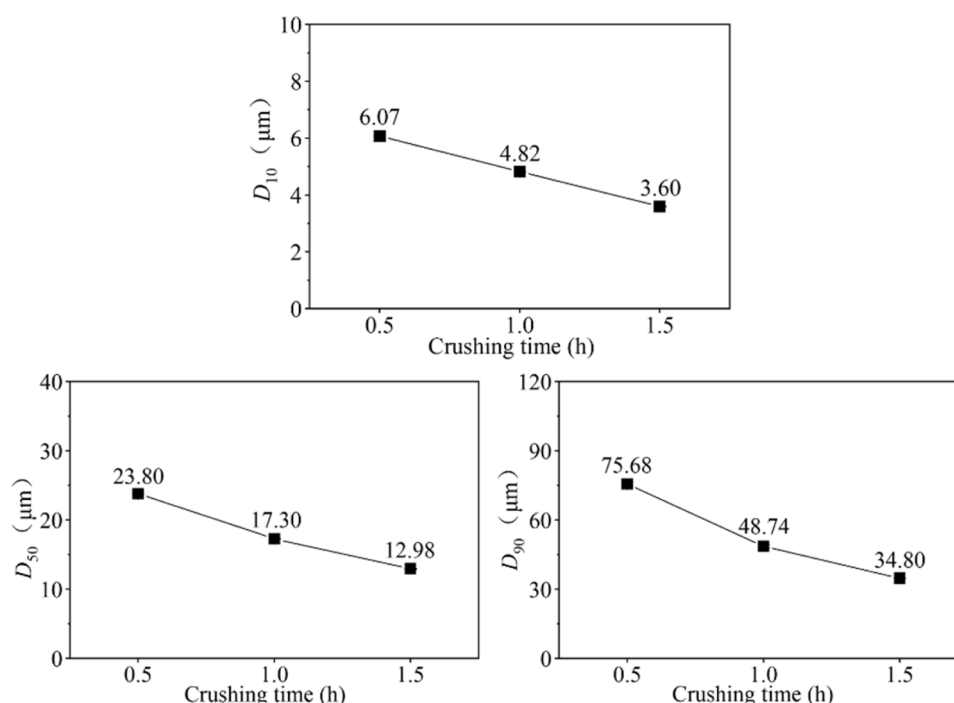
Fig. 3. Particle size distribution of WUG-pretreated corn stover at different grinding times.
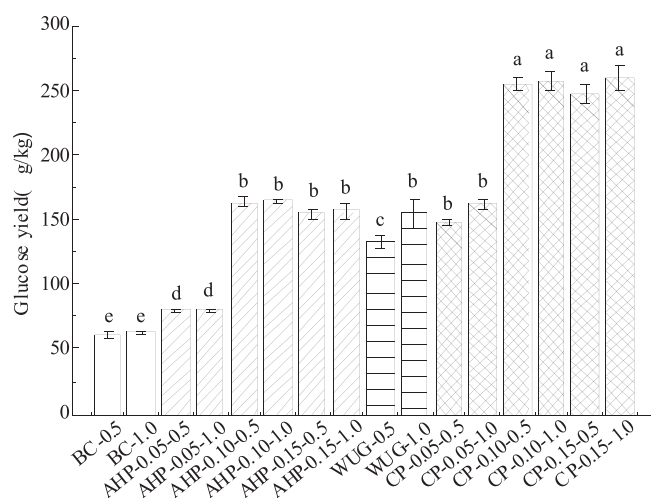

Fig. 4. Glucose yield from enzymatic saccharification of corn stover samples with different pretreatments.

and improved pretreatment efficiency.

