Effects of Pretreatment on the Microcharacterization and Fermentation of Bamboo Shoot Shells

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This study focuses on the pretreatment and characterization of natural fibers from the bamboo shoot shell (BSS) of *Phyllostachys heterocycla* to determine their suitability as biorefining materials. The discarded bamboo shoot shell was used as a source of fibers, which were analyzed for their physical, chemical, and microstructure properties. Fourier transform infrared spectroscopy, X-ray diffraction spectra, and scanning electron microscopy confirmed that a mixture of sodium hydroxide immersion plus high-pressure steam treatment allowed the cellulose structure to be disrupted, providing more adsorption sites for cellulases. Gas chromatography mass spectrometry (GC-MS) also showed that the pretreatment exposed the internal structure of the fibers and that high-mass silicon compounds were present in the eluted solution. After adding the cellulase produced by *Trichoderma viride* and *Aspergillus niger*, the reducing sugar yield was increased by 268% and 251%, compared to unpretreated BSS fibers. This strategy may apply to many industries, especially biorefining and lignocellulose biotransformation technology.

DOI: 10.15376/biores.19.3.4604-4618

Keywords: Bamboo shoot shell; FT-IR; Pretreatment; XRD; GC-MS; Cellulose

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INTRODUCTION

Biomass has received widespread attention in recent years due to its usability, low cost, and renewable properties (Lu *et al*. 2019). Biofuels and biobased products converted from lignocellulose biomass are expected to reduce global dependence on fossil fuels and greenhouse gas emissions (Qing *et al*. 2016). Lignocellulose is one of the most abundant renewable resources, with more than 40 million tons of lignocellulose feedstock produced globally each year, accounting for 50 percent of the global biomass, including agroforestry waste, paper waste, energy crops, *etc*., most of which go unused and discarded (Sanderson 2011; Mathews *et al*. 2015; Yin *et al.* 2018; Kalsoom *et al*. 2019). Bamboo is a perennial herb, rich in cellulose, hemicellulose, and lignin. It is estimated that the annual production of bamboo shoot shells (BSS) in China exceeds about 20 million tons (Ye *et al*. 2014; Archila *et al*. 2018; Huang *et al*. 2019). While small amounts of BSS are used for functional foods or natural fibres, most are thrown away as agricultural waste (Luo *et al*. 2018). There is a lot of interest in not only converting BSS into products of added economic value but

also benefiting the environment by decreasing the agri-waste load (Seidl and Goulart 2016; Zhang *et al*. 2017; Goodman 2020).

Pretreatment of forest wood fibers has an important impact on their biodegradation and efficient utilization. Likewise, they are mainly composed of cellulose, hemicellulose, and lignin in a tightly cross-linked structure, which is one of the main reasons why lignin fibers are not readily biodegradable (Yuan *et al.* 2021). In addition, their biodegradation tends to be impeded by non-productive bonding of the cellulase through hydrophobic, electrostatic, and hydrogen bonding interactions (Oliveira *et al.* 2020), a phenomenon of adsorption that further contributes to an increase in the cost producing biofuels. Therefore, rationally changing the natural structure of lignin and reducing the polymerization of cellulose and hemicellulose can improve the degradation efficiency of bamboo shoot hull cellulose. However, lignin was still present in the pretreated lignin matrix, and the ineffectual adsorption of lignin to cellulase resulted in a decrease in cellulase lysis efficiency. Many studies have found that adding a surfactant to the enzyme lysis process can significantly increase the conversion rate of lignin lysis, decrease the adsorption of lignin to the enzyme, and thus reduce the amount of cellulose (Zhou *et al.* 2015; Bajaj and Mahajan 2019; Ralph *et al.* 2019; Ejaz *et al.* 2021). At the same time, most cellulase preparations are the product of microbial fermentation, such as *Trichoderma viride* and Aspergillus niger (Sukumaran *et al.* 2009; Kucharska *et al.* 2018; Srivastava *et al*. 2018).

In this study, BSS associated with agricultural production were pretreated with a combination of physical, chemical, and biological methods to reduce the lignin content, and the pretreated materials were characterized and analyzed by X-ray diffraction (XRD), gas chromatography – mass spectrometry (GC-MS), zeta potential, Fourier transform infrared (FT-IR), and scanning electron microscopy (SEM). The effects of metal ions and surfactants on cellulase activity in fungi were investigated. These results may contribute to the industrialization of biorefining and lignocellulosic bioconversion technologies.

