Assessing the availability of two bamboo species for fermentable sugars by alkaline hydrogen peroxide pretreatment

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Highlights

AHP significantly enhanced the enzymatic saccharification of bamboo.

PEB has a relatively rigid structure with stronger recalcitrance than NAB.

The lignin behaviors of two bamboo samples were investigated and compared.

Abstract

This study comprehensively investigated two bamboo species (*i.e.*

Neosinocalamus affinis and *Phyllostachys edulis*) in terms of their cell wall ultrastructure, chemical compositions, enzymatic saccharification, and lignin structure before and after alkaline hydrogen peroxide pretreatment (AHP). During AHP, *Neosinocalamus affinis* (NAB) had higher delignification than *Phyllostachys edulis* (PEB), and thus showed better enzymatic digestibility (93.05% *vs* 53.57% for glucan). The fundamental chemical behavior of the bamboo lignins was analyzed by fluorescence microscope (FM), confocal Raman microscope (CRM), molecular weight analysis, and 2D HSQC-NMR. Results indicated that the PEB has thicker cell wall and more concentrated lignin in its compound middle lamella and cell corner middle lamella than NAB. Moreover, PEB lignin contains more G units (S/G of 0.95), in evident contrast to that of NAB lignin (S/G of 1.30), which favor the formation of C-C linkages, thus impeding its degradation during the AHP.

Keywords: *Neosinocalamus affinis*, *Phyllostachys edulis*, Alkaline hydrogen peroxide pretreatment, Enzymatic saccharification, lignin

1. Introduction

Due to the over-exploitation and overuse of fossil fuels, environmental issues and resource scarcity are becoming more and more serious, which necessitate the development of sustainable and renewable energies and materials (Chen et al., 2017; Xiang and Runge, 2014). Lignocellulose is a renewable, abundant, and inexpensive resource, which holds the potential to be processed into various chemicals and materials. Lignocellulose is mainly made up of cellulose, hemicellulose and lignin, promoting the large-scale, sustainable production of biofuels. The biorefinery of lignocellulose mainly includes six processes: feedstock collection, chipping, pretreatment, enzymatic saccharification, microorganism fermentation, and distillation (Meng and Ragauskas, 2014; Rubin, 2008). Lignocellulose, including energy crops, forestry, and agricultural residues, can be hydrolyzed into monomeric sugars *via* enzymes and further converted to ethanol which has garnered growing attention as a promising alternative to gasoline (Ragauskas et al., 2006). In lignocellulosic biomass, cellulose is tightly enwrapped with hemicellulose and lignin, forming a compact defensive structure which is resistant to biological degradation (Chen et al., 2011; Huang et al., 2020b). Therefore, it is necessary to alter the chemical compositions or physicochemical structures of lignocellulose by pretreatment in order to increase the substrates accessibility to cellulolytic enzymes, as well as enable the high-efficiency utilization of lignin and hemicellulose (Asada et al., 2005; Zhao et al., 2012).

Since then, various pretreatment techniques, such as physical, chemical, physicochemical, and biological pretreatments, have been investigated in detail (Li et al., 2018; Sun and Cheng, 2002). Although these pretreatment methods have been tested extensively, it is still far from a standard industrialization practice because of the inherent drawbacks of the pretreatment process (Geng et al., 2014). For example, dilute acid pretreatment can cause corrosion to processing equipment, and also can lead to the production of fermentation inhibitors. Alkaline pretreatment may cause peeling reaction of cellulose, and thus lower its yield. In the case of liquid hot water pretreatment, it needs to be performed at high temperature and high pressure. Thus, advanced pretreatment mediums that can remedy these challenges are desired.

Alkaline hydrogen peroxide pretreatment (AHP), a low-environmental-threat pretreatment technology compared to traditional thermochemical processes, is feasible in improving the enzymatic digestibility of substrate due to its extraordinary performance in removing lignin (Li et al., 2012). AHP is derived from the pulp bleaching industry. During AHP, hydrogen peroxide under alkaline conditions can decompose to generate strong hydroxyl and superoxide anion radicals, which oxidize and fragment lignin, leading to delignification (Zhang et al., 2019). Simultaneously, hemicellulose is partially degraded and removed from the biomass by the hydrogen peroxide action in addition to delignification (Klinke et al., 2002). Different from hemicellulose and lignin, cellulose was reported to be almost unaffected in AHP (Huang et al., 2020c). According to Yamashita et al. (2010), AHP could perform well at mild processing conditions without losing its strong delignification compared to other thermochemical methods like alkali or organosolv pretreatment. In addition, the degradation products of hydrogen peroxide are water and oxygen. Thus no residual H_2O_2 could be found in the pretreated samples (Rabelo et al., 2008). However, the efficiency of AHP seems to vary significantly from biomass to biomass. Previous work has demonstrated that AHP could perform well for poplar, wheat straw, and reed, with glucan saccharification higher than 90%. While for softwood, such as masson pine and redwood, glucose yield was only 62.5% and 22.2%, respectively, far from the results acquired with hardwoods and grass (Huang et al., 2020a).