Furthermore, when the treatment time was extended from 0.5 h to 1.0 h, there was no notable change in the enzymatic hydrolysis efficiency of AHP-pretreated corn stover with the same dosage of H_2O_2. In contrast, the enzymatic hydrolysis efficiency of WUG-pretreated corn stover increased with longer treatment times as shown in Fig. 4 (P < 0.01). This enhancement could be attributed to the ultrafine grinding of lignocellulosic material down to the cellular level. The prolonged pulverization continued to disrupt the crystalline properties of cellulose and the polymerization degree of polysaccharides, thereby improving enzymatic saccharification (Zhang et al., 2021a; Liu et al., 2019; Yang et al., 2022). In the case of CP pretreatment with the same dosage of H_2O_2, extending the milling time did not lead to a significant improvement in the enzymatic saccharification of corn stover. Comparatively, CP treatment of 0.5 h can more effectively and comprehensively disrupt the spatial structure of corn stover when

compared to WUG pretreatment. It is plausible that the maximum level of disruption may have been achieved at this duration. Additionally, the pretreatment sample with the best enzymatic hydrolysis numerically was CP-0.15–1.0, which had an enzymatic glucose yield of 259.30 g/kg ± 9.57 g/kg, and glucose conversion of 62.89 %. However, the enzymatic glucose yield of the CP-0.10–0.5 samples did not show a significant difference (P > 0.01) compared to the former, with a yield of 254.23 g/kg ± 5.28 g/kg, resulting in a 58.76 % glucose conversion. These results suggest that within the studied range of CP pretreatment, the optimal enzymatic glucose yield for corn stover was achieved at a dose of 0.10 g/g of H_2O_2 and a treatment time of 0.5 h.

In a study by Banerjee et al., the enzymatic glucose conversion of corn stover after 24 h of AHP pretreatment with an H_2O_2 dosage of 0.125 g/g of corn stover, 10 % solids loading, and a treatment temperature of 23°C was 51 % (Banerjee et al., 2011). Similarly, Mittal et al. reported an enzymatic glucose conversion of corn stover after 3 h of AHP pretreatment with an H_2O_2 dosage of 0.09 g/g of corn stover, 10 % solids loading, and treatment temperature of 50°C, which was below 60 % (Mittal et al., 2017). Notably, the CP pretreatment in our study utilized lower H_2O_2 dosage, higher solid-to-liquid ratio, and shorter treatment time compared to the AHP conditions of Banerjee's. Furthermore, our CP pretreatment featured a higher solid-to-liquid ratio, shorter treatment time, and did not require heating in contrast to the AHP pretreatment conditions of Mittal's. These findings indicate that the combination of AHP pretreatment and WUG could effectively enhance pretreatment efficiency for corn stover.

3.3. Major components content change in solid residues and supernatants with different pretreatments

The changes in the content of major components in the solid residue and supernatant of different pretreatments are shown in Table 1. Overall, the influence of WUG on the content of each component of the samples was found to be not substantial, which is similar to the results of the previous study on dry ultra-fine grinding conducted in our laboratory. Previous research indicated that ultra-fine grinding for 0.5 h and 1.0 h reduced corn stover size below the cellular scale but had little impact on polysaccharide degradation (Qu et al., 2024). Both AHP and

Table 1
The contents of the main components in corn stover samples after different pretreatments.