EXPERIMENTAL

Materials

The bamboo shoot cultivation base is in Longhui County, Hunan province, China. It is provided by Long Hui County Golden Bamboo Sprouts Development Co. Ltd. Plant logs are naturally air-dried and crushed into 60-order BSS sawdust for dry storage. Cellulase was purchased from Xia Sheng Enzyme Biotech (from *Trichoderma viride*) and SIGMA (from *Aspergillus niger*). Figure 1 shows a photograph of bamboo shoot plant.

Fig. 1. Bamboo shoot plant

BSS Pretreatment

The bamboo shoot shells (BSS) was thoroughly washed, dried in an oven at 80 ℃ for 24 h, crushed and sifted with a grinder, and then put into a conical bottle for separate pretreatment (Yang *et al*. 2023).

Characterization

Chemical analysis

The decomposed powder was extracted with boiling water for 20 min. The mixture was filtered, and the residue was dried in a hot air oven at 105 ℃ to a constant weight. The lignin and acid and acid detergent fibre (ADF) contents were determined according to the modified method proposed by the NREL, USA (Pradipta and Irawati 2020). ADF refers to the fibres that could be dissolved by sulphuric acid.

Fourier transform infrared spectroscopy (FT-IR) analysis

The free functional groups in the BSS were detected as an infrared absorbance band using an FT-IR spectrometer (IRTracer 100). The powdered sample was placed over potassium bromide pellets, and the infrared spectrums were recorded within the range of 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1} signal to noise ratio (Vinod *et al*. 2021).

Gas chromatography-mass spectrometry (GC-MS) analysis

First, 300 mL of ultra-pure water was added to the sample, which was subjected to oscillation at a speed of 150 revolutions per min for 1 h. Subsequently, it was filtered using 8 layers of sterile gauze and extracted three times with methylene chloride (approximately 10 mL each time). The resulting extraction liquids were combined and concentrated to approximately 1 mL through nitrogen blowing at room temperature. Finally, the concentrated solution was tested chromatographically. The chromatographic column used in this process was DB-5MS (0.25 mm × 60 m, 0.25 μm), while high purity helium served as the carrier gas flowing at a rate of 1.00 mL/min. The temperature was maintained at 50 ℃ for a duration of 2 min before being increased by an increment of 5 ℃ /min until reaching a final temperature of 280 ℃ for an additional period of 3 min (Zhang *et al*. 2017).

Particle size and zeta potential

The 0.2 g BSS samples were placed in a 50 mL conical bottle and treated with citric acid-sodium citrate buffer solution (50 mM, pH 4.8). Subsequently, the conical bottle was

incubated in a constant temperature shaker at 50 ℃ and 180 rpm for 2 h, followed by an additional hour of rest. Afterward, 2 to 3 mL of the supernatant was extracted for particle size and potential analysis using a DLS analyzer.

X-ray diffraction (XRD) analysis

The crystallinity properties of BSS were investigated using an X'Pert-Pro diffractometer with an X-ray generator set at 30 mA and 40 kV. X-rays at a wavelength of 0.154 nm were passed through the fiber sample, and the diffracted X-rays were recorded by a detector, which was rotated at a scanning speed of 5°/min in the 2θ range from 10° to 80°. According to the Siegel empirical method, the crystallinity index (CI) of BSS is calculated by Eq. 1 (Ramaiah *et al*. 2020),

$$\mathrm{CI} = \frac{H_{22}-H_{16}}{H_{22}} \quad (1)$$

The crystal size is calculated using Eq. 2, λ

$$\mathrm{CS} = \frac{K\lambda}{\beta \cos\theta} \quad (2)$$

where Scherrer's constant is given as K = 0.89. Here wavelength is denoted by λ, and β denotes the peak's full width at half-maximum.

Fermentation

Preliminary enzymatic hydrolysis fermentation of BSS

One gram of BBS chips was transferred into a 50 mL and allowed to react with 30 mg cellulase made by *Trichoderma viride* and *Aspergillus niger* (later A and B enzymes), and hydrolyzed at pH 4.8 and 40 ℃ with a liquid-solid ratio of 30: 1 mL/g for 48 h.