Bamboo, which is widely distributed in China with an annual production of three billion, is a promising starting material for bioethanol production due to its perennial nature, fast growing and less require of water and fertilizer supply. According to Gao et al. (2021), bamboos with different growing time showed different behavior during the pretreatment, and the bamboo with short growing time clearly had better enzymatic digestibility than that with long growing time, due to its low lignin content and thinner cell wall structure. However, bamboo includes many species, such as *Neosinocalamus affinis*, *Phyllostachys edulis*, *Dendrocalamus farinosus*, and so forth, which differ in chemical and physical structures, showing completely different recalcitrance towards pretreatment. In this study, two most widely planted bamboo species in China, *i.e.*, *Neosinocalamus affinis* and *Phyllostachys edulis*, were subjected to AHP to evaluate their physicochemical behavior and enhance the enzymatic digestibility. The physical and chemical structures of pretreated bamboo were investigated with Fourier transform infrared spectroscopy, X-ray diffraction, confocal Raman microscopy, fluorescence microscopy, gel permeation chromatography, and HSQC NMR spectra, in an attempt to unveil the difference of AHP in improving the enzymatic digestibility of different bamboo species. The pretreatment of bamboo has been widely published recently (Lu et al., 2022), based on these studies, this research investigated the performance of different bamboo species in the AHP. Thus, it may offer a guide for the bamboo biorefinery in different regions in China.

2. Materials and methods

2.1 Materials

Bamboo samples (*Phyllostachys edulis and Neosinocalamus affinis,* both with 5-

year growing time) were harvested, respectively, in Guizhou province and Fujian province of China, which were termed as PEB and NAB. Enzymes, including cellulase (CTec2) with an activity of 250 FPU/mL, and xylanase (X2753) with an activity of 3490 U/g, were purchased from Novozymes. All other chemicals were of analytical grade, and purchased from Sinopharm Chemicals (Beijing, China).

2.2 Bamboo pretreatment

Prior to the AHP, the obtained bamboo culms were mechanically treated by a twin-screw extruder and disk mill in order to reduce the size and improve heat transfer in the pretreatment. The detailed size reduction method was illustrated previously (Huang et al., 2020a).

AHP of bamboo was carried out at a consistency of 10% in a 350 mL glass flask. The pretreatment medium was prepared by mixing H_2O_2 (0-3wt%), NaOH (2.2 wt%), Na_2SiO_3 (0.4 wt%), and diethylenetriaminepentaacetic acid (DTPA, 0.1 wt%) into DI water. The medium was blended sufficiently with 10 g bamboo (dry weight) in glass beaker. Next, the mixture was loaded into pretreatment flask, sealed with a cap, and placed in a 90 °C water bath maintaining for 60 minutes. The flasks were immediately immersed into cool water upon the pretreatment. The solid residues were filtered (using filter paper) and rinsed by DI water until it became pH neutral. Finally, the pretreated samples were stored at 4 °C for the following experimentations. The AHP pretreated residues of NAB and PEB were labeled as RNAB and RPEB, respectively.

2.3 Enzymatic saccharification

Enzymatic saccharification was carried out with a consistency of 2.5% (w/v). In detail, weighing 0.5 g samples (dry weight) into the glass flask, followed by acetate buffer to control the pH around 4.8. Next, cellulase and xylanase were supplemented at the dosages of 15 FPU/g-glucan and 150 U/g-xylan. Finally, DI water was added to set system volume of 20 mL. The flasks were then placed in an orbital shaker at 50 °C and 150 rpm for enzymatic saccharification. After 72 h of enzymatic hydrolysis, the supernatant was obtained by centrifugation, and then diluted and subjected to high performance liquid chromatography (HPLC) analysis.

2.4 Preparation of cellulolytic enzyme lignin (CEL)

To prepare the CEL in raw and pretreated samples, dried samples were milled using the QM-QX4 planetary ball mill (Nanjing University Capital Management Co., Ltd, Nanjing, China) for 24 h. The milled sample was then conducted an enzymatic hydrolysis to remove the carbohydrates. In detail, the ball-milled bamboos were first dispersed in acetate buffer solution at pH 4.8. Cellulase was followed with 250 FPU/g-substrate, and the mixture was then placed at 50 °C and 150 rpm for 48 h. Subsequently, the substrate was isolated by centrifugation and washed using DI water. The residuals relived the processes of enzymatic hydrolysis for another 48 h and then freeze-dried. After that, the samples were extracted twice in the dark with 96% aqueous dioxane/water solution (v/v). This extraction process was performed two times. All supernatants were combined, filtered, then rotary-evaporated to remove the solvents. Finally, the CELs were regenerated in DI water and then centrifuged and freeze-dried.

2.5 Analytical tests

The chemical compositions, including glucan, xylan, arabinan, acid-soluble (ASL) and acid-insoluble lignins (AIL), were quantified by the NREL standard sulfuric acid hydrolysis procedure (Sluiter et al., 2008). Monomeric sugars from the sulfuric acid hydrolysis were quantified with an Agilent 1260 HPLC. Different components in samples were isolated by a Bio-Rad Aminex PHX-87H column. The HPLC used 5mM H_2SO_4 as the eluent at a 0.6 mL/min flowing rate at 55 ℃.

The enzymatic hydrolysis yields were calculated as follows:

$$\text{enzymatic hydrolysis yield (\%)} = \frac{\text{glucose/xylose in enzymatic hydrolyzate (g)}}{\text{initial glucose/xylose in substrate (g)}} \times 100\%$$

The fluorescence microscope (FM) images of untreated and pretreated cell wall structure were captured by the Olympus BX51 microscope. The excitation wavelength of 330-385 nm, and the emission wavelength of 400-420 nm were applied to visualize lignin autofluorescence.