Sample	Solid Recovery Rate (g/100 g)	Solid residues				Supernatant			
		Cellulose content (%)	Hemicellulose content (%)	Lignin content (%)	Acetyl content (%)	Glucose + Oligoglucose content (g/100 g)	Xylose+ Oligosaccharide content (g/100 g)	Arabinose + oligosaccharide content (g/100 g)	Acetic acid content(g/100 g)
BC−0.5	70.73	37.63 ± 0.52[cd]	21.31 ± 0.33[bcd]	22.50 ± 0.19[a]	5.41 ± 0.13[a]	5.41 ± 0.09[e]	0.50 ± 0.02[i]	0.33 ± 0.02[j]	0.26 ± 0.00[g]
BC−1.0	72.13	37.69 ± 0.78[cd]	21.56 ± 0.70[abcd]	22.43 ± 0.22[a]	5.49 ± 0.05[a]	5.59 ± 0.01[d]	0.50 ± 0.02[i]	0.33 ± 0.02[j]	0.25 ± 0.00[g]
AHP−0.05−0.5	72.89	39.16 ± 0.34[bc]	22.22 ± 0.16[abc]	22.66 ± 0.06[a]	3.98 ± 0.30[cd]	5.41 ± 0.01[e]	0.51 ± 0.01[i]	0.44 ± 0.01[h]	1.22 ± 0.07[f]
AHP−0.05−1.0	70.46	38.76 ± 0.84[bc]	21.99 ± 0.53[abcd]	21.80 ± 0.16[ab]	4.19 ± 0.04[c]	5.59 ± 0.00[d]	0.47 ± 0.00[i]	0.39 ± 0.00[i]	1.30 ± 0.05[ef]
AHP−0.10−0.5	67.34	42.30 ± 0.20[a]	23.58 ± 0.45[a]	18.65 ± 0.36[ef]	1.51 ± 0.10[ghi]	5.42 ± 0.00[e]	0.99 ± 0.00[c]	0.78 ± 0.01[d]	3.16 ± 0.04[ab]
AHP−0.10−1.0	65.83	42.19 ± 1.38[a]	23.21 ± 0.91[ab]	18.53 ± 0.18[ef]	1.60 ± 0.02[fghi]	5.29 ± 0.00[f]	0.99 ± 0.00[c]	0.75 ± 0.00[d]	3.01 ± 0.09[cd]
AHP−0.15−0.5	66.44	42.12 ± 0.77[a]	23.41 ± 0.91[a]	19.14 ± 0.42[e]	1.71 ± 0.02[fgh]	5.37 ± 0.01[e]	0.88 ± 0.00[e]	0.68 ± 0.00[e]	3.01 ± 0.03[cd]
AHP−0.15−1.0	65.62	41.20 ± 0.69[ab]	23.02 ± 0.86[ab]	18.88 ± 0.80[ef]	1.82 ± 0.02[f]	5.39 ± 0.01[e]	0.94 ± 0.01[d]	0.69 ± 0.00[e]	2.96 ± 0.03[cd]
WUG−0.5	71.19	35.67 ± 1.50[d]	20.31 ± 1.17[cd]	21.46 ± 0.52[bc]	5.19 ± 0.19[b]	5.87 ± 0.00[a]	0.58 ± 0.00[h]	0.46 ± 0.00[h]	0.22 ± 0.00[g]
WUG−1.0	78.17	35.32 ± 0.77[d]	20.06 ± 0.13[cd]	20.31 ± 0.34[d]	5.04 ± 0.03[b]	5.81 ± 0.02[ab]	0.58 ± 0.00[h]	0.46 ± 0.01[h]	0.20 ± 0.00[g]
CP−0.05−0.5	73.68	37.00 ± 0.98[cd]	20.87 ± 0.69[cd]	20.93 ± 0.27[cd]	3.91 ± 0.02[d]	5.75 ± 0.01[bc]	0.64 ± 0.00[g]	0.50 ± 0.01[g]	1.27 ± 0.02[f]
CP−0.05−1.0	76.40	36.54 ± 0.35[cd]	20.52 ± 0.11[cd]	20.12 ± 0.29[d]	3.63 ± 0.02[e]	5.80 ± 0.00[ab]	0.70 ± 0.00[f]	0.55 ± 0.00[f]	1.40 ± 0.01[e]
CP−0.10−0.5	68.13	38.94 ± 0.89[bc]	20.60 ± 0.58[cd]	17.30 ± 0.22[gh]	1.60 ± 0.01[fghi]	5.54 ± 0.00[d]	1.46 ± 0.01[b]	0.97 ± 0.01[ab]	3.09 ± 0.00[bc]
CP−0.10−1.0	72.95	37.63 ± 0.17[cd]	20.21 ± 0.42[cd]	16.86 ± 0.14[h]	1.32 ± 0.01[i]	5.56 ± 0.00[d]	1.53 ± 0.03[a]	0.99 ± 0.01[a]	3.24 ± 0.01[a]
CP−0.15−0.5	69.68	39.22 ± 0.93[bc]	21.22 ± 0.17[bcd]	17.95 ± 0.22[fg]	1.76 ± 0.08[fg]	5.76 ± 0.01[bc]	1.46 ± 0.01[b]	0.93 ± 0.00[c]	2.93 ± 0.00[d]
CP−0.15−1.0	73.13	37.11 ± 1.26[cd]	19.89 ± 1.12[d]	17.24 ± 0.26[gh]	1.45 ± 0.02[hi]	5.70 ± 0.02[c]	1.47 ± 0.01[b]	0.95 ± 0.02[bc]	3.17 ± 0.00[ab]