Determination of the enzyme activity

Cellulase A and B were determined by hydrolysis of 50 mg filter paper at pH 4.8 and 50 °C. The amounts of enzyme required to hydrolyse the filter paper to produce 1 μmol glucose per h was defined as 1 unit of enzyme activity (U). The specimens of cellulase A and B were determined by filter paper enzyme activity (FPA) at 1000 and 1200 U/g, respectively (Silveira *et al*. 2012).

Determination of reducing sugar

The fermentation solution was centrifuged at 5000 rpm for 10 min, the recovered supernatant was diluted 3 times, and the reduction sugar content was determined by DNS colorimetry and standard glucose curves, as described previously (Jain *et al*. 2020). Reducing sugar conversion was calculated using Eq. 3,

$$\omega = \frac{C \times V}{M_0} \times 100\% \quad (3)$$

where ω is reduced sugar conversion extent (mg/g), C is enzymatic hydrolysis concentration (mg/mL), V is the volume of enzymatic hydrolysis liquid product (mL), and M_0 is BSS dry mass (g).

RESULTS AND ANALYSIS

Determination and Characterization of Components in BSS

Table 1. Comparison of BSS Pretreated by Different Methods

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	40.22	31.92	21.33
NaOH	45.89	32.48	11.73
NaOH＋H_2O_2	52.40	35.08	9.14
NaOH+Autoclaving	48.14	33.77	9.33
NaOH+Autoclaving＋H_2O_2	53.52	35.59	7.59
NaOH+Autoclaving＋ laccase	50.81	33.96	8.29

Note: The pretreatment conditions were: 4%NaOH, 4%H_2O_2, 121 °C, Steam pressure 205.8 kPa, 1 g BSS, and 50 mg/glaccase 3h.

Table 1 shows that pretreatment significantly reduces lignin content, exposes cellulose, and is more involved in enzymatic lysis and glycosylation. Scanning electron microscope (SEM) showed significant differences in the surface morphology of bamboo shoots with different treatment methods (Fig. 2).

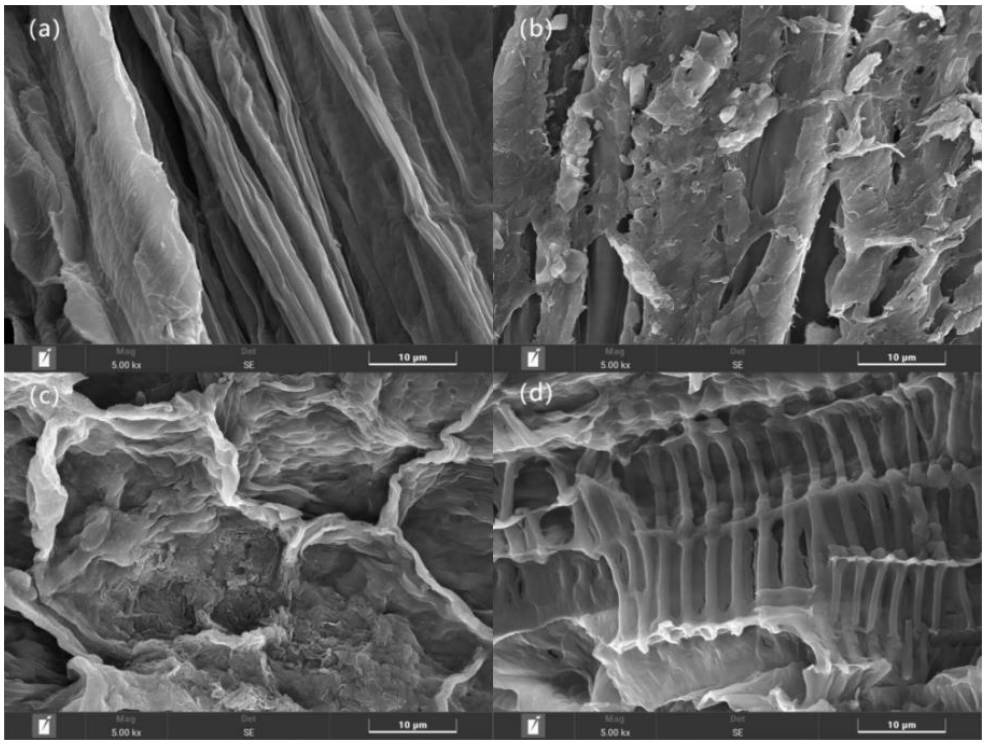


Fig. 2. Scanning electron microscope images of BSS with different pretreatment methods: (a) Untreated; (b) NaOH pretreated; (c) NaOH+Autoclaving pretreated; (d) H_2O_2 pretreated