The crystallinity index (CrI) of raw and pretreated bamboo samples was measured by a Bruker D8 advanced X-ray diffraction (XRD) instrument. The XRD test used Cu Kα X ray source at 40 kV and 40 mA. The recorded 2θ region was scanned from 10° to 40°, and the CrI of samples was calculated by the following equation (Creely and Conrad, 1958):

$$\text{Crystallinity index(CrI)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100\%$$

where I_{002} is the diffraction intensity of the crystalline regions at 2θ of ~22.5° and I_{am} is the diffraction intensity of amorphous regions at 2θ of ~18.5°.

The FTIR test were conducted with a Nicolet Is50 FTIR spectrometer, which was applied to reveal the changes of the functional groups in biomass. The spectra pattern was in the range of 4000 -500 cm^{-1} with a 4 cm^{-1} resolution, and each test was scanned

32 times.

For Raman imaging, a confocal Raman microscope (CRM, DXR2xi, Thermo scientific) was used. The CRM test was performed under a ×100 oil immersion microscope objective (Olympus, NA = 0.25) and a laser in the visible wavelength (λ=532 nm) was used at RT. Raman test were scanned within the wavenumber of 3400-200 cm^{-1} (Ji et al., 2015).

The molecular weight distribution (weight-average (M_w) and number-average (M_n) molecular weights) of the lignins was determined with gel permeation chromatography (GPC), equipped with a UV detector at 260 nm. Samples for GPC test were first acetylated with pyridine/acetic anhydride, and then dissolved in tetrahydrofuran for the molecular weight measurement.

For the two-dimensional heteronuclear single-quantum coherence NMR (2D-HSQC NMR) test, 150 mg of lignin was first dissolved in 0.5 mL of DMSO-d_6, and then the samples were subjected to a Bruker Ascend^{TM} 600 MHz spectrometer with a cryoprobe at RT, and the details for the test can be found in the previous report (Meng et al., 2019).

3. Results and discussion

3.1 Chemical composition analysis in raw and pretreated bamboo

In China, bamboo is a potential resource for biorefinery processing in term of the tachyauxesis, wide distribution and high productivity (Dong et al., 2020), meanwhile bamboo is highly lignified with strong recalcitrance to physical and chemical attack. Indeed, some studies have achieved high saccharification of cooked bamboo, but these researches were derived from pretreatments with severe conditions (Ali et al., 2020). AHP has been proven an effective method in substantially delignifying lignocellulose and improve the enzymatic saccharification of pretreated substrate (Soltanian et al., 2020; Zhang et al., 2019). In this study, two kinds of bamboo (*i.e.* NAB and PEB) were investigated, and their behaviors during the AHP were compared. The solid yield, main composition, and lignin removal of raw bamboo and AHP treated bamboo are shown in Fig. 1.

After the AHP, it was observed that the two bamboos were recovered in different yields. As shown in Fig. 1a, the pretreated solid recovery of NAB and PEB was decreased by 9.59% and 9.22% as the H_2O_2 loadings increased from 0% to 3.0%, respectively. Moreover, it can be clearly observed that the solid recovery of PEB was higher than NAB. At the H_2O_2 loading of 3%, the solid recovery of PEB was 79.15%, 7.51% higher than that of NAB. The data indicated that, as the H_2O_2 loading increased, the dissolution of the lignocellulosic component was enhanced, and the NAB degradation seems to be more pronounced than PEB. Furthermore, the compositions of untreated and pretreated bamboo (NAB, PEB, RNAB, and RPEB) were investigated. As can be seen in Fig. 1b, the untreated NAB contains 44.23% glucan, 17.53% xylan, and 26.08% lignin (ASL and AIL). As to raw PEB (Fig. 1c), it contains 41.00% glucan,12.78% xylan, and 30.99% lignin. It can be found that the PEB has more lignin content while less hemicellulose, which endows its higher recalcitrance. After pretreatment, AHP resulted in the degradation of xylan and lignin of both cases. Specifically, xylan content of pretreated bamboos was almost unchanged with increasing H_2O_2 loading. On the contrary, the glucan content of pretreated bamboos increased gradually with increasing H_2O_2 loading and finally reached 56.71% (NAB) and 55.13% (PEB), respectively, at H_2O_2 loading of 3.0%. This result is because of the selective removal of hemicellulose and lignin, and thus enriched cellulose in the pretreated bamboos. The delignification of NAB was found to be improved with the increase of H_2O_2 dosage (Fig. 1d), which increased gradually from 48.88% (0 wt% H_2O_2) to 68.17% (3.0 wt% H_2O_2). The same trends were also found for PEB (from 36.52% to 53.72%). It can be concluded that the NAB lignin was more readily degraded than PEB lignin, which may be related to its physical and chemical structures and will be analyzed in the following sections.

3.2 The effect of AHP on the enzymatic saccharification of bamboos

Enzymatic saccharification is a crucial metric to evaluate the pretreatment efficacy. The pretreated bamboo samples (RNAB and RPEB) were thus conducted enzymatic hydrolysis at 2.5% consistency for 72 h. As can be seen in Fig. 2, the untreated bamboo without any pretreatment had very poor enzymatic hydrolysis efficiency, which was 11.14% (glucan) and 1.43% (xylan) for NAB, and 8.76% (glucan) and 1.37% (xylan) for PEB. This is actually caused by the enwrapping and sealing of lignin and hemicellulose on cellulose, which as a result inhibited the enzymatic digestibility. While after the AHP, the enzymatic hydrolysis yield was observed to be reinforced considerably, with glucan hydrolysis yields of 68.73% (NAB) and 34.13% (PEB), and xylan hydrolysis yields of 79.10% (NAB) and 75.60% (PEB), respectively, even without the addition of H_2O_2. With the introduction of H_2O_2, enzymatic hydrolysis efficiency continued increasing for both NAB and PEB. Specifically, glucan hydrolysis yield of NAB increased from 68.73% (0 wt% H_2O_2) to 83.35% (0.5wt% H_2O_2), 88.26% (1.0 wt% H_2O_2), 88.74% (1.5 wt% H_2O_2), 88.46% (2.0 wt% H_2O_2) and 90.62% (3.0 wt% H_2O_2), when increasing the H_2O_2 from 0% to 3%, indicating that AHP was powerful in removing the recalcitrance of NAB. While in the case of PEB, the AHP seems to be less efficient, as the glucan hydrolysis yield only reached a maximum of 55.83% as increasing the H_2O_2 dosage (Fig. 2b). These results revealed that the PEB possesses more resistance than NAB, which may be related to its compact physicochemical structure.