The content of each component in the solid residue was based on the corresponding solid residue, and the content of each component in the supernatant was based on the corn stover raw material samples. In cases where different lowercase letters were employed within the same column, it signified highly significant variances (P < 0.01). Notably, both furfural and hydroxymethylfurfural were absent in the supernatant across all samples.

CP pretreatments demonstrated efficacy in eliminating lignin and acetyl groups from the corn stover (P < 0.01). The acetyl groups removed were predominantly transformed into acetic acid present in the supernatant (Saini et al., 2024). Increasing the H$_2$O$_2$ dosage from 0.10 to 0.15 g/g during AHP and CP pretreatments did not lead to significant alterations (P > 0.01) in the removal of lignin and acetyl groups by the pretreatments. Notably, furfural and hydroxymethylfurfural were not generated by any of the pretreatment methods. Although AHP, WUG, and CP pretreatments all markedly boosted the soluble monosaccharides and oligosaccharides content in the supernatant (P < 0.01), the increase was limited compared to the polysaccharide content in the solid residue. Extending the pretreatment time from 0.5 h to 1.0 h did not lead to significant changes (P > 0.01) in the content of wood fiber components for each pretreated sample.

Combined with Fig. 4, it is evident that AHP pretreatment, aided by H$_2$O$_2$, effectively removed lignin and acetyl groups from corn stover. This process initially disrupted the network structure of cellulose-hemicellulose-lignin, thereby enhancing the accessibility of cellulose and subsequently improving the enzymatic saccharification efficiency (Ho et al., 2019). Conversely, WUG pretreatment, while not impacting the main components of corn stover, was able to disrupt the cellular structure of the biomass through mechanical action, exposing more cellulose for enzymatic breakdown. Building upon the lignin and acetyl group removal achieved by AHP pretreatment, the subsequent CP pretreatment further disrupted the cell structure of corn stover. By employing ultra-fine grinding, CP treatment increased enzyme accessibility to cellulose, effectively combining the benefits of AHP and WUG pretreatments to enhance enzymatic hydrolysis. Interestingly, despite

the higher cellulose content in AHP-pretreated corn stover compared to CP-pretreated samples when the H$_2$O$_2$ dosage was increased to 0.10 g/g corn stover, the final enzymatic glucose yield was nearly 100 g/kg lower. This discrepancy suggests that the enzymatic hydrolysis pattern of cellulose may differ between AHP and CP pretreated samples.

3.4. Mechanism analysis of the enhancement of enzymatic hydrolysis effect of AHP/WUG composite pretreatment

The relationship between the cellulose content of different pretreated corn stover and its enzymatic glucose yield is illustrated in Fig. 5. Given that BC and WUG pretreatments can be considered as variations of AHP and CP pretreatments with an H$_2$O$_2$ dosage of 0.00 g/g corn stover, quantitative fitting analysis was conducted on the cellulose content and enzymatic glucose yield of BC+AHP pretreated samples and WUG+CP pretreated samples, respectively. The analysis revealed a notable linear positive correlation between the cellulose content and enzymatic glucose yield of the two sets of pretreated samples:

AHP: $GY = 23.26 \times$ Cellulose content - 817.64 ($r^2 = 0.97$, $P = 0.00$)

CP: $GY = 31.78 \times$ Cellulose content - 979.40 ($r^2 = 0.61$, $P = 0.02$)