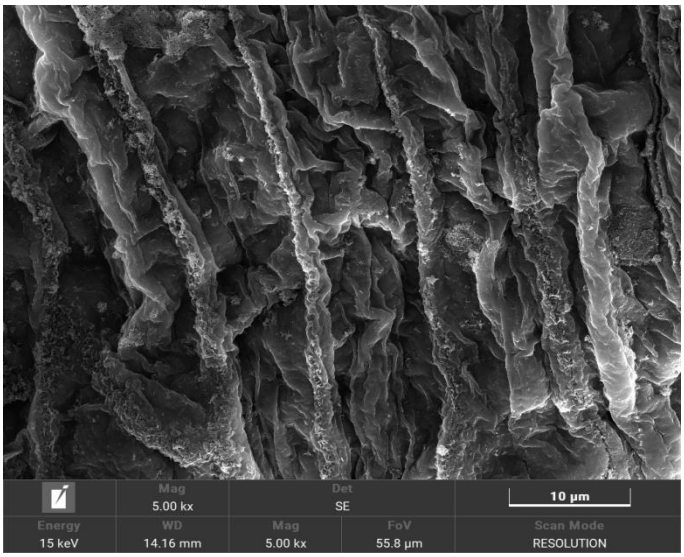


Fig. 3. Scanning electron microscope images of BSS with NaOH-Autoclaving-H_2O_2 pretreatment method

In Fig. 2(a), the surface structure of the unpretreated BSS appears smooth and dense, and the fibers are arranged in an orderly manner. In part (b), surface roughness was apparent after the BSS was treated with sodium hydroxide. There appeared to be broken holes, increasing the material surface area, which might be attributed to the significant dissolution of biodegradable components and the degradation of hemicellulose and lignin on the BSS. Lowering the lignin content to some extent destroys the original structure, increasing the availability of cellulose to the cellulase enzyme (Chandra *et al*. 2009; Yuan *et al*. 2021). However, pretreatment also results in the transfer of lignin from the wall of cellulose to the surface of cellulose, causing a physical barrier to cellulose binding to cellulose (Li and Zheng 2017).

In Fig. 3, it was apparent that oxidation of BSS with hydrogen peroxide and sodium hydroxide corrosion, combined with high pressure steam treatment, not only destroyed the original structure, thus increasing the availability of cellulase on cellulose, but it also contributed to degradation of the surface lignin, thus increasing enzymatic efficiency.

FT-IR Analysis

Based on Table 1, when considering economic and environmental factors, NaOH+Autoclaving pretreatment achieved the best combined effect, so the BSS processed by it was tested. Figure 3 shows FT-IR results before and after NaOH+Autoclaving treatment. In both spectra, strong absorbance peaks were apparent at 3422 cm^{-1}, which is the absorbance peak of the hydroxyl group (Balaji and Nagarajan 2017), while 1643 and 1063 cm^{-1} are the absorbance peaks of C=O and C-O-C, respectively. The number of hydrogen bonds in lignocellulose substrates is associated with the absorbance peaks of hydroxyl groups (Jayaramudu *et al*. 2010; Yang *et al*. 2020). In the experiment, NaOH+Autoclaving decreased the intensity of C=O and C-O-C peaks, correspondingly increased the intensity of the peak at 3422 cm^{-1}, thus enhancing the number of hydroxyl groups. It is proposed here that the change can contribute to the hydrogen bonding with cellulase. Thus, NaOH + Autoclaving is expected to reduce the negative charge of the BSS substrate and increase the number of hydroxyl groups in the

BSS substrate, improving the electrostatic and hydrogen bonding interaction between the BSS substrate and cellulase, and increasing the binding strength of the cellulase and BSS.