In addition, the enzymatic sugar yield, which was calculated by incorporating the solid recovery, was further investigated (Fig. 3). It can be found that the enzymatic sugar yield has similar trend with enzymatic hydrolysis yield. In detail, the enzymatic glucose yield of NAB increased from 57.61% to 75.66% with increasing H_2O_2 dosage from 0 to 3%, higher than that of PEB (33.93%-53.25%). However, NAB exhibited a lower enzymatic xylose yield than PEB (Fig. 3b), in contrast to the enzymatic hydrolysis yield, which is due to the significant degradation of NAB hemicellulose during the AHP. Overall, the enzymatic saccharification of AHP treated substrates was observed to be significantly enhanced when increasing the H_2O_2 loading, which was mainly because of the degradation of lignin during the AHP that led to the exposure of more glucan/xylan to cellulolytic enzymes, as demonstrated by the decreased lignin content in Fig. 1b and c. Among two bamboos, NAB displayed higher lignin removal and higher enzymatic hydrolysis efficiency, which indicated that NAB is a promising feedstock to produce monomer sugars.

Recently, Gao et al. (2021) reported that except bamboo species, the bamboos with different growing years showed completely different recalcitrance towards pretreatment, with the one-year-old bamboo possessing highest enzymatic digestibility than older ones. This is due to its low lignin content and thinner cell wall structure in one-year-old sample which is readily to be penetrated and then degraded by various chemicals. In this study, 5-year-old NAB and PEB were used, which have lignin content of 26.08% and 30.99%, respectively, and theoretically possess lower enzymatic digestibility than the one-year-old one. However, one-year-old bamboo has less biomass productivity, while the 5-year-old samples has a constant high productivity, thus increasing its application value.

Overall, the aforementioned results revealed that the lignin removal considerably contributed to enzymatic saccharification of glucan. To evaluate the significance of lignin degradation, the glucan hydrolysis yield was plotted against the lignin removal in the AHP process (Fig. 4). It can be found that strong positive correlation existed between lignin removal and glucan hydrolysis yield for both samples (R^2=0.96 and 0.95 for NAB and PEB, respectively), which suggested lignin as the main factor impeding the enzymatic saccharification of two bamboo species.

3.3 Structural variations before and after AHP

To better understand the effect of AHP on the cellulose structure of two bamboos, the cellulose crystallinity under different H_2O_2 dosages was measured, and its relationship with enzymatic hydrolysis yield was analyzed, and the results are shown in Table 1. The CrI value of raw NAB bamboos was 53.59%, clearly lower than that of PEB (59.36%). It can be seen that the overall trend of relative crystallinity was increased after AHP. Specifically, with the addition of H_2O_2 (from 0 % to 3.0%), the CrI of NAB increased gradually from 53.59% to 70.09% (3.0 wt% H_2O_2). However, the CrI of PEB increased and reached its highest (66.96%) at 1.0% H_2O_2. Thereafter, the cellulose CrI slightly decreased (from 66.96% to 66.09%) when increasing the H_2O_2 loading to 3.0%. The CrI increase was caused by the degradation of lignin and hemicellulose which can be validated by the strong relationship between CrI and lignin removal (see supplementary materials), resulting in the increase of crystalline cellulose in pretreated bamboo (Li et al., 2017). While the slight decrease in PEB CrI at high H_2O_2 dosage indicated that the crystalline cellulose in PEB may also degrade at high H_2O_2 conditions (Kim et al., 2003). According to Alam et al. (2020), CrI alone cannot reflect the relative cellulose content in substrate because of the interference of other hexoses in hemicellulose which may cause the overestimation of CrI. Thus ratio of CrI and cellulose (*i.e.* glucan) was introduced (Table 1). As can be seen, although the CrI increased with the increasing H_2O_2 dosage, the CrI/cellulose value suffered from a slight decrease, indicating the AHP caused a transformation of crystalline cellulose to other crystal structures. The enzymatic hydrolysis was then plotted against CrI/cellulose values (see supplementary materials), and a negative correlation was found, revealing the cellulose crystalline structure impeded its enzymatic digestibility.