The slope of the linear regression equation for CP-pretreated corn stover was found to be higher than that for AHP-pretreated corn stover. This suggests that the enzymatic hydrolysis of cellulose in CP-pretreated corn stover was more effective compared to AHP-pretreated corn stover. While AHP pretreatment is capable of removing some lignin and acetyl groups, the potency of AHP pretreatment utilized in this study was
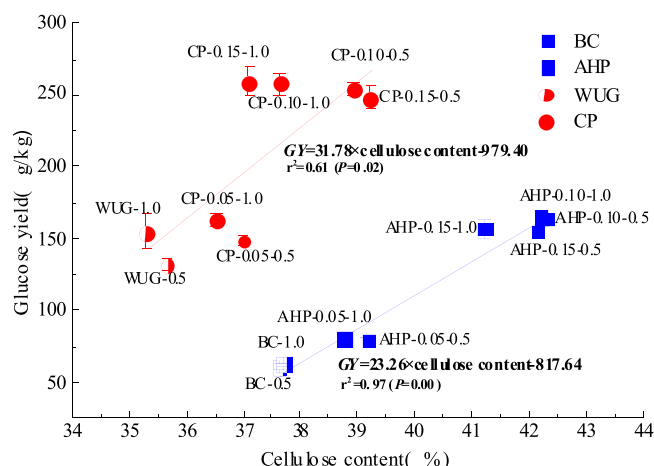
Fig. 5. Relationship between enzymatic hydrolysis of glucose yield and cellulose content in AHP-pretreated and CP-pretreated samples.

relatively low, resulting in limited damage to the corn stover. Previous studies conducted in our laboratory have shown that ultra-fine grinding can reduce the size of lignocellulosic materials to below the cellular scale (30–50 μm) (Yang et al., 2022; Lin et al., 2024; Gao et al., 2020). This process helps in breaking down the dense network structure of cellulose-hemicellulose-lignin in corn stover, leading to an increase in the specific surface area and porosity of the materials. Additionally, ultra-fine grinding enhances the exposure of cellulose on the material surface, disrupts the crystalline structure of cellulose, and reduces the degree of polymerization of cellulose. These effects collectively contribute to improving the enzyme accessibility of cellulose for enhanced enzymatic hydrolysis efficiency (Liu et al., 2019; Qu et al., 2024; Ntakirutimana et al., 2023). At the same time, ultra-fine grinding plays a crucial role in reducing the diffusion resistance of cellulase within the internal pores of the material during enzymatic digestion, facilitating enhanced adsorption of cellulase (Zhang et al., 2023). The CP pretreatment method involves the partial removal of lignin and acetyl groups using AHP. This process disrupts the cellulose-hemicellulose-lignin network, complementing the effects of ultra-microcrushing. The synergistic action of AHP and WUG pretreatment further enhanced the disruption of the cellular and cellulose crystal structures in maize straw, leading to improved efficiency in enzymatic cellulose digestion. This collaborative approach significantly boosts the enzymatic efficiency of cellulose by enhancing the destruction of cellular and cellulose crystal structures.

4. Conclusions

To reduce energy consumption and improve efficiency, a novel one-step composite pretreatment that combines wet ultrafine grinding and AHP pretreatment with a low H_2O_2 dose in a high solid loading was developed in this study. The CP pretreatment not only removed lignin and acetyl groups but also disrupted the cell structure of corn stover, which effectively superimposes the enhancement effects of AHP and WUG on the enzymatic hydrolysis of corn stover. The optimized enzymatic glucose yield reached 254.23 g/kg after CP pretreatment of 1:2 solid-to-liquid ratio, 0.10 g/g corn stover H_2O_2 loading, and treatment time of 0.5 h. The results of the quantitative relationship between cellulose content and glucose yield of AHP-pretreated corn stover and CP-pretreated corn stover suggested that cellulose in CP-pretreated corn stover has a higher enzymatic hydrolysis efficiency.

Ethics approval and consent to participate

Not applicable.

CRediT authorship contribution statement

Lujia Han: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Yuke Han:** Writing – review & editing, Visualization. **Junbao Li:** Writing – original draft, Validation, Formal analysis, Data curation. **Weihua Xiao:** Methodology. **Suan Shi:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

JB Li performed the experiments and drafted the manuscript. YK Han took part in data analysis and figure drawing. WH Xiao advised experiment design. SA Shi and LJ Han suggested the article's topic and critically revised the paper. All authors have read and approved to publish the final manuscript.

Data Availability

Data will be made available on request. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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