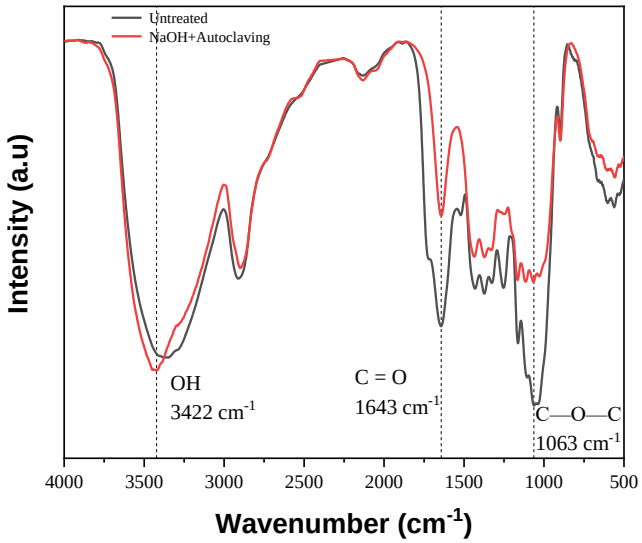


Fig. 4. FT-IR spectra of raw materials and NaOH + autoclaving

GC—MS Analysis

The surface of bamboo shell is coated with a protective waxy film, which primarily consists of aldehydes, fat-soluble fatty acids, lipids, alkanes, ketones, and fatty alcohols. Silicon is the second most abundant element in the Earth's crust. In addition to being present as silicates, silicon can also form covalent bonds with plant cell wall components such as hemicellulose, pectin, and lignin (Sheng and Chen 2020). The untreated bamboo shell eluent (Fig.5.S1) contains silicones, long-chain fatty acids and long-chain aliphatic hydrocarbons that confirm these structures. However, the proportion of silicones in the eluent is relatively low, and most of them exist as low molecular weight compounds including $C_8H_{24}O_4Si_4$, $C_{10}H_{30}O_5Si_5$, and $C_{12}H_{36}O_6Si_6$. This suggests that mechanical crushing only disrupts the cellulose structure on the surface while exposing some cellulose structures that are then dissolved in pure water during extraction.

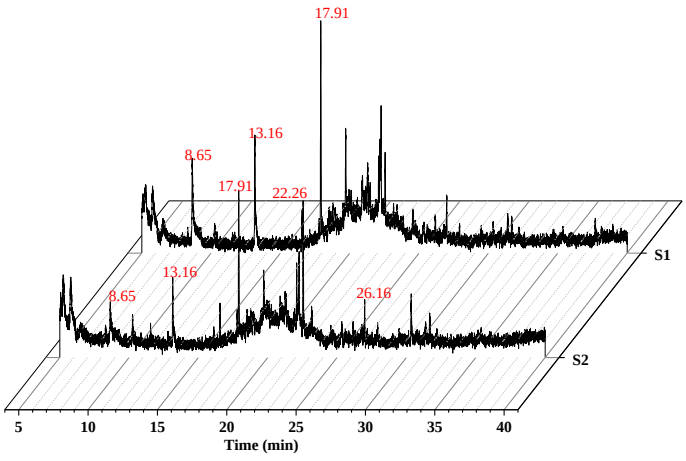


Fig. 5. GC-MS of raw materials and NaOH + autoclaving

Compared to the original bamboo shells, pre-treated bamboo shell elution (Fig. 5. S2) exhibited a wider range of higher molecular weight silicon-containing compounds such as $C_{14}H_{42}O_7Si_7$ and $C_{16}H_{48}O_8Si_8$. The proportion of silicon-containing compounds also increased. This is because when wood fibers detach from the surface of BSS due to soaking in sodium hydroxide and high pressure steam treatment process, their internal silica-rich structure becomes exposed and a small amount gets washed away through mechanical oscillation. The presence of higher molecular weight silica compounds in the eluent confirms effective destruction of fiber structure within bamboo shells by this pretreatment method.

XRD Analysis

Figure 5 clearly shows the XRD diffractogram of BSS before and after pretreatment. In Fig. 6, both samples show two different peaks. Generally speaking, plant fibers have two identical peaks near 2θ =15° and 2θ =22°, which correspond to the peaks of type I and type IV cellulose (Sreenivasan *et al*. 2011; Senthamaraikannan *et al*. 2016; Madhu *et al*. 2018). The peak near 15° is relatively less intense, and the peak near 22° is more intense, which shows that the BSS contains more type IV cellulose.