The FTIR spectra were further carried out to reveal the chemical structure changes of untreated and pretreated bamboo during the AHP (see supplementary materials). The signals at 3334 cm^{-1} and 2894 cm^{-1} corresponded to the hydroxyls stretching and the C-H stretching vibration of methoxy groups (OCH_3), respectively (Pandiyan et al., 2014). The signal at 1736 cm^{-1} was from the C=O stretching vibration in acetyl groups of hemicelluloses, and it can be seen that its intensity suffered a shape decrease after AHP. The peak located at 1246 cm^{-1}, which was assigned to the ester linkage between hemicellulose and lignin, also decreased after AHP (Hou et al., 2017; Ren et al., 2016; Sun et al., 2015). These results indicated that the hemicellulose and lignin-carbohydrate complex depolymerization occurred during the pretreatment (Qing et al., 2016). The characteristic peaks at 1599, 1506, and 1450 cm^{-1} were attributed to the skeletal vibrations of the benzene ring, and the intensity significantly reduced after AHP. The phenomena were because of the considerable degradation of lignin, in good agreement with the composition analysis. The characteristic peak related to cellulose β-glycosidic linkages at 896 cm^{-1} enlarged after the AHP, indicating cellulose preservation (Liang et al., 2020). The result showed partial lignin and hemicellulose were removed, and the cellulose was retained during the AHP, which was consistent with the aforementioned conclusion.

3.4 Morphological and topochemical characteristics

The anatomical features of bamboo are associated with the physicochemical natures of lignocellulose, which are strongly related to its enzymatic digestibility. Therefore, investigation of morphological and topochemical changes was crucial to evaluate the application prospects of bamboo biomass. The transverse sections of two bamboo samples before and after AHP were investigated to illustrate the bamboo cell morphology and distribution of lignin in different cell types by lignin autofluorescence (see supplementary materials).

Microscopy images showed the typical morphological regions of the herbaceous plant, in which the vascular bundles (VB) were surrounded by parenchyma (Par). The VB is composed of protoxylem vessel (Pxv), metaxylem vessel (Mxv), sclerenchyma fiber (Sf), and sieve tube (St). For both raw samples, it is evident that the cell wall structures were compactly stacked and well-organized, and the slight damage was probably due to mechanical damage in the cutting process. After pretreatment, the cell structure of both samples was observed to be broken down. Notably, the St and Pxv in the VB were swollen, and the Par was seen to be isolated from each other, and some were broken. The changes of cell walls were caused by the effective delignification and partial removal of hemicelluloses, resulting in the swelling of bamboo cell wall. By ultraviolet irradiation, the distribution of lignin after the sample characterization was observed. FM images showed that the raw bamboo of NAB and PEB had the intensest lignin autofluorescence compared to the AHP-pretreated samples. These results indicated that after being subjected to AHP, a significant amount of lignin was removed, accompanied by the bamboo structure becoming loose, which could lead to the exposure of cellulose-accessible surface area, thus increasing the substrate accessibility.

Changes in the spatial distribution of cellulose, hydroxycinnamic acids (HCA) and lignin in the Par and SF of raw and pretreated bamboos were investigated *in situ* by confocal Raman microscopy (CRM), which varied with bamboo species, morphological regions, and cell types (see supplementary materials). The main components of bamboo (NAB, PEB) have heterogeneous distributions in cell walls, in which the diverse intensities insinuated the concentrations. In Par (both samples), the highest concentrations of the three main components were found in the compound middle lamella (CML), while cell corner middle lamella (CCML) regions have the lowest intensity. On the contrary, the signals in SF were more pronounced in the CCML than that in the CML. At the same time, it can be observed that the differences in the physical structure of the two bamboos, PEB SF has a thicker cell wall (*i.e.*, smaller cell lumen) than NAB. After the AHP, it can be seen that signals of the cellulose, HCA and lignin in the pretreated bamboos were decreased to different extents. Particularly, the signals of lignin and HCA were found to decrease substantially, suggesting the breakage of ester and ether bonds between hemicelluloses and lignin (Ji et al., 2015). Furthermore, the delignification of NAB was more significant, and the cell profile of its SF became almost unidentifiable. On the contrary, signals in pretreated PEB SF were partially preserved, especially in the CCML. It can be concluded that the lower removal rates of lignin and hemicellulose in PEB was probably because of the thick cell wall and concentrated lignin content in its CCML and CML that is resistant to AHP degradation.

3.5 Characterization of lignin

Previously, it was demonstrated that lignin is the main factor impeding cellulose digestibility (Li et al., 2022). Thus it is pivotal to analyze the residual lignin characterization after the AHP. The molecular weight of lignin is strongly associated with its physicochemical structures, which is related to the biomass's natural recalcitrance and the valorization of lignin (Allison et al., 2012). In order to obtain the molecular weight distribution, CELs of two bamboos were extracted from raw and AHP treated bamboo (3% H_2O_2) and evaluated by GPC, and the result are shown in Table 2. It was found that the raw CELs of NAB and PEB have very similar M_w, which are 12111 g/mol and 12163 g/mol, respectively. Also, the PDI of the two raw lignins are close (4.2 and 4.0, respectively). After AHP, M_w and M_n values of the two samples decreased, but to different extents it was observed. Particularly, a 46.53% reduction in M_w was observed for NAB, while that of PEB was only 14.63%. This result indicated that the PEB lignin possesses more resistance to the AHP, which may associate with its chemical structure and was investigated with state-of-art characterization technology. After the AHP, the PDI was also decreased, indicating the degradation of large lignin moiety and thus leaving behind the uniform lignin residue.

The detailed chemistry structures of the CELs were studied using the HSQC NMR technology, which could help to further understand the fundamental lignin changes in the AHP. The aromatic (δ_C/δ_H 90-150/6.0-8.0 ppm) and side-chain regions (δ_C/δ_H 50-90/2.5-6.0 ppm) in the HSQC spectra of all the CELs obtained from NAB, PEB, RNAB, and RPEB were analyzed (see supplementary materials). The semiquantitative results are given in Table 3. The detailed cross-signals assignments were referred to these publications (Kellock et al., 2019; Wen et al., 2015).

In aromatic region, NAB and PEB lignins showed major aromatic units signals of syringyl (S), guaiacyl (G), and hydroxyphenyl (H) units, indicating bamboo lignin is mainly G-S-H lignin, in contrast to the G-S lignin in woody biomass (Zhao et al., 2020).

The normal S units showed major cross-peak at δ_C/δ_H 103.8/6.70 ppm ($C_{2,6}$-$H_{2,6}$ correlations), and that in the C_α-oxidized S (S’) unit signal was found at δ_C/δ_H 106.2/7.32 ppm in two bamboos’ lignins. Signals of the G unit could be centered at 110.6/6.99 (C_2-H_2), 114.8/6.77 (C_5-H_5), and 118.7/6.82 (C_6-H_6) ppm, and the $C_{2,6}$-$H_{2,6}$ correlations of the H unit located at 127.5/7.18 ppm. In addition, a substantial amount of *p*-coumarate (PCE) and ferulic acid (FA) were observed in two bamboo lignins, which revealed the existence of lignin-carbohydrate complex (LCC) structures in bamboo (Wen et al., 2013). Moreover, clear signals at δ_C/δ_H 106.2/7.05 (C_3-H_3), 98.7/6.23 ($C_{2,6}$-$H_{2,6}$), 94.2/6.57 (C_8-H_8), and 103.8/7.32 ($C'_{2,6}$-$H'_{2,6}$) ppm were attributed to the existence of tricin (T), which was widely reported to be a unique structure of grass, especially in less lignified area (Zhao et al., 2020). Other signals from the aromatic spectrum region were derived from the unsaturated side chains of cinnamaldehyde end-groups (J). From the semiquantitative data of raw CELs (Table 3), it can be seen that the NAB lignin possesses 54.0% S unit, higher than that of 45.5% in PEB lignin, while the G and H units contents were lower in NAB (42.8% and 3.1% *vs* 48.1% and 6.4%). As known, G unit contains only one methoxy group, and H unit is absent in that group, in contrast to the two methoxy groups in S unit. Thus the low S/G ratio (0.95 in PEB *vs* 1.3 in NAB) and more H unit in PEB may favor to form various C-C linkages with the adjacent lignin unit due to the low steric hindrance and will be discussed later (Cao et al., 2022). After the AHP, the 2D-HSQC spectral patterns in two CELs showed different variations. Specifically, in RNAB CEL, the signals of PCE decreased considerably, especially that of PCE_7 (144.5/7.42ppm) and PCE_8

(113.5/6.27ppm), which almost disappeared, revealing the cleavage of LCC linkages during AHP. At the same time, signals of the H unit were also weakened, and the signals of T were totally disappeared. Furthermore, the AHP reduced the content of S and H units from 54.0% and 3.1% to 40.7% and 1.4%, respectively, and increased the content of G from 42.8% to 57.9%, which decreased the S/G ratio from 1.3% to 0.7%. Besides, contents of FA and PCE also decreased extensively from 3.5% and 28.8% to 1.0% and 5.0%, respectively. As to RPEB CEL, the signals of PCE and FA were also weakened (from 3.9% and 19.9% to 3.3% and 5.6%, respectively). While the signals of T were primarily preserved. Different from RNAB, the content of S unit in PEB lignin increased from 45.5% to 54.7%, but the G and H units reduced from 48.1% and 6.4% to 41.6% and 3.7%, which as a result increased S/G ratio from 0.95% to 1.3%.

In the side-chain region, the information relating to the interunit linkages in lignin were provided, like *β*-O-4 aryl ether (A), *β*-*β* bonds (B), and *β*-5 bonds (C), which were the primary interunit linkages of bamboo lignin. The A_α signal appeared at δ_C/δ_H 71.7/4.86 ppm, while the A_β for S and G units was observed at δ_C/δ_H 86.1/4.05 and 83.4/4.33 ppm, respectively. The signals at δ_C/δ_H 59.5/3.51 and 63.2/4.26 ppm were derived from the A_γ and A'_γ (γ-acylated A structure), respectively. The signals of C_α, C_β and C_γ were centered at 86.8/5.46, 52.6/3.45 and 62.1/3.76 ppm. In addition, a trace amount of the spirodienone substructure (*β*-1, D) and *p*-hydroxycinnamyl alcohol end groups (I) were also observed. Both the two raw bamboo CELs have comparable *β*-O-4 content of 36.3% (NAB) and 37.4% (PEB), as can be seen in Table 3. However, the PEB CEL clearly contains more *β*-*β* and *β*-5 linkages (4.1% and 3.9% *vs* 3.7% and

2.1%), which is due to its low S/G ratio and high H unit content (as illustrated previously). The β-O-4 linkage is brittle and is liable to be cleaved during the pretreatment, while the C-C bond has very high bond energy and is difficult to degrade (Tsai et al., 2020). This is the reason why PEB possesses much lower delignification and lower enzymatic saccharification than NAB. After AHP, signals of all the subunits decreased, indicating the cleavage of lignin linkages. For NAB CEL, the β-O-4 contents significantly decreased from 36.3% to 23.8% (see Table 3). Besides, the β-β and β-5 contents also decreased from 3.7 % and 2.1% to 2.5% and 0.8%, respectively. However, for PEB CEL, both the β-O-4 and β-5 linkages slightly increased from 37.4% and 4.1% to 38.7% and 4.9%, agreeing with the GPC results that PEB lignin is relatively stable during the AHP. In sum, the dominant interunit bonds of β-O-4, β-β and β-5 were well preserved in PEB CEL, and it suggested that PEB has the relatively rigid structure with stronger recalcitrance compared with NAB, which was consistent with the FM and CRM results.