XRD also can be used to analyze the crystallinity of lignocellulose. The higher the crystallinity, the lower the efficiency of cellulase hydrolysis (Prithiviraj *et al*. 2016). The crystallinity index CI of BSS was calculated using Eq. 1. The crystallinity of untreated BSS substrate was 77.7%, and the crystallinity of NaOH+Autoclaving treated BSS substrate was 50.9%. This finding indicates that NaOH+Autoclaving treatment can improve the hydrolysis efficiency of BSS substrate by affecting its crystallinity.

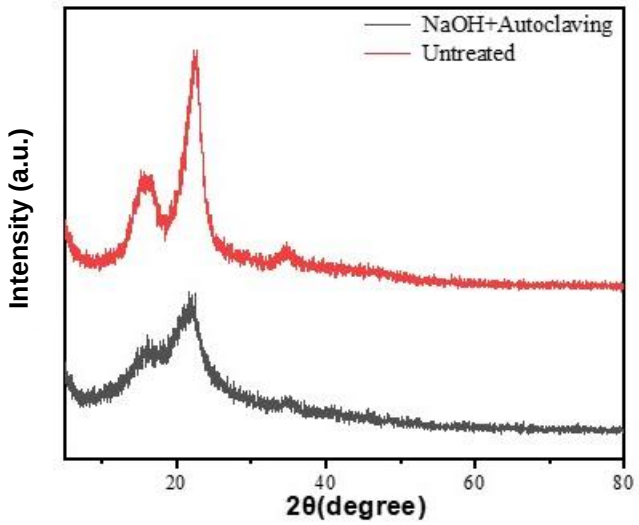


Fig. 6. XRD diffractogram for BSS

Zeta Potential Analysis and Particle Size Analysis

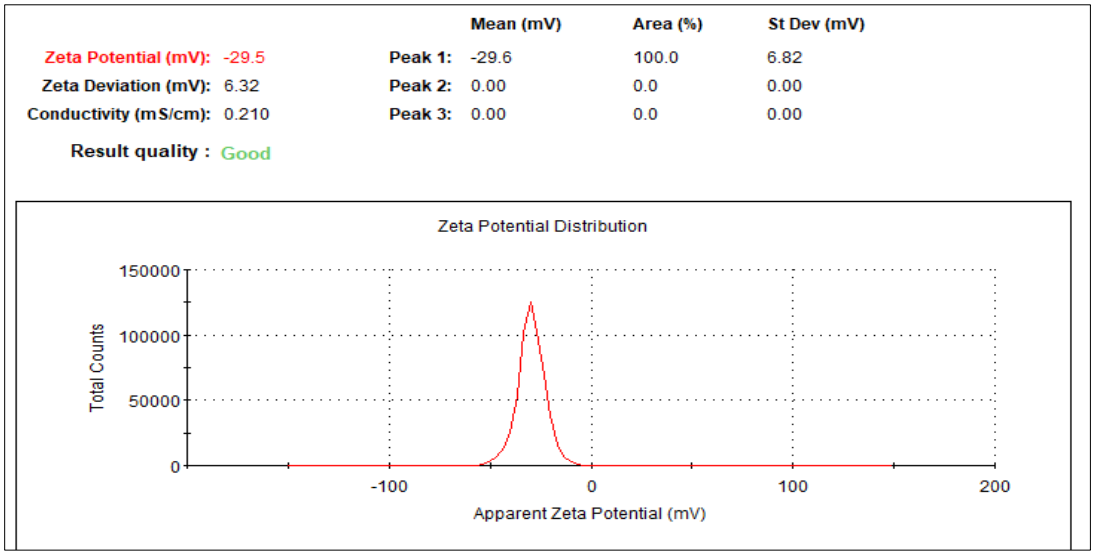
Zeta potential and FT-IR analysis can be employed to investigate the electrostatic and hydrogen bond interactions between cellulase and lignocellulosic substrates (Mahendraprabu *et al*. 2020). As depicted in Table 2 and Fig. 7, the zeta potential of bamboo shell substrate became less negative, changing from -29.5 to -21.6 mV after being soaked in sodium hydroxide and subjected to high pressure steam treatment, while cellulase itself exhibited a negative charge (Sammond *et al*. 2014). In essence, there exists a repulsive relationship between cellulase and bamboo shell substrate; however, the

combined treatment of sodium hydroxide soaking and high pressure steam reduced the absolute value of zeta potential of BSS, thereby mitigating its repulsive effect on cellulase and enhancing the electrostatic interaction between substrate and enzyme.

The particle size of lignocellulosic substrates can significantly influence cellulase adsorption capacity. Smaller particle sizes result in larger relative specific surface areas, providing more binding sites for cellulase (Yao *et al*. 2021; Daoud *et al*. 2010; Lu *et al*. 2019). Table 2 presents the average particle size of BSS before and after undergoing sodium hydroxide soaking combined with high pressure steam treatment. Prior to pretreatment, the average particle size was measured at 612 nm; however, post-treatment reduced it to 190 nm. These findings demonstrate that combining sodium hydroxide soaking with high pressure steam treatment effectively decreases substrate particle size, increases available binding sites for cellulase adsorption, thus augmenting its overall adsorption capacity.

Table 2. Particle Size, Zeta Potential, Hydrogen Bond and Crystallinity of Bamboo Shell Substrates

	Particle Size (nm)	Zeta Potential (mV)	Crystalline (%)	Hydrogen Bond
Untreated	612.3	-29.5	77.71	Less
NaOH+Autoclaving	190.3	-21.6	50.93	More



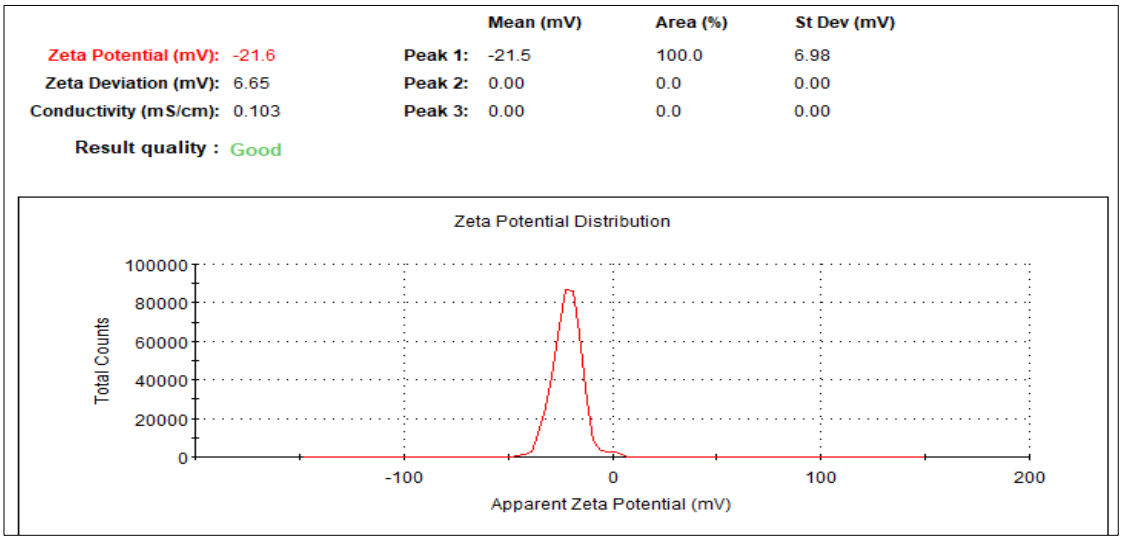


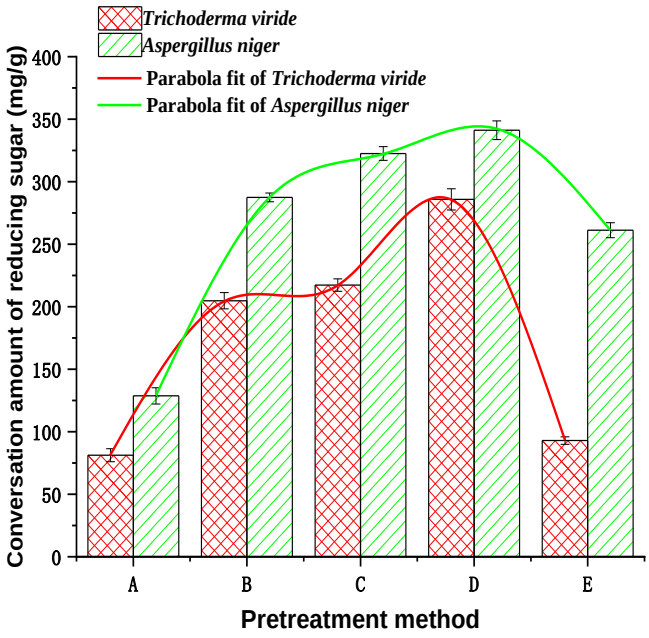
Fig. 7. Zeta potentiogram of raw materials and NaOH + autoclaving