3.6 Hypothesis model of the enhanced enzymatic hydrolysis by AHP

Based on the analysis of the pretreated samples, a hypothesis model was proposed to correlate substrate features with the enzymatic hydrolysis, as shown in Fig. 5. It can be seen that the AHP degraded both hemicellulose and lignin and thus increased the cellulose content, but the cellulose crystalline structure was destroyed during the AHP. The removal of hemicellulose and lignin, and the break of cellulose crystalline structure substantially increased the digestibility of pretreated bamboo, which overall enhanced the sugar yield after the enzymatic hydrolysis. In addition, the lignin M_w, S/G ratio, β-

O-4, β-β and β-5 contents in NAB CEL decreased, while S/G ratio, β-O-4 and β-β contents in PEB CEL suffered from an increase. This is ascribed to the abundant G unit and C-C linkages in PEB lignin which was resistant to AHP degradation and thus resulted in lower enzymatic hydrolysis yield than NAB.

4. Conclusion

The enzymatic digestibility of two samples was improved during AHP with removing most of the lignin and partial hemicelluloses, preserving almost all the cellulose. FM and CRM images results showed that the bamboo structure became loose, accompanied by the breakage of ether and ester bonds between hemicelluloses and lignin. Residual CELs analysis indicated that PEB contains more G units which could establish more C-C linkage between lignin units, making the PEB lignin more resistant to AHP. Overall, *Neosinocalamus affinis* (NAB) is more suitable for biorefinery than *Phyllostachys edulis* (PEB) based on the evaluation of cellulose conversion and delignification effect.

E-supplementary data for this work can be found in e-version of this paper online

Declaration of Competing Interest

There are no known competing financial interests.

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Table 1 CrI and the CrI/cellulose value of the pretreated samples.

H_2O_2 dosage (wt%)	NAB		PEB	
	CrI (%)	CrI/cellulose	CrI (%)	CrI/cellulose
Raw	53.59	1.21	59.36	1.45
0	64.05	1.28	63.92	1.33
0.5	66.82	1.24	64.40	1.21
1.0	68.14	1.26	66.96	1.22
1.5	68.73	1.19	66.56	1.25
2.0	69.32	1.21	66.19	1.20
3.0	70.09	1.24	66.09	1.20

Table 2 Number-average (M_n), weight-average (M_w) molecular weight and polydispersity index (PDI) of the raw bamboo CEL and residual lignin in pretreated samples.

Sample	M_w (g/mol)	M_n (g/mol)	PDI
NAB	12111 ± 165	2858 ± 18	4.2 ± 0.1
PEB	12163 ± 31	3008 ± 4	4.0 ± 0.0
RNAB	6476 ± 67	2394 ± 4	2.7 ± 0.0
RPEB	10384 ± 27	3468 ± 2	3.0 ± 0.0

Table 3 Semiquantitative result for lignin subunits and interunit linkages of the two bamboo CELs (NAB and PEB) and residual lignin in pretreated bamboo.

	NAB	RNAB	PEB	RPEB
Lignin subunits (%)				
Syringyl (S)	54.0	40.7	45.5	54.7
Guaiacyl (G)	42.8	57.9	48.1	41.6
p-Hydroxyphenyl (H)	3.1	1.4	6.4	3.7
S/G	1.3	0.7	0.95	1.3
End groups (%)				
Ferulic acid (FA)	3.5	1.0	3.9	3.3
p-Coumarate (PCE)	28.8	5.0	19.9	5.6
Interunit linkages (%)				
β-O-4	36.3	23.8	37.4	38.7
β-*β*	3.7	2.5	4.1	4.9
β-5	2.1	0.8	3.9	3.1

Figures Legends

Fig. 1. Effect of AHP on solid recovery (a), chemical composition of NAB (b) and PEB (c), and lignin removal (d) of two bamboos.

Fig. 2. Glucan (a) and xylan (b) hydrolysis yields before and after AHP with different H_2O_2 loadings.

Fig. 3. Effect of H_2O_2 concentration on the enzymatic sugar yield.

Fig. 4. Correlation between glucan hydrolysis yield and lignin removal (a: NAB; b: PEB).

Fig. 5. A hypothesis model of the integrated impacts on sugar yield during the bamboo AHP (Green/red arrows and "+"/"-" mark as negative/positive impacts, respectively).

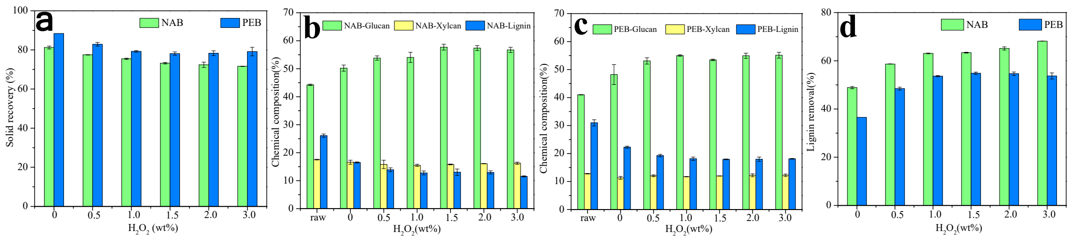

Fig. 1.