Comparison of substrate reducing sugar yields

According to the reaction conditions that were derived from the experimental results in Table 3, the conversion extents of A enzyme reductsugar were 207, 193, and 189 mg/g, respectively. The conversion extents of B enzyme reductase were 330, 332, and 337 mg/g, respectively.

Table 3. Enzymatic System of BSS

Name	pH	Temperature	Time	Enzyme addition	Liquid to material ratio	Shaker speed
A	4.6	50 °C	56 h	50 mg	20:1	0
B	4.8	50 °C	56 h	50 mg	20:1	100 r/min



A: Untreated; B: NaOH; C: NaOH+Autoclaving; D: NaOH+Autoclaving+ H_2O_2 ; E: NaOH+Autoclaving+Laccase

Fig. 8. Comparison of reducing sugar production by different pretreatment methods

Under the same optimal conditions, BSS with no pretreatment, NaOH treatment, Autoclaving-NaOH treatment, H_2O_2-Autoclaving-NaOH treatment, and Laccase-Autoclaving-NaOH treatment were subjected to enzymatic fermentation. As can be seen from (Fig. 8), both the A and B enzyme reaction systems showed better enzymatic hydrolysis of BSS treated with Autoclaving-NaOH and H_2O_2-Autoclaving-NaOH compared to unpretreated BSS, but Autoclaving-NaOH was preferred for cost of production and environmental pollution. Finally, the yields of A and B enzymes were increased by 268% and 251%, respectively, compared to untreated raw materials.

CONCLUSIONS

1. Some microscopic tests such as scanning electron microscopy (SEM) showed that, as a result of pretreatment with NaOH and high-pressure steam, the hemicellulose and lignin in the bamboo shoot shell (BSS) were partially dissolved, the surface structure was destroyed, more cellulose was exposed, and the relative content of cellulose was increased.
2. The reduction of carbonyl group and the increase of hydroxyl group in BSS increased the electrostatic interaction force between substrate and cellulase and strengthened the adsorption of cellulase and contact site. The zeta potential of the substrate was optimized from -29.5 to -21.6 mV, and the repulsion of cellulase and BSS decreased. At the same time, the average particle size of the substrate decreased from 612 nm to 190 nm, and the relative increase of specific surface area would provide more adsorption sites for cellulase. After treatment, the crystallinity of BSS decreased from

77.7% to 50.9%, which made cellulase and substrate better binding. Silicon is one of the main elements of plant cell wall. Silica compounds with higher molecular weight such as $C_{14}H_{42}O_7Si_7$ and $C_{16}H_{48}O_8Si_8$ were found in the pre-treated BSS elution, indicating that the cell wall was loose and the fiber structure was further damaged.

3. NaOH + Autoclaving treated BSS increased the yield of reducing sugars of both enzymes by 268% and 251%, respectively, compared to the untreated raw materials.

ACKNOWLEDGMENTS

This work was supported by grants from Scientific Research Project of Department of Education of Hunan Province (No.20K144); Hunan Forestry Science and Technology Research and Innovation Fund Project (XLK202407); and Technology Innovation Fund for Graduate Students (No.2023CX02003).

Author Contributions

TZ and ML conceived and designed the project; TZ, MY and YM treated experimental materials; TZ, JC， CH and CX analyzed and interpreted the data; TZ, ML and BZ wrote the paper.

Data Availability

The data supporting the findings of this study are available from the corresponding author BZ upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Article submitted: February 7, 2024; Peer review completed: April 9, 2024; Revised version received and accepted: May 4, 2024; Published: May 28, 2024.
DOI: 10.15376/biores.19.3.4604-4618

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