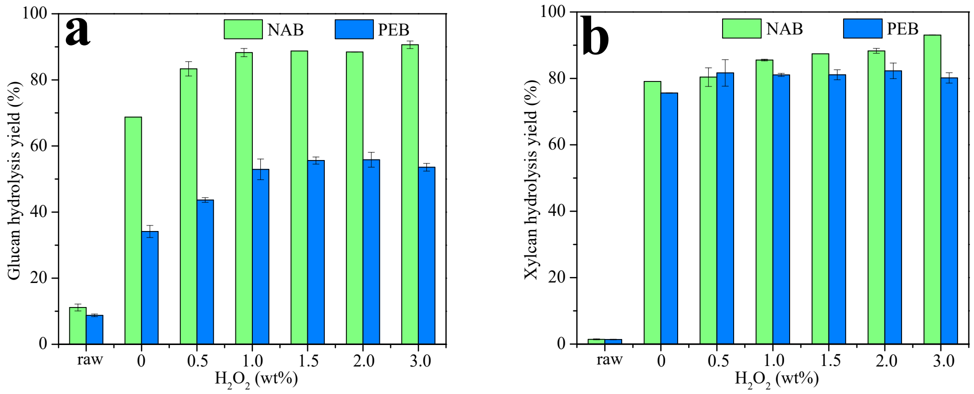

Fig. 2.

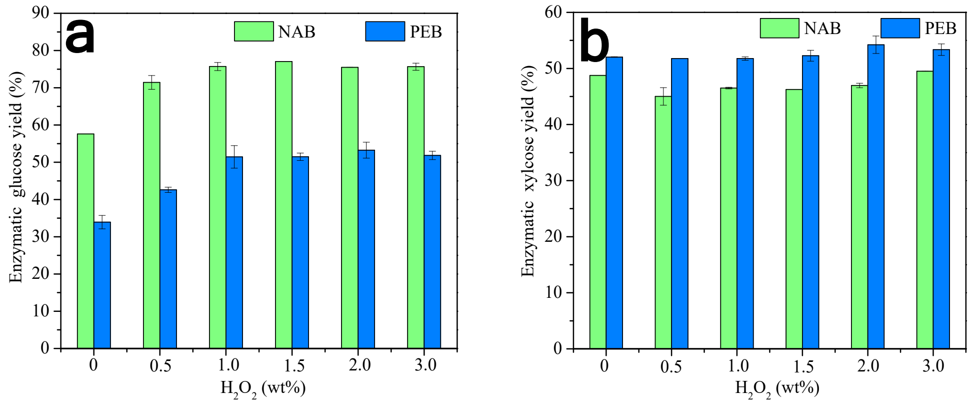

Fig. 3.

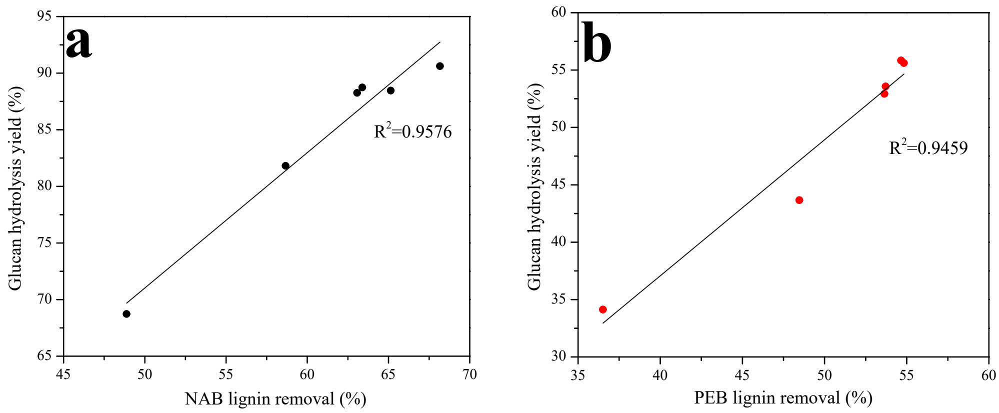

Fig. 4.

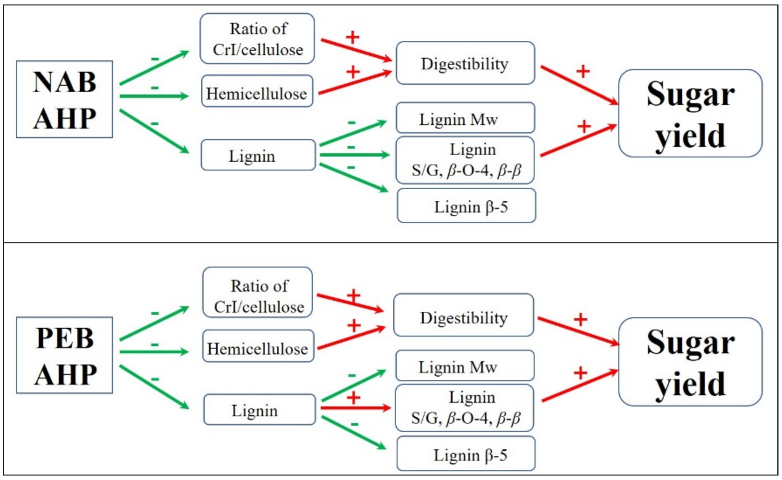
NAB
AHP
Ratio of
CrI/cellulose
Hemicellulose
Lignin
Digestibility
Lignin Mw
Lignin
S/G, β-O-4, β-β
Lignin β-5
Sugar
yield
PEB
AHP
Ratio of
CrI/cellulose
Hemicellulose
Lignin
Digestibility
Lignin Mw
Lignin
S/G, β-O-4, β-β
Lignin β-5
Sugar
yield

Fig. 